



DOUGLAS MACHADO LEITE

**OTIMIZAÇÃO DA PROPAGAÇÃO *in vitro* E
ACLIMATIZAÇÃO DE *Paliavana sericiflora* Benth.**

**LAVRAS-MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Engenharia Florestal, área de concentração Silvicultura, para a obtenção do título de Doutor.

Prof. Dr. Gilvano Ebling Brondani
Orientador

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*Aos que sempre estiveram comigo.
Dedico*

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*“Mas hoje eu queria muito agradecer a mim!
Porque eu não desisti.” (Anitta, 2019)*

RESUMO

Os campos rupestres configuram-se como geossistemas singulares, ostentando rica biodiversidade vegetal com notáveis adaptações morfológicas, anatômicas, fisiológicas e reprodutivas. Tais adaptações conferem resiliência a essas espécies diante das condições ambientais adversas que caracterizam esses ambientes, como baixos teores de matéria orgânica, solos rasos e déficit hídrico acentuado. Dentre a flora campestre rupestre, destaca-se a *Paliavana sericiflora*, espécie ameaçada de extinção conforme a Lista Vermelha da Flora do Brasil. Essa planta não apenas possui alto potencial ornamental, mas também se revela atrativa para a fauna local, podendo inclusive ser empregada em projetos de restauração ecológica desses frágeis ecossistemas. O estabelecimento de protocolos eficientes para a micropropagação e aclimatização de *P. sericiflora* assume papel crucial na estratégia de conservação da espécie. Essa técnica viabiliza a produção em massa de mudas com qualidade superior em relação a outros métodos tradicionais de propagação por sementes, contribuindo significativamente para a preservação dessa espécie. A micropropagação de *P. sericiflora* oferece diversas vantagens em relação ao cultivo convencional, como a produção rápida de mudas uniformes e livres de doenças. No entanto, a otimização dos componentes e condições de cultivo é crucial para maximizar os resultados e favorecer a aclimatização das mudas. Nesse sentido, este estudo investigou o efeito dos carboidratos, sistemas de trocas gasosas e espectro luminoso, na micropropagação de *P. sericiflora*. A aclimatização, etapa crítica na micropropagação, representa um grande desafio devido às mudanças bruscas de ambiente que podem levar à perda significativa de mudas. Para superar esse desafio, o estudo também propõe a utilização de um sistema de microtubetes enriquecidos com nanopartículas de zinco (ZnO-NPs-NPs) para auxiliar na produção e aclimatização de mudas micropropagadas de *P. sericiflora*, promovendo maior sobrevivência e crescimento.

Palavras-chave: Micropropagação. Nanomateriais. Óxido de zinco. Campo rupestre.

ABSTRACT

Rock fields stand out as singular geosystems, boasting rich plant biodiversity with remarkable morphological, anatomical, physiological, and reproductive adaptations. These adaptations confer resilience to these species in the face of the harsh environmental conditions that characterize these environments, such as low organic matter content, shallow soils, and accentuated water deficit. Among the rupestrian campestrian flora, *Paliavana sericiflora* stands out, a species threatened with extinction according to the Red List of the Flora of Brazil. This plant not only has high ornamental potential but also proves to be attractive to local fauna, and can even be used in ecological restoration projects for these fragile ecosystems. The establishment of efficient protocols for micropropagation and acclimatization of *P. sericiflora* plays a crucial role in the species conservation strategy. This technique enables the mass production of seedlings with superior quality compared to other traditional seed propagation methods, significantly contributing to the preservation of this species. Micropropagation of *P. sericiflora* offers several advantages over conventional cultivation, such as the rapid production of uniform and disease-free seedlings. However, optimizing the components and *in vitro* cultivation conditions is crucial to maximize results and favor seedling acclimatization. In this sense, this study investigated the effect of carbohydrates, gas exchange systems, and light spectrum on the micropropagation of *P. sericiflora*. Acclimatization, a critical stage in micropropagation, represents a major challenge due to the abrupt environmental changes that can lead to significant seedling loss. To overcome this challenge, the study also proposes the use of a microcontainer system enriched with zinc nanoparticles (ZnO-NPs-NPs) to assist in the production and acclimatization of micropropagated *P. sericiflora* seedlings, promoting greater survival and growth.

Keywords: Micropropagation. Nanomaterials. Zinc oxide. Campo rupestre.

INDICADORES DE IMPACTO

A pesquisa sobre a “Otimização da propagação *in vitro* e aclimatização de *Paliavana sericiflora* Benth” apresenta um amplo espectro de impactos que se estendem ao âmbito científico, ambiental, social e econômico. No campo científico: desenvolvimento do protocolo de propagação permitindo a conservação *in vitro* da espécie, maior compreensão do efeito das ZnO-NPs em plantas e contribuição para conservação do campo rupestre e a possível criação de bancos de germoplasma da espécie. No âmbito social e ambiental: preservação da biodiversidade por meio da restauração e recuperação de área degradadas, podendo ser utilizando com exemplos em aulas de educação ambiental, ecoturismo e projetos de conscientização para produção de mudas de forma sustentável da agricultura ao paisagismo aplicando o uso de recipientes biodegradáveis, reduzindo o microplásticos no ambiente. O estudo impacta a sociedade local em contato com o campo rupestre e a nível global com a conservação da biodiversidade. No âmbito econômico possui potencial uso comercial (espécie e recipiente), apoio no ecoturismo com a restauração de ecossistemas e contribui para desenvolvimento sustentável, além da técnica permitir a produção de mudas durante todo o ano em grandes quantidades e com qualidade. Pode ser classificado dentro da política nacional de extensão nas áreas de Educação e Meio Ambiente. A pesquisa está alinhada com alguns Objetivos do Desenvolvimento Sustentável (ODS) da ONU sendo eles: 2 fome zero e agricultura sustentável, 12 consumo e produção responsáveis, 13 ação contra a mudança global do clima e 14 vida terrestre.

IMPACT INDICATORS

The research on "Optimization of *in vitro* propagation and acclimatization of *Paliavana sericiflora* Benth" presents a wide range of impacts that encompass scientific, environmental, social, and economic spheres. In the scientific realm, the development of a propagation protocol facilitates the *in vitro* conservation of the species, enhances our understanding of the effects of ZnO-NPs-NPs on plants, and contributes to the preservation of the rocky field ecosystem and the potential establishment of germplasm banks for the species. In the social and environmental domain, the research promotes biodiversity conservation through the restoration and recovery of degraded areas, serving as an exemplary tool for environmental education classes, ecotourism initiatives, and awareness campaigns. Furthermore, it advocates for sustainable seedling production methods in agriculture and landscaping, utilizing biodegradable containers to reduce microplastic pollution. The study positively impacts both local communities engaged with the rocky field ecosystem and the global community through biodiversity conservation. From an economic perspective, the research holds potential for commercial utilization of both the species and the container, supports ecotourism by restoring ecosystems, and contributes to sustainable development. Additionally, the developed technique enables year-round production of high-quality seedlings in large quantities. The research aligns with the Brazilian National Extension Policy in the areas of Education and Environment and resonates with the United Nations Sustainable Development Goals (SDGs) 2 (Zero Hunger and Sustainable Agriculture), 12 (Responsible Consumption and Production), 13 (Climate Action), and 14 (Life Below Water).

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PRIMEIRA PARTE

1 INTRODUÇÃO

O Brasil detém um conjunto abrangente de Unidades de Conservação (UCs) federais, ultrapassando 334, conforme dados do ICMBio. Essas UCs assumem um papel crucial na preservação da biodiversidade brasileira, assegurando a proteção de habitats naturais, espécies endêmicas, ameaçadas ou vulneráveis. Além das Ucs federais, o país conta com áreas protegidas sob a gestão de estados, municípios e até mesmo empresas privadas. Todas essas áreas compartilham o mesmo objetivo fundamental, a qual se refere a conservação da biodiversidade brasileira e a manutenção de espécies com alto valor ecológico. A proteção desses espaços naturais é essencial para garantir o bem-estar da sociedade (Carvalho; Backx, 2018).

Inseridas no mosaico diversificado de áreas naturais do Brasil, os campos rupestres se distinguem como formações vegetais únicas. Caracterizados por sua associação a afloramentos quartzíticos, graníticos ou ferruginosos, esses campos apresentam uma composição florística variável, moldada pela geologia do substrato e pelo grau de desagregação da rocha (Santos *et al.*, 2018). Entre os tipos de campos rupestres, destaca-se o campo ferruginoso, reconhecido por sua alta biodiversidade e elevada proporção de espécies endêmicas e raras (Jacob *et al.*, 2007; Messias *et al.*, 2012). No entanto, a biodiversidade rica em espécies arbustivas, herbáceas e gramíneas desses campos enfrenta ação antrópica devido a atividade de extração de ferro.

A fisionomia dos campos rupestres é caracterizada predominantemente por um estrato arbustivo e herbáceo. As plantas que habitam esses ambientes oligotróficos, com baixos teores de nutrientes e matéria orgânica, solos rasos, déficit hídrico e altas concentrações de ferro oxidado, desenvolveram notáveis adaptações morfológicas, anatômicas, fisiológicas e reprodutivas para sobreviver e prosperar nessas condições desafiadoras (Jacob *et al.*, 2007; Mendonça *et al.*, 2019). Apesar da grande diversidade e do alto grau de endemismo presentes nos campos rupestres, a escassez de estudos sobre a propagação representa um entrave para a implementação de medidas eficazes de conservação. Dificultando a aplicação de técnicas para a preservação de táxons com alto grau de vulnerabilidade de extinção, e impede a identificação de outras espécies ainda desconhecidas.

Entre as espécies dos campos rupestres ferruginosos, destaca-se *Paliavana sericiflora*, arbusto endêmico do Brasil que compõe as paisagens de campo sujo e campo limpo. Essa planta, que pode alcançar até três metros de altura, se destaca por suas flores atrativas à fauna,

além de seu potencial paisagístico, caracterizado por flores com aspecto ceroso. A espécie também apresenta grande potencial para projetos de recuperação e restauração de campos rupestres (Messias *et al.*, 2012; Santos *et al.*, 2018).

Apesar do vasto potencial de *P.sericiflora* para diferentes usos, os estudos sobre sua propagação seminal e/ou vegetativa são recentes. inclusive para o gênero ao qual pertence, a restrita obtenção de sementes devida a sazonalidade da floração, a dificuldade de coleta por ocorrer em paredões rochosos, a pouca quantidade de sementes produzidas e o tamanho das sementes, além de ser classificada como vulnerável à extinção no Sistema de Informação sobre a Biodiversidade Brasileira (Rossini *et al.*, 2020; SiBBR, 2022), os estudos de micropropagação podem minimizar as problemáticas quanto a produção de mudas da espécie.

A micropropagação, técnica de cultivo *in vitro* de tecidos sob condições assépticas, é uma ferramenta promissora para a propagação vegetativa de espécies (George *et al.*, 2008). Nessa técnica, o material é inicialmente estabelecido em meio de cultura adequado, seguido de multiplicação, alongamento e enraizamento, culminando na geração de novas plantas geneticamente idênticas ao explante original seja ele oriundo de sementes ou não. Diversos fatores influenciam o sucesso da micropropagação, incluindo o teor de açúcares no meio de cultura, a utilização de sistemas de ventilação e a qualidade da luz (Ferreira *et al.*, 2016). A otimização desses parâmetros é crucial para maximizar os resultados do cultivo *in vitro* e, conseqüentemente, aumentar as chances de sucesso na aclimatização. Ajustes no ambiente de cultivo garante a adaptação das plantas às condições *ex vitro*, elevando a sobrevivência (Tronco *et al.*, 2015; Ferreira *et al.*, 2016; Vahdati *et al.*, 2017).

Os carboidratos “açúcares” irão fornecer energia auxiliado no crescimento dos tecidos e no desenvolvimento *in vitro* das plantas, podendo afetar a qualidade e o estado fisiológico das plantas a depender do tipo de espécie trabalhada, carboidratos exógenos podem ainda melhorar o desempenho fotossintético das plantas *in vitro* (Barbosa *et al.*, 2021; Saez *et al.*, 2016). Já as trocas gasosas com o ambiente podem auxiliar o amadurecimento dos tecidos tornando-os mais resistentes a aclimatização, reduzindo o teor de etileno, regulando a umidade e temperatura dentro do frasco, favorecendo as diferentes fases de cultivo (Leite *et al.*, 2024).

O espectro luminoso pode influenciar as respostas, assim como luz insuficiente pode ser prejudicial para as plantas, pois pode retardar o crescimento, reduzir a fotossíntese e aumentar a suscetibilidade das plantas a doenças. A luz excessiva também pode ser prejudicial para as plantas, pois pode danificar as estruturas das plantas, como as folhas e as flores. Portanto, é importante fornecer o espectro luminoso adequado de luz para as plantas durante as diferentes fases de cultivo (Oliveira *et al.*, 2021; Souza *et al.*, 2022).

A aclimatização de mudas micropropagadas é uma etapa crucial para produção de mudas. Nessa fase, as mudas são gradualmente expostas às condições do ambiente externo, minimizando perdas e promovendo seu desenvolvimento. O uso de microtubetes (tubetes com aproximados 4 cm³) em um sistema de mini-estufim se destaca como uma estratégia eficaz nesse processo (Brondani *et al.*, 2017). É um sistema compacto e eficiente permite otimizar o espaço físico e os recursos utilizados, tornando a aclimatização mais econômica e viável, especialmente para o cultivo de espécies em pequena escala ou para projetos com recursos limitados (Brondani *et al.*, 2012).

A utilização de microtubetes apresenta diversas vantagens para o enraizamento e aclimatização. O tamanho reduzido dos microtubetes otimiza o espaço necessário para o cultivo, permitindo que essa etapa seja realizada em laboratório, sob condições controladas de temperatura e luminosidade (Ferraz *et al.*, 2015; Faria *et al.*, 2021, 2022). Microtubetes confeccionados com materiais biodegradáveis como o poliláctico (PLA) dispensam a necessidade de remoção das mudas para o plantio. O PLA se destaca como um bioplástico biodegradável, com ampla disponibilidade e capacidade de decomposição sem causar danos ao meio ambiente (Taib *et al.*, 2022). Sua alta durabilidade e resistência o posicionam como uma das principais alternativas aos produtos derivados do petróleo como o PET ou PP.

Além disso, o PLA é produzido a partir de subprodutos agrícolas a base de milho, cana de açúcar ou beterraba, tornando-o uma opção viável por sua versatilidade e mínimo impacto ambiental (Taib *et al.*, 2022), principalmente na produção de microplásticos, um problema ambiental crescente associado ao uso dos polímeros tradicionais. Pode se decompor em meses, de acordo com sua composição, sendo rapidamente absorvido pelo ambiente, sem causar danos ecológicos (Taib *et al.*, 2022). O microtubetes podem ser enriquecidos com nutrientes, facilitando a absorção pelas raízes das mudas, otimizando o crescimento e desenvolvimento das plantas. Essa estratégia contribui para a aclimatização das microestacas, aumentando suas chances de sobrevivência após o transplante para o ambiente *ex vitro*.

A incorporação de nutrientes ao microtubete apresenta vantagens e desvantagens que devem ser cuidadosamente consideradas. A adição de nutrientes pode aumentar o custo do produto, mas também o torna mais competitivo no mercado, além de possibilitar melhorias nos tratamentos culturais e na produção de mudas. Uma das formas mais promissoras de incorporar nutrientes ao microtubete é a utilização de nanomateriais. Estes materiais, compostos por partículas com tamanhos entre 0 e 100 nanômetros, podem aprimorar a absorção de nutrientes pelas plantas, especialmente aqueles com baixa disponibilidade no solo (Natarelli *et al.*, 2021, Jakhar *et al.*, 2022; Haydar *et al.*, 2024). A utilização de materiais em escala nanométrica

apresenta grande potencial em diversas áreas, e abre novas perspectivas para a otimização do cultivo de mudas e o aumento da produtividade agrícola (Haydar *et al.*, 2024).

A aplicação de nutrientes na forma de nanopartículas em culturas agrícolas apresenta diversos benefícios (Haydar *et al.*, 2024). O zinco (Zn), em particular, é um micronutriente essencial para o desenvolvimento das plantas, mas apresenta baixa disponibilidade em solos tropicais (Araujo *et al.*, 2016; Taiz *et al.*, 2017). O zinco possui um papel importante em diversas funções fisiológicas das plantas. Sua participação em reações redox garante a transferência de elétrons e a transformação de energia, processos essenciais para o metabolismo vegetal. Além disso, o zinco contribui para a síntese de clorofila em algumas espécies, otimizando a fotossíntese e o crescimento das plantas (Taiz *et al.*, 2017).

A aplicação de Zn na forma de nanopartículas oferece diversas vantagens em comparação com formas tradicionais de fertilizantes. As nanopartículas apresentam maior superfície específica, permitindo uma melhor absorção do nutriente pelas raízes das plantas. Além disso, a liberação gradual do zinco a partir das nanopartículas pode garantir um fornecimento mais eficiente e prolongado do nutriente, minimizando perdas por lixiviação ou evaporação (Silva *et al.*, 2016). A utilização de nanopartículas de zinco em culturas agrícolas representa uma estratégia inovadora e promissora para otimizar o crescimento e a produtividade das plantas, especialmente em solos com baixa disponibilidade desse micronutriente essencial.

O Zn desempenha um papel essencial na síntese do triptofano. Este aminoácido, por sua vez, é um precursor das auxinas, hormônios vegetais que regulam diversos processos de crescimento, incluindo a formação de novas brotações e o desenvolvimento radicular (Brondani *et al.*, 2012). A exposição das células vegetais ao ZnO-NPs aumenta a absorção celular de Zn^{2+} , promovendo um fornecimento mais eficiente desse nutriente essencial (Natarelli *et al.*, 2021; Yoshihara *et al.*, 2021). Essa otimização na absorção de zinco pode contribuir para o crescimento inicial e a aclimatização das plantas micropropagadas. Uma vez que as auxinas, sintetizadas a partir do triptofano, podem estimular o desenvolvimento radicular, fortalecendo as plantas e aumentando suas chances de sobrevivência após o transplante para o ambiente *ex vitro*.

Os benefícios do uso de microtubetes enriquecidos com ZnO-NPs não se limitam à aclimatização de espécies ameaçadas. Essa tecnologia também pode ser aplicada com sucesso na produção de mudas florestais, otimizando o crescimento e a qualidade das plantas, e contribuindo para o reflorestamento e a recuperação de áreas degradadas.

A combinação de micropropagação, conservação e utilização de tecnologias inovadoras abre novas perspectivas para a preservação da biodiversidade e o desenvolvimento sustentável. Essa abordagem integrada garante a perpetuação de espécies vegetais ameaçadas, contribuindo para

a produção eficiente de mudas florestais e promove a recuperação de áreas degradadas, assegurando um futuro mais verde para o planeta.

O conhecimento sobre as espécies do campo rupestre ferruginoso, um bioma de extrema importância ecológica, ainda é bastante limitado. Diante desse cenário, o objetivo dessa pesquisa foi produzir protocolos eficientes de micropropagação e aclimatização, verificando o efeito de trocas gasosas e espectro luminoso para *Palaiaana sericiflora* Benth.

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SEGUNDA PARTE

**ARTIGO 1 - IMPROVEMENT IN THE PRODUCTION OF MICROPROPAGATED
SEEDLINGS OF *Paliavana sericiflora* Benth.: AN ENDANGERED SPECIES**

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Improvement in the production of micropropagated seedlings of *Paliavana sericiflora* Benth: an endangered species

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Abstract

This study focused on the species *Paliavana sericiflora* Benth., considered vulnerable to extinction. The species is endemic to *Campo Rupestre Ferruginoso* habitats, which are geosystems with specific soil and climate characteristics that result in a flora that is strictly adapted to these conditions. Due to the importance of conservation of the species and the need to produce seedlings for the establishment of germplasm banks, the objective of this study was to investigate the effect of active chlorine on seed disinfection, the best source of carbohydrates and the use of gas exchange systems in the in vitro culture of *P. sericiflora*. The seeds were immersed for different times in sodium hypochlorite (NaOCl, 1.00–1.25% active chlorine) for disinfection and exposed to different gas exchange systems and carbohydrate concentrations during in vitro multiplication. In the elongation and rooting stages, different systems for gas exchange with the external environment were tested. The results indicated that immersion of seeds in active chlorine for 10 min was effective in obtaining the largest number of germinated plants free of contamination. Supplementation of 30 g L⁻¹ sucrose to the culture medium promoted greater bud sprouting during multiplication. The use of one membrane favoured the growth and emergence of new shoots. The mini-incubator system was considered efficient for acclimatization, favouring the production of micropropagated *P. sericiflora* plants.

Key message

The study presents unpublished results on in vitro culture for the seedling production of *Paliavana sericiflora*, an endangered species of Brazilian flora.

Keywords Gesneriaceae · Campo rupestre · In vitro culture · Vulnerable to extinction

Abbreviations

MS	Murashige and Skoog culture medium (Murashige and Skoog 1962)
NAA	α -naphthaleneacetic acid
BAP	6-benzylaminopurine
IBA	indole-3-butyric acid
NME	rigid lids with no membrane
1ME	rigid lids with one membrane
3ME	rigid lids with three membranes

Introduction

Campo Rupestre Ferruginoso (i.e., ferruginous rocky fields or cangas) habitats are geosystems that have specific soil and climate characteristics (Messias et al. 2012). They harbour a variety of endemic species adapted to the specific environmental conditions of this habitat. This unique vegetation structure occurs on rocky outcrops and has the ability to survive environments with high light and thermal intensity and low water, soil and organic matter availability. Because plants growing in these habitats develop under adverse conditions and in areas subject to mining, it is essential to conduct conservation studies on these species to aid in their maintenance and propagation (Messias et al. 2012).

Among the species endemic to canga fields, some occur in areas with economic importance for the extraction of ore, such as iron. The anthropogenic activities in these areas weaken the survival of several species (Saraiva et al.

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2020), placing them at risk of extinction or vulnerability. To mitigate this impact, it is essential to conduct conservation, collection and propagation studies of the species found in these locations (Saraiva et al. 2020; Veloso et al. 2022).

Vulnerable species that occur in *Campo Rupestre* habitats include the endemic *Paliavana sericiflora* Benth., which grows in crevices of rocky outcrops, where organic matter is found in greater quantity (Anastácio et al. 2018; Rossini et al. 2020). Characterized as a shrub, it maintains its leaves during flowering. These are opposite, verticillate, and serrated. Its cymose inflorescences range from 1 to 4 flowers in the axillary position, its flowers are reddish or brown on the inner side and cream-green on the outer side, and may have purple spots or vinaceous striations. Its calyx is triangular, and its corolla is funnel-shaped with a cylindrical base. The fruits are of the ellipsoid capsule type. Flowering occurs from March to December, and pollination and seed dispersal are carried out by animals. In addition to its scenic value, *P. sericiflora* also plays an important role in environmental recovery, attracting fauna to the region (Anastácio et al. 2018; Rossini et al. 2020; WFO 2023).

In addition to their relevance for conservation, some studies have identified compounds with cytotoxic, allopathic, and antifungal activities in species of the *Paliavana* genus (Sales et al. 2018). However, information on the propagation strategies and seedling production of these species is still scarce in the literature. Thus, considering the lack of information on the propagation of *P. sericiflora*, it is necessary to conduct studies to fill this gap.

Micropropagation is a technique for the large-scale propagation of plants that provides time and space savings and optimal phytosanitary conditions for uniform development of seedlings (George et al. 2008). Micropropagation also allows the production of plants regardless of soil and climatic conditions and seed quantity or availability (Davey and Anthony 2010). Some adjustments to propagation protocols can be made to improve and maximize the results of micropropagation, such as seed disinfection techniques and adequate choice of carbohydrate source which vary according to the studied plant and the propagation stage that it is, thus favouring morphogenic responses (George et al. 2008; Zahara et al. 2017).

Some plants depend on carbohydrates for in vitro survival and for faster responses in the multiplication and elongation stages, since the exogenous source of carbohydrates maintains metabolic processes and supports plant growth (Gonçalves et al. 2023). Another approach to maximize production and reduce losses during acclimatization is the use of systems that allow gas exchange with the external environment, making plants better adapted to external environmental conditions and improving morphogenic responses, especially elongation and adventitious rooting.

The possibility of gas exchange with the environment outside the container may favour tissue elongation, while the maintenance of CO₂ inside the culture flask may stimulate an increase in the photosynthetic rate while reducing the accumulation of ethylene, which, in high concentrations, can trigger senescence in the cultivated plants (Molinari et al. 2021, 2023).

Given the importance of conserving the species and optimizing plant production through the establishment of germplasm banks, the objective of this study was to evaluate the effect of active chlorine on seed disinfection, the best carbohydrate source for in vitro multiplication and the use of gas exchange systems for the cultivation of *P. sericiflora* with the aim of producing micropropagated seedlings.

Materials and methods

Study site and source of plant material

The experiments were conducted at the Laboratory of in vitro Culture of Forest Species, Department of Forestry Sciences, School of Agricultural Sciences of Lavras, Federal University of Lavras (UFLA), Lavras, Minas Gerais State, Brazil. The species is distributed exclusively in the Minas Gerais region (Fig. 1) and is classified as vulnerable to extinction according to the Brazilian biodiversity information system (SIBBR 2020). The plant material was provided by Empresa GERDAU Açominas SA Company, Research and Innovation Unit in Ferruginous Rupestrian Grassland (*Unidade de Pesquisa e Inovação em Campos Rupestres Ferruginosos*), Ouro Branco, Minas Gerais State, Brazil, and was collected from individuals growing in *Campo Rupestre Ferruginoso* habitats, with registration number ACF88E2 in SisGen.

In vitro germination and establishment

Seeds were washed in running water for approximately 5 min, immersed in a 1.00–1.25% sodium hypochlorite (Clarix®) solution for 5, 10, and 15 min in a horizontal laminar flow chamber, followed by five washes in autoclaved deionized water, and inoculated with one seed per test tube (25 × 150 mm) containing 10 mL of the culture medium. The culture medium used was MS (Murashige and Skoog 1962) supplemented with 30 g L⁻¹ sucrose (Synth Ltda) and 6 g L⁻¹ agar (Merck SA), with the pH adjusted to 5.8 ± 0.05 before adding the agar. The culture medium was autoclaved at 121 °C and 1.0 kgf cm⁻² for 20 min. The seeds were conditioned for 30 d in a growth chamber at 24 ± 1 °C under a 16-h photoperiod and 40 μmol m⁻² s⁻¹ of irradiance.

The experimental design used was completely randomized with three treatments and 20 replicates per treatment.

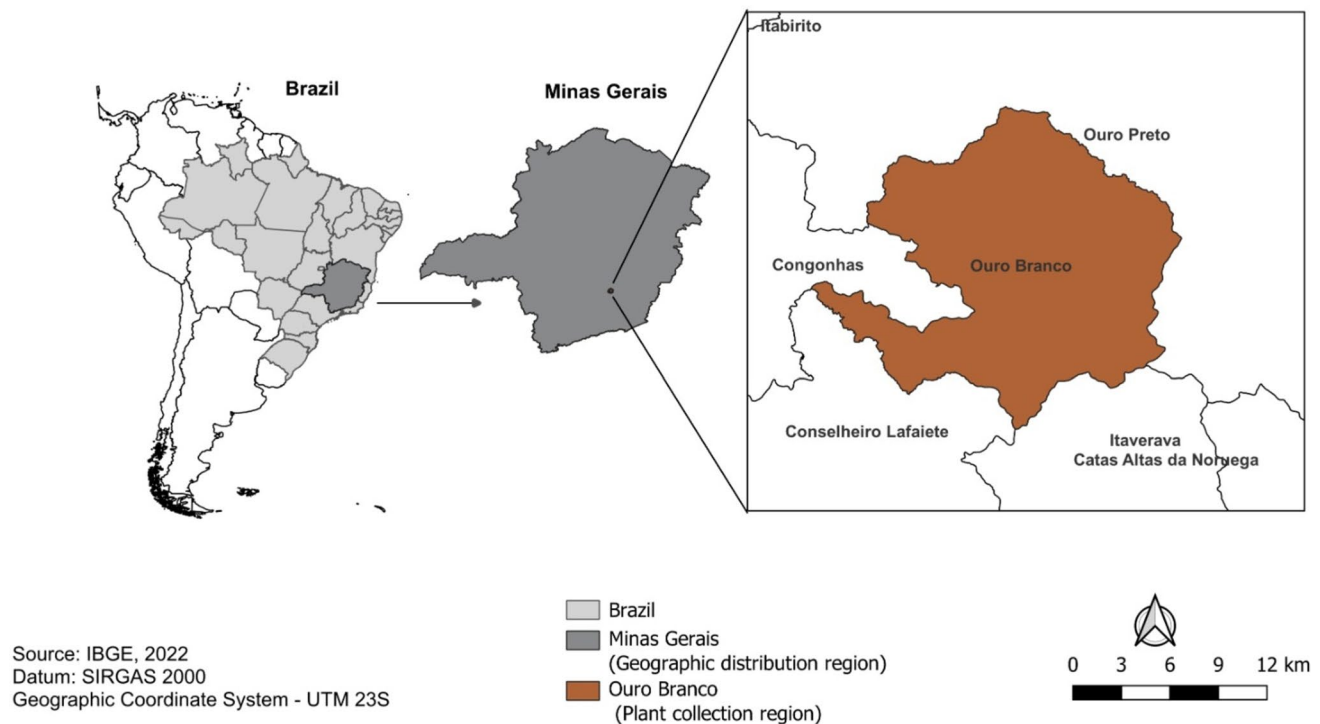


Fig. 1 Map of the geographical distribution and collection area of *Paliavana sericiflora* Source: IBGE (2022)

The percentage of germination and contamination were evaluated at 30 d, and germinated seeds free of contamination were considered established, which were used in the remaining experiments (Fig. 2).

Effect of gas exchange and sucrose concentrations on in vitro multiplication

The plant material used was in vitro germinated seedlings with an average size of 0.5 cm. The MS culture medium was used in the experiments, added with 15 and 30 g L⁻¹ sucrose supplemented with 0.05 mg L⁻¹ NAA (Sigma®), 0.50 mg L⁻¹ BAP (Sigma®) and 6 g L⁻¹ agar. The experiment was conducted in 250-mL flasks containing 40 mL of culture medium with three different test sealing systems: (i) rigid polypropylene lids with no membrane (NME); (ii) polypropylene lids with a one-hole (1.0 cm diameter) covered with a 1.0-cm² membrane (1ME); and (iii) polypropylene lids with three holes (1.0 cm in diameter) covered with a 1.0-cm² membrane (3ME) (Saldanha et al. 2012). After subculture, the flasks were kept in a growth room at 24 ± 1 °C under a 16-h photoperiod and 40 μmol m⁻² s⁻¹ of irradiance.

Evaluations were performed 30 d after set up. The experiment was conducted in a completely randomized design in a 2 × 3 double factorial (2 carbohydrate concentrations and 3 sealing systems) with 15 replicates per treatment. At 30 d of cultivation, oxidation, vigour, number of leaves, number of

shoots, shoot length (cm) and photosynthetic pigment contents [chlorophyll *a*, *b*, *alb*, total (*a* + *b*), and carotenoids] were evaluated. Oxidation evaluations were performed using presence and absence, and vigour evaluations were performed according to the rating scale of Souza et al. (2021) with modifications.

Effect of gas exchange on in vitro elongation

In the in vitro elongation stage, the shoots produced in the multiplication stage were prepared by isolating four clusters containing shoots with an average height of 1 cm, 2 to 3 pairs of expanded leaves, and excellent vigor. These shoots were subcultured for 30 d in 250-mL flasks containing 40 mL of 30 g L⁻¹ sucrose, 6 g L⁻¹ agar, 0.05 mg L⁻¹ BAP, and 0.5 mg L⁻¹ NAA added to MS culture medium.

The experiment was conducted in a completely randomized design with 15 replicates per treatment. The gas exchange system consisted of three different types of sealing (NME, 1ME and, 3ME) (Saldanha et al. 2012). After subculture, the flasks were kept in a growth room at 24 ± 1 °C under a 16-h photoperiod and 40 μmol m⁻² s⁻¹ of irradiance for 30 d for further evaluation.

At 30 d of cultivation, oxidation, vigour, number of shoots, number of elongated shoots (> 1 cm), number of roots, shoot length (cm), leaf area (cm²) (Souza et al. 2020) and photosynthetic pigment contents [chlorophyll *a*, *b*, *alb*,

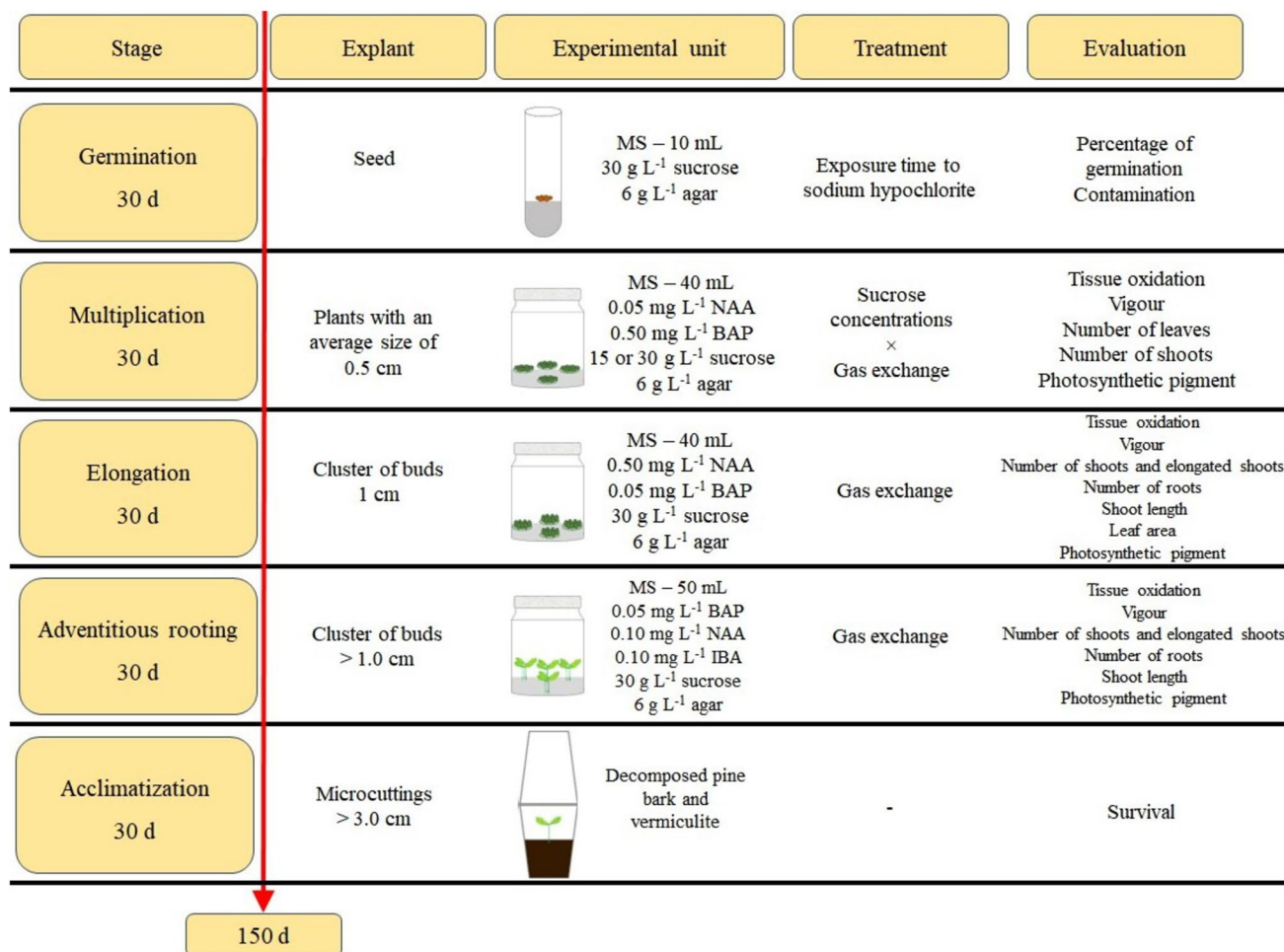


Fig. 2 Flowchart summary of the experiments carried out with *Paliavana sericiflora*

total ($a + b$), and carotenoids] were evaluated. Oxidation evaluations were performed using presence and absence, and vigour evaluations were performed according to the rating scale of Souza et al. (2021) with modifications.

Effect of gas exchange on in vitro adventitious rooting

In the in vitro rooting stage, the shoots produced during elongation were prepared by isolating four clusters with standardized shoots larger than 1 cm and with excellent vigour and inoculated under aseptic conditions in 250-mL glass flasks. They were cultivated for 30 d in 50 mL of MS culture medium supplemented with 30 g L⁻¹ sucrose, 6 g L⁻¹ agar, 0.05 mg L⁻¹ BAP, 0.1 mg L⁻¹ NAA, and 0.1 mg L⁻¹ IBA (Sigma®).

The experiment was conducted in a completely randomized design with 15 replicates per treatment. The gas exchange system consisted of three different types of sealing (NME, 1ME and, 3ME) (Saldanha et al. 2012).

After subculture, the flasks were kept in a growth room at 24 ± 1 °C under a 16-h photoperiod and 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of irradiance for 30 d for further evaluation.

At 30 d of cultivation, the following were evaluated: vigour, oxidation, number of roots, shoot length (cm), number of shoots, number of elongated shoots (> 1 cm) and photosynthetic pigment contents [chlorophyll *a*, *b*, *alb*, total ($a + b$), and carotenoids]. Oxidation evaluations were performed using presence and absence, and vigour evaluations were performed according to Souza et al. (2021) with modifications.

Acclimatization

Microcuttings with a length of more than 3 cm and with roots were transplanted into polypropylene cups in a mini-incubator system following with modifications. The commercial substrate used was composed of decomposed pine bark and regular vermiculite at 1:1 (v/v). The microcuttings were grown under a 16-h photoperiod and 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$

irradiance in a laboratory environment, with irrigations made by manual spraying with a sprayer every 48 h for 30 d for subsequent evaluation of survival.

Analysis of photosynthetic pigments

At the end of the cultivation period, leaf discs (25 mg of fresh leaf matter) were sampled, added to 5 mL of dimethyl sulfoxide (Sigma-Aldrich) and preserved for 48 h in the dark (Lichtenthaler 1987). The absorbance of the samples was determined in triplicate in a 10-mm quartz cuvette in a Genesys 10UV spectrophotometer (ThermoScientific, USA). The wavelengths (665, 649 and 480 nm) and the equations for calculating the chlorophyll *a*, *b*, and carotenoid contents were based on the method described by Wellburn (1994).

Statistical analysis

Each experiment had 15 to 20 replicates per treatment tested, which were obtained after 30 d of cultivation. The data of the response variables were checked for homoscedasticity and normal distribution of residuals using the Hartley and Shapiro–Wilk tests, respectively, adopting a 5% error probability level. The results were evaluated by analysis of variance (ANOVA, $P < 0.05$), followed by Tukey's test ($P < 0.05$) to compare means. The R program version 4.0.3 and the functions contained in the ExpDes.pt (Ferreira et al. 2021) and easyanova (Arnhold 2013) packages were used. The Kruskal–Wallis test at the 5% significance level was

applied for non-parametric data (Giraudoux 2022; Hothorn et al. 2008; Rigby and Stasinopoulos 2005).

Results

In vitro germination and establishment

Under the experimental conditions, a difference in the percentage of contamination of *P. sericiflora* seeds was observed among the different times of exposure to active chlorine (i.e., concentration 1.00–1.25%). The highest means were obtained in the treatment with 5 min of exposure to active chlorine (15% contamination), which differed significantly from the treatments with 10 and 15 min of exposure (Fig. 3a). For in vitro germination, the highest values were observed with a time of exposure to active chlorine of 5 and 10 min (100% germination), but there was no significant difference from the treatment with 15 min of exposure (Fig. 3b).

Effect of gas exchange and sucrose concentrations on in vitro multiplication

In the in vitro multiplication of the material from germination, only the variable shoot length significantly differed among the treatments tested. When the multiplication was evaluated separately, the treatment without gas exchange with the external environment (Fig. 4a) provided the largest shoots. Regarding the concentration of carbohydrates, there was no significant difference between the two amounts

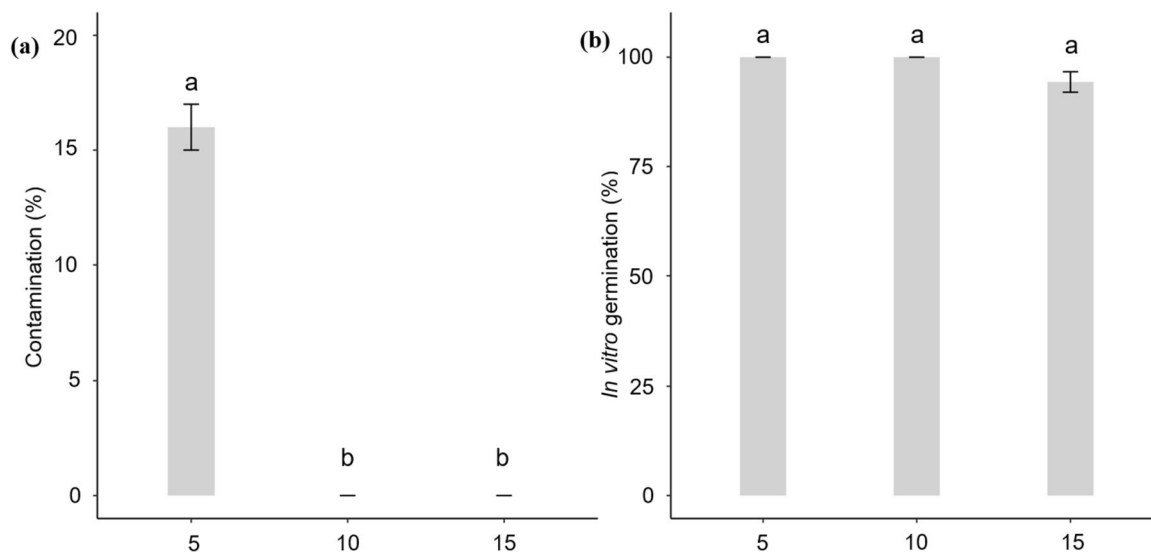


Fig. 3 Characteristics observed at 30 d of in vitro cultivation of *Palisavana sericiflora* as a function of exposure time to sodium hypochlorite (5, 10, and 15 min). **a** Contamination; **b** In vitro germination.

Means followed by the same letter do not differ significantly according to Tukey's test at the 5% significance level. Bars represent the sample standard error

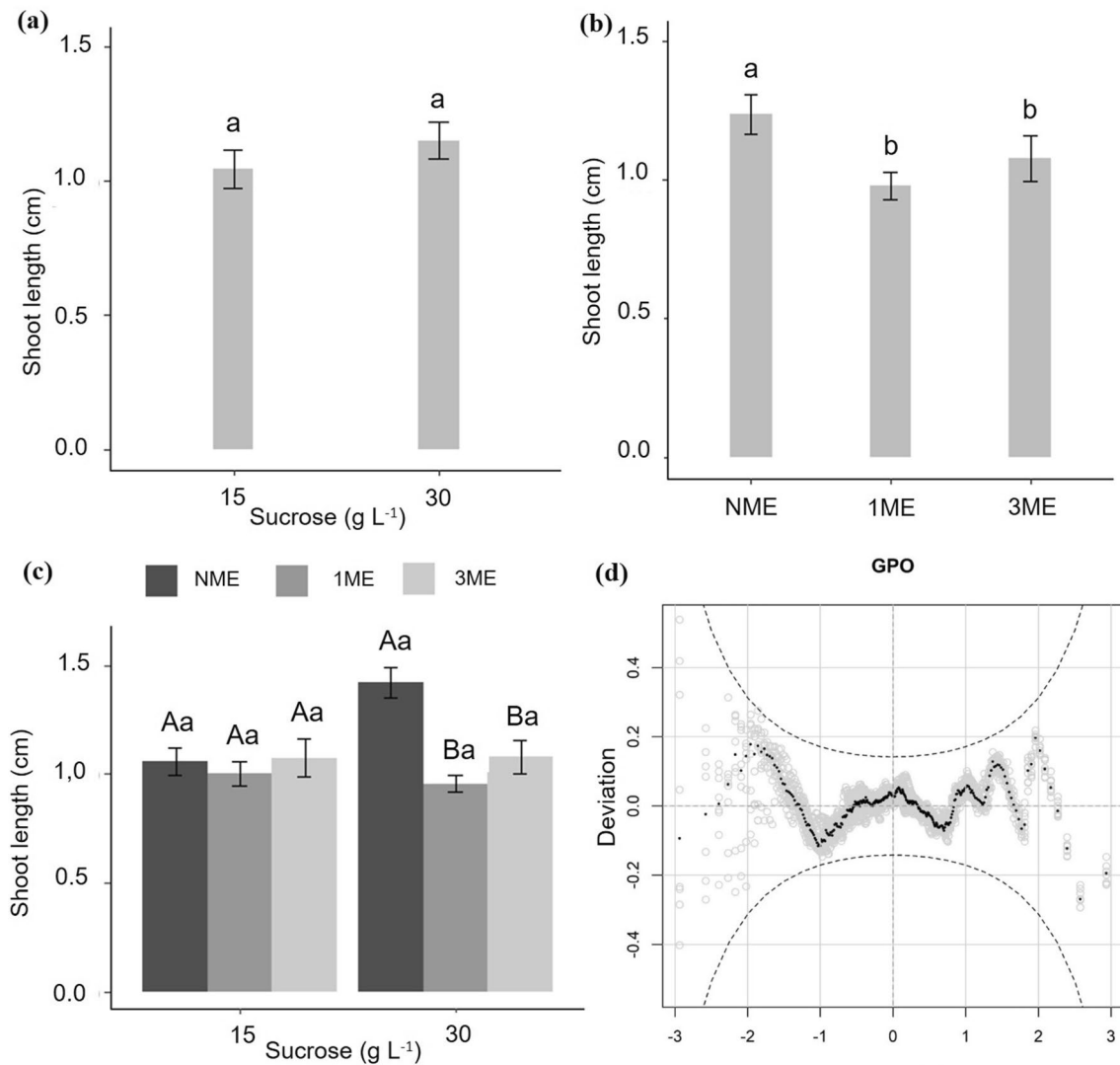


Fig. 4 Characteristics with significant differences observed at 30 d of in vitro multiplication of *Paliavana sericiflora* as a function of the number of membranes used for gas exchange (NME, 1ME, and 3ME membranes) and carbohydrate concentration (15 and 30 g L⁻¹). **a** shoot length as a function of carbohydrate concentration, **a** shoot length as a function of the number of membranes, **c** shoot length as

a function of carbohydrate concentration and number of membranes and **d** worm plot of residuals adjusted to the Poisson distribution for the variable number of shoots. Means followed by the same letter do not differ significantly according to Tukey's test at the 5% significance level. Bars represent the sample standard error

used (Fig. 4b). When observing both sucrose concentrations (Fig. 4c), the non-use of membranes for gas exchange presented the best results for shoot length.

The worm plot (Fig. 4d) shows the distribution of residuals for the variable number of shoots, the variable of interest for multiplication. The model that was fitted to the data assumed a generalised Poisson distribution (GPO), and the data points were level above the origin, resulting in residuals with very high means and underestimated locations, evidencing the non-normality of the data. The

highest mean number of shoots (5) was observed for the sucrose concentration of 30 g without the use of membranes, with some replicates reaching up to 30 shoots.

Effect of gas exchange on in vitro elongation

In the elongation stage, four variables showed significant differences. In this stage, mixotrophism (gas exchange) was tested. For the variables number of roots and shoot length (Fig. 5a, d), the treatment without the use of membranes

showed the highest values, differing from the other treatments. When observing the number of shoots and the number of elongated shoots (Fig. 5b, c), the presence of membranes (one or three membranes) favoured the responses for both variables, for leaf area, the use of three membranes produced leaves with an area greater than 2 cm² (Fig. 5e).

Effect of gas exchange on in vitro adventitious rooting

For in vitro rooting, an experiment was performed regarding mixotrophism. The treatments without membranes and with three membranes for gas exchange showed the highest mean number of roots (Fig. 6a). For shoot length and number of shoots (Fig. 6b, d), the presence of one membrane for gas exchange led to the best results, with the longest shoot reaching 3.0 cm and an average of 6 shoots. For the number of elongated shoots (Fig. 6c), there was no difference among the treatments tested. For vigour, the treatments with one and three membranes produced the best plant score of 3, corresponding to excellent vigour. However, the non-use of membranes led to plants with only good (2) and excellent (3) vigour. Lower oxidation values were also observed for the use of three membranes, with an average close to 0 and an absence of oxidation (Fig. 6e, f).

The analysis of the content of chlorophyll *a*, chlorophyll *a/b* ratio, total chlorophyll (*a + b*) and carotenoids (Fig. 7a–d, respectively) showed significant differences among the treatments tested for rooting. For all variables, the non-use of membranes or the use of three membranes did not differ from each other and presented the best means for chlorophyll and carotenoids.

Acclimatization

Seedling survival was evaluated at 30 d. of a total of 170 seedlings acclimatized, 102 survived the mini-incubator system and 68 showed fungal contamination (discarded material), totalling 60% survival of acclimatized plants. The use of the mini-incubator system was efficient for acclimatization (Fig. 8a–f) and production of *P. sericiflora* seedlings through in vitro cultivation.

Discussion

The establishment of micropropagation protocols adjusted to each species is important, especially when the species has occurrence restricted to anthropized areas of economic interest or presents some level of vulnerability to extinction.

One of the main problems faced in in vitro establishment is contamination, be it fungal or bacterial infection, often resulting from organisms endogenous to the seed, which

makes in vitro propagation difficult because these organisms can consume the seed. In some cases, protocols of immersion in sodium hypochlorite are efficient in the asepsis of the seeds, generating satisfactory results, as seen in the in vitro germination of *Raulinoa echinata* (Hoffmann et al. 2022). In this study, all treatments were efficient in disinfection without affecting seed viability. In studies with mahogany where the immersion time in hypochlorite reached 40 min, there was also no significant difference between the treatments, with adequate asepsis results (Pereira et al. 2021).

In the multiplication stage, the concentration of carbohydrates and gas exchange with the external environment can maximize shoot production or produce more vigorous shoots suitable for the next stages of in vitro cultivation (Martins et al. 2015). However, the response to this gas exchange with the environment may vary depending on the species. For *Vaccinium ashei* Reade, where the membrane system, when compared to the conventional system, favored the size and number of shoots, leaf area and chlorophyll content (Hung et al. 2016).

In the in vitro elongation stage, the responses of the aerial part length and root number variables remained similar to those of the multiplication and rooting stages. However, in the elongation stage, the number of shoots, the number of elongated and leaf area shoots showed maximized responses with the use of porous membranes. Similar responses were also reported in a study with the olive cultivar 'Arbequina', where the use of porous membranes favored gas exchange (Pinheiro et al. 2013). With emphasis on the leaf area, the use of three membranes doubled the size of the leaves, favoring the growth of in vitro plants at this stage. During the elongation phase, approximately 20 shoots were produced per cluster, in this stage, there was greater growth of these shoots, with emphasis on the use of one membrane where the elongation of the shoots may have been maximized by the higher relative humidity inside the flask since gas exchanges with the medium are reduced when compared to the use of three membranes and lower ethylene content and CO₂ maintenance when compared to non-use of membranes as well as *Corymbia maculata* (Molinari et al. 2023). This effect may have maximized the morphophysiological responses of the treatment producing larger more elongated shoots that facilitate the next stages of micropropagation.

In clonal propagation, rooting is a major challenge, whether in vitro or *ex vitro*, for several species. *P. sericiflora*, it is possible to observe the formation of roots since multiplication when the hormonal stimulus is directed exclusively to production and shoots. In the multiplication stage, the concentration of carbohydrates and gas exchange with the external environment can maximize shoot production or produce more vigorous shoots suitable for the next stages of in vitro cultivation (Martins et al. 2015). However, the response to this gas exchange with the environment may

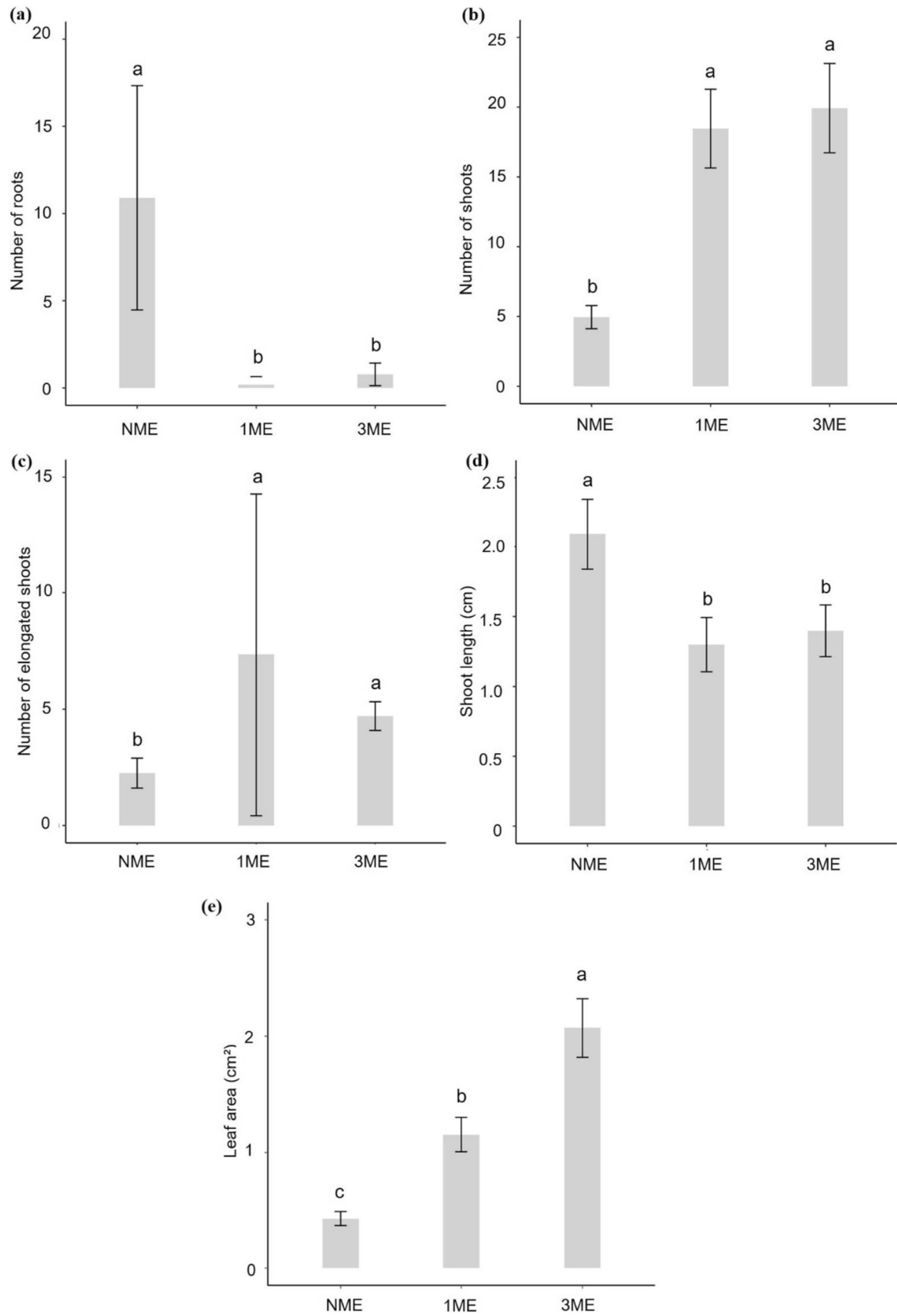


Fig. 5 Characteristics with significant differences observed at 30 d of in vitro elongation of *Paliavana sericiflora* as a function of the number of membranes used for gas exchange (NME, 1ME, and 3ME membranes). **a** number of roots; **b** number of shoots, **c** number of elongated shoots (Kruskal–Wallis test), **d** shoot length and **e** leaf area (cm²). Means followed by the same letter do not differ significantly according to Tukey’s test at the 5% significance level. Bars represent the sample standard error

vary depending on the species. For *Vaccinium ashei* Reade, where the membrane system, when compared to the conventional system, favored the size and number of shoots, leaf area and chlorophyll content (Hung et al. 2016).

At rooting, the highest means for the variables that differed significantly were observed with the presence of membranes, producing more vigorous plants with a greater ability to survive acclimatization (Moreira et al. 2013). Higher oxidation was observed in treatments using membranes, which was also observed in olive cultivars. This oxidation can be avoided by reducing the period between subcultures or by using products that minimize oxidation, such as

polyvinylpyrrolidone (PVP) (Stefanel et al. 2021). However, oxidation did not affect acclimatization.

The pigment contents during the rooting stage may have been favoured by the greater number of roots and possibly a greater amount of products to be transformed into carbohydrates for plant growth. The composition of the light spectrum, the nutrient composition, and some mineral elements can cause changes in the pigment content in leaves (Moura et al. 2023).

In the acclimatization stage, all plants already had roots, which came from the base of the cuttings and from axillary buds. This type of system was also efficient in the production of *E. benthamii* seedlings (Brondani et al. 2012), presenting good results without the use of regulators and with direct rooting in the substrate.

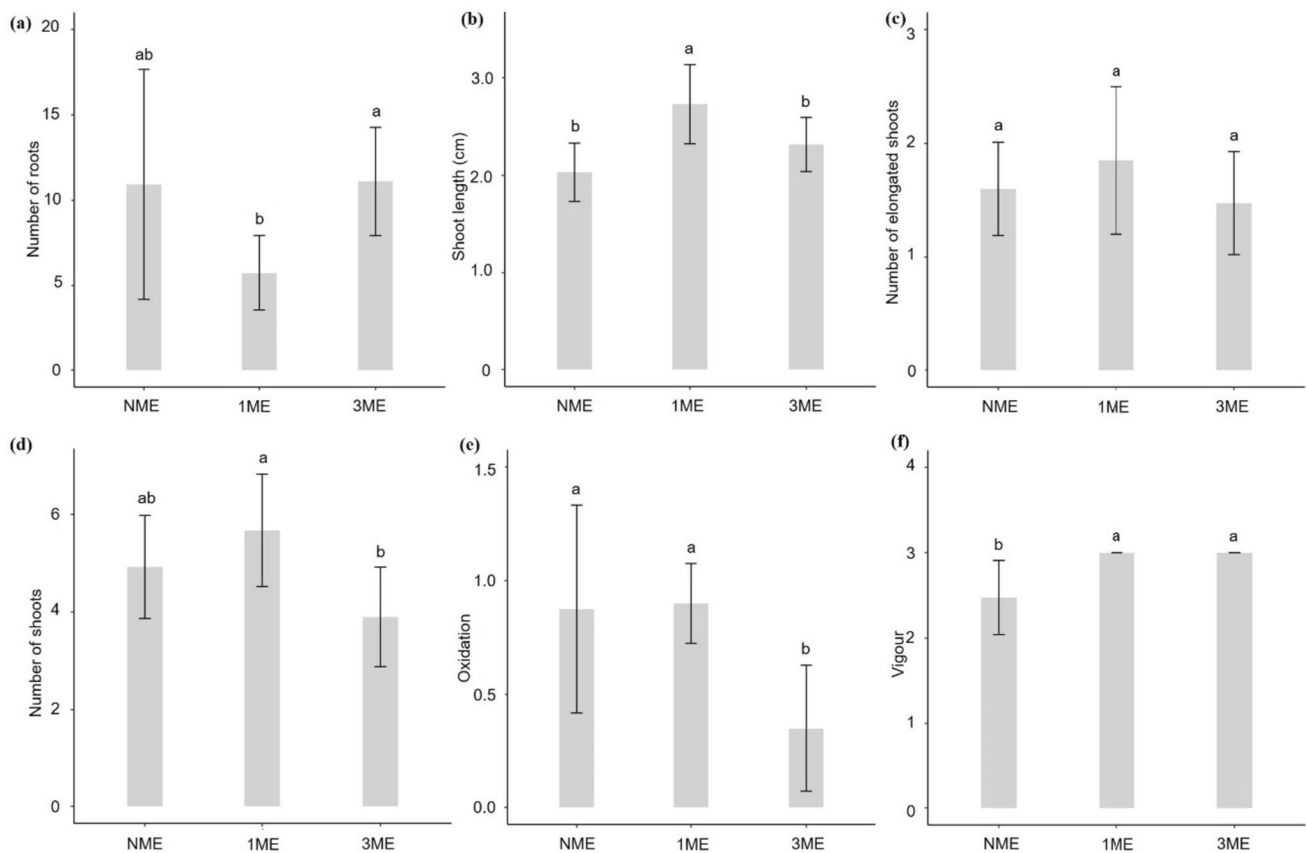


Fig. 6 Characteristics with significant differences observed at 30 d of in vitro rooting of *Paliavana sericiflora* as a function of the number of membranes used for gas exchange (NME, 1ME, and 3ME membranes). **a** Number of roots; **b** shoot length (cm) (Tukey’s test, 5%

significance), **c** number of elongated shoots, **d** number of shoots, **e** oxidation and **f** vigour. Means followed by the same letter do not differ significantly according to the Kruskal–Wallis test at the 5% significance level. Bars represent the sample standard error

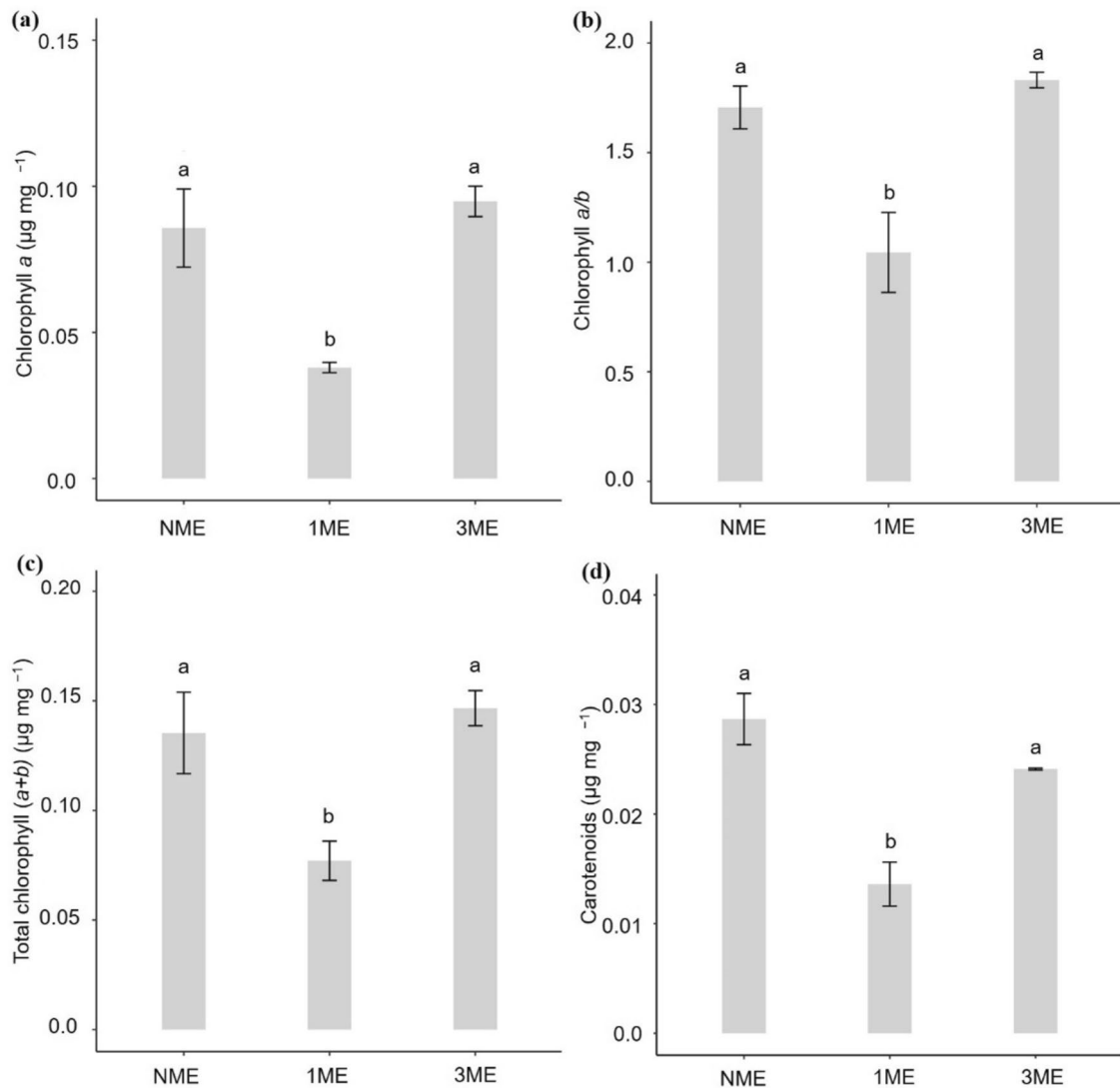


Fig. 7 Characteristics with significant differences observed at 30 d of in vitro rooting of *Paliavana sericiflora* as a function of the number of membranes used for gas exchange (NME, 1ME, and 3ME membranes). **a** chlorophyll *a*; **b** chlorophyll *a/b*, **c** total chlorophyll (*a*+*b*)

and **d** carotenoids. Means followed by the same letter do not differ significantly according to Tukey's test at the 5% significance level. Bars represent the sample standard error

Conclusion

For the in vitro establishment of a greater number of plants free of contamination, active chlorine can be used for 10 min. The multiplication of shoots established in vitro can be performed by supplementing the culture medium with 30 g of sucrose, and at this stage, the use of membranes for gas exchange with the external environment can be dispensed.

During in vitro elongation, the use of one or three membranes promotes shoot growth and the emergence of new shoots. In the adventitious rooting stage, one or three membranes could also be used, as this was the treatment where the plants were more vigorous, with 3ME having lower tissue oxidation, which may contribute to a higher survival rate in the acclimatization phase.

The mini-incubator system used in acclimatization was efficient to produce *P. sericiflora* plants and could be improved further to reduce contamination and improve *ex vitro* survival.

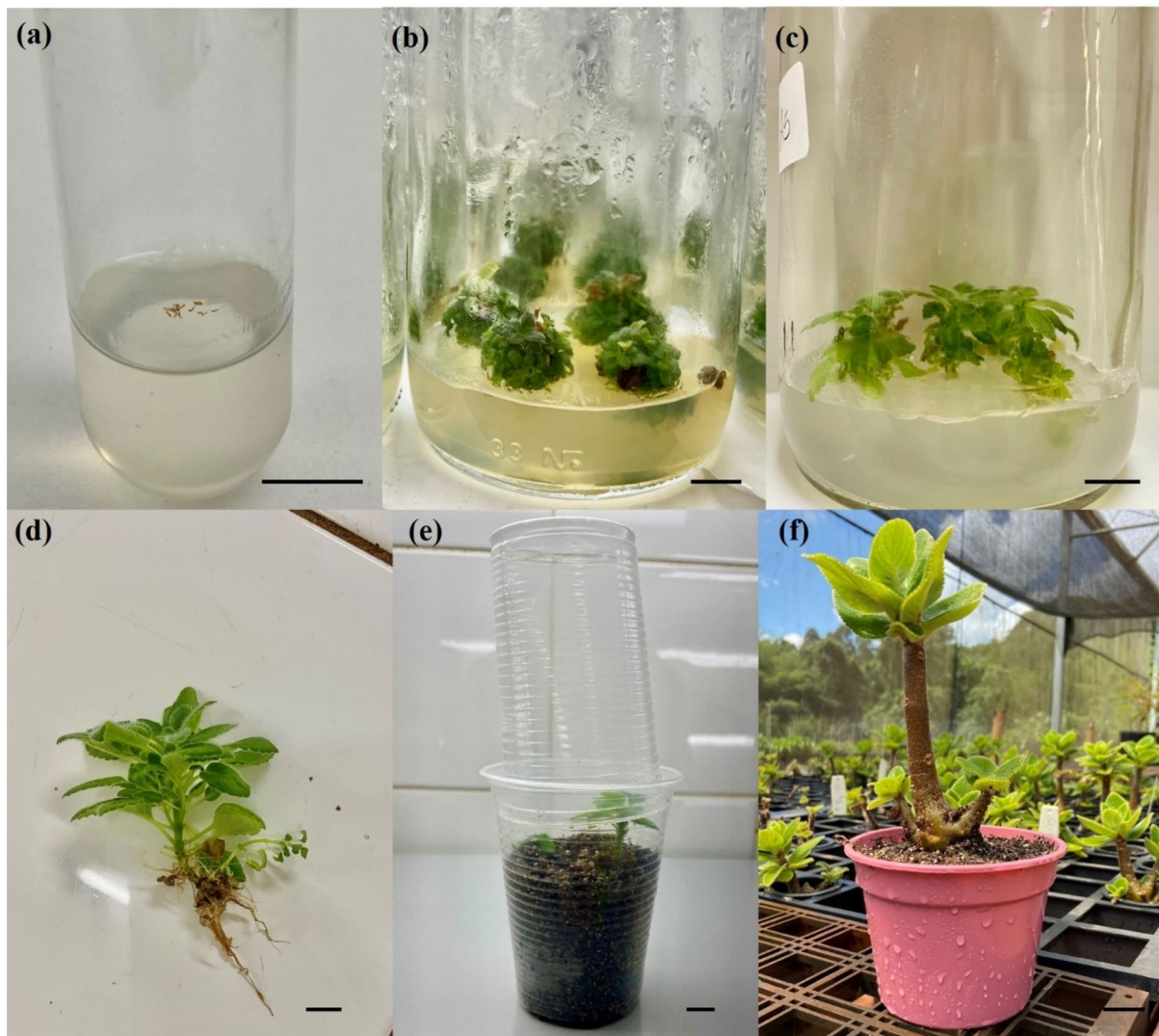


Fig. 8 Stages of in vitro seedlings production of *Paliavana sericiflora* by micropropagation technique: **a** in vitro introduction of *Paliavana* seeds, **b** multiplication of germinated plants (30 d), **c** elongation of shoots (30 d), **d** adventitious rooting of the elongated clusters (30 d),

e start of acclimatization in mini-incubator system and **f** 120 d after the beginning of acclimatization in a mini-incubator system. Bar: 1 cm

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Author contributions DML conducting the experiment, statistical analysis, writing, and discussion. SBF review, and interpretation of results. KIRS review and interpretation of results. GEB supervisor, was responsible for the obtention of funds and the administration of project, review, and discussion. All authors revised the manuscript and approved the final version.

Data Availability All data generated or analyzed during this study are included in this published article or are available from the corresponding author on a request.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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ARTIGO 2 - MICROPROPAGATION AS A CONSERVATION TOOL FOR *Paliavana sericiflora* Benth.: EVALUATING LIGHT SPECTRUM FOR EFFICIENT PLANT PRODUCTION

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Micropropagation as a Conservation Tool for *Paliavana sericiflora* Benth.: Evaluating Light Spectrum for Efficient Plant Production

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Abstract

Paliavana sericiflora Benth., a vulnerable, wind-dispersed, and seasonally-fruiting Brazilian endemic from the rupestrian fields, lacks established seedling production methods. Micropropagation offers a promising alternative for its conservation. This study evaluated the influence of light spectrum on *in vitro* multiplication, elongation, rooting and acclimatization microcontainer of *P. sericiflora*. Established shoots were subcultured in test tubes containing MS medium with growth regulators for bud multiplication under four light spectra: white, red, blue, and red/blue combination. The same spectra were used during elongation and rooting. All spectra supported bud multiplication with good vigor. Elongation and rooting were most successful under the red/blue combination or white light, yielding the highest number of roots. The microcontainer improved seedling survival and acclimatization, resulting in more vigorous plants for nursery hardening-off.

Keywords: *In vitro* culture, Light spectrum, Biodegradable container, Rupestrian field.

Introduction

In the ‘canga’ areas, also known as ‘campos rupestres’, rocky outcrops that characterize their phytophysiology can be observed. From these, a specialized ecosystem has developed and adapted to specific soil and climate conditions. The rupestrian grasslands grow herbaceous and shrubby vegetation with a high capacity to withstand extreme temperatures and to develop under a small layer of organic matter and with low fertility (Negreiros *et al.* 2009; Messias *et al.* 2012). The soil is very shallow, and the water availability is low, in addition to having high heat and light intensity. However, these rocky outcrops are of great economic interest, as many are a source of minerals, such as iron. Therefore, endemic species are under great anthropogenic pressure and are threatened with extinction (Messias *et al.* 2012).

The species *Paliavana sericiflora* Benth. occurs in a ‘canga’ area and is categorized as endangered (SiBBR 2020). It is a shrub species that occurs between rocks under the organic

matter present, with potential in landscaping and ecological restoration (Anastácio *et al.* 2018; Rossini *et al.* 2020).

The production of seedlings for many plant species can be hampered by various factors, such as low viability and number of seeds, short seed production periods, seed collection difficulties and different types of seed dormancies (Lara *et al.* 2021). Thus, one of the methods that can facilitate the production of *P. sericiflora* seedlings and overcome these problems is the use of a micropropagation technique.

Micropropagation enables the mass production of seedlings of different forest species, which facilitates endangered plant reintroduction into the ecosystem. In addition to enabling the mass production of seedlings and improving morphophysiological features, this technique also makes it possible to produce seedlings free from pathogens or diseases and to create germplasm banks for the genetic conservation of endangered or economically important plants. Conservation with micropropagation guarantees the permanence of the species genetic material in a small space, allowing seedlings to be produced regardless of seed sourcing and climatic conditions, producing seedlings with a uniform pattern (George *et al.* 2008). The production of micropropagated seedlings can be maximized or improved by refining cultivation protocols, both in terms of internal and external conditions.

Improving protocols can minimize costs and maximize seedling production. Adjustments can range from changing the source of carbohydrates and antioxidant components and nutritional adjustments to altering cultivation conditions, such as by using different light spectra. This can promote morphogenic responses at different stages of cultivation (Souza *et al.* 2022; Hashim *et al.* 2021).

The light spectrum can influence growth and development *in vitro*, and it is important to determine the best spectrum to promote physiological activities. Excessive light can also be harmful to plants, as it can damage plant structures such as leaves and flowers. Therefore, it is important to provide the right light spectrum for plants to optimize the different stages of *in vitro* cultivation (Oliveira *et al.* 2021; Souza *et al.* 2022).

Light is a fundamental factor in the micropropagation of plant species. The light spectrum and intensity used in cultivation provide different responses in terms of growth and development (Frade *et al.* 2023). White fluorescent lamps are commonly used on *in vitro* culture, whereas LED lamps are a promising alternative because they are more efficient in terms of energy consumption and induce desired responses in plant tissue growth (Erig and Schuch 2005; Pasa *et al.* 2012). For the white, blue, and red-light spectra, the responses observed have been found to differ depending on the stage and species being studied, and the specificity of the response can help optimize *in vitro* cultivation.

Considering the need for studies to improve the protocol for producing seedlings of endangered species and to optimize the production of micropropagated seedlings, the aim of this work was to evaluate the effect of the light spectrum on the multiplication, elongation, and *in vitro* rooting stages of *Paliavana sericiflora*.

Materials and methods

Research site and plant material

Plant material was provided by GERDAU Açominas S.A., Ouro Branco, MG, Brazil. Shoots used in the experiments made up the established *in vitro* plant material bank (Leite *et al.* 2024; Fig. 1; code ACF88E2 in SisGen).

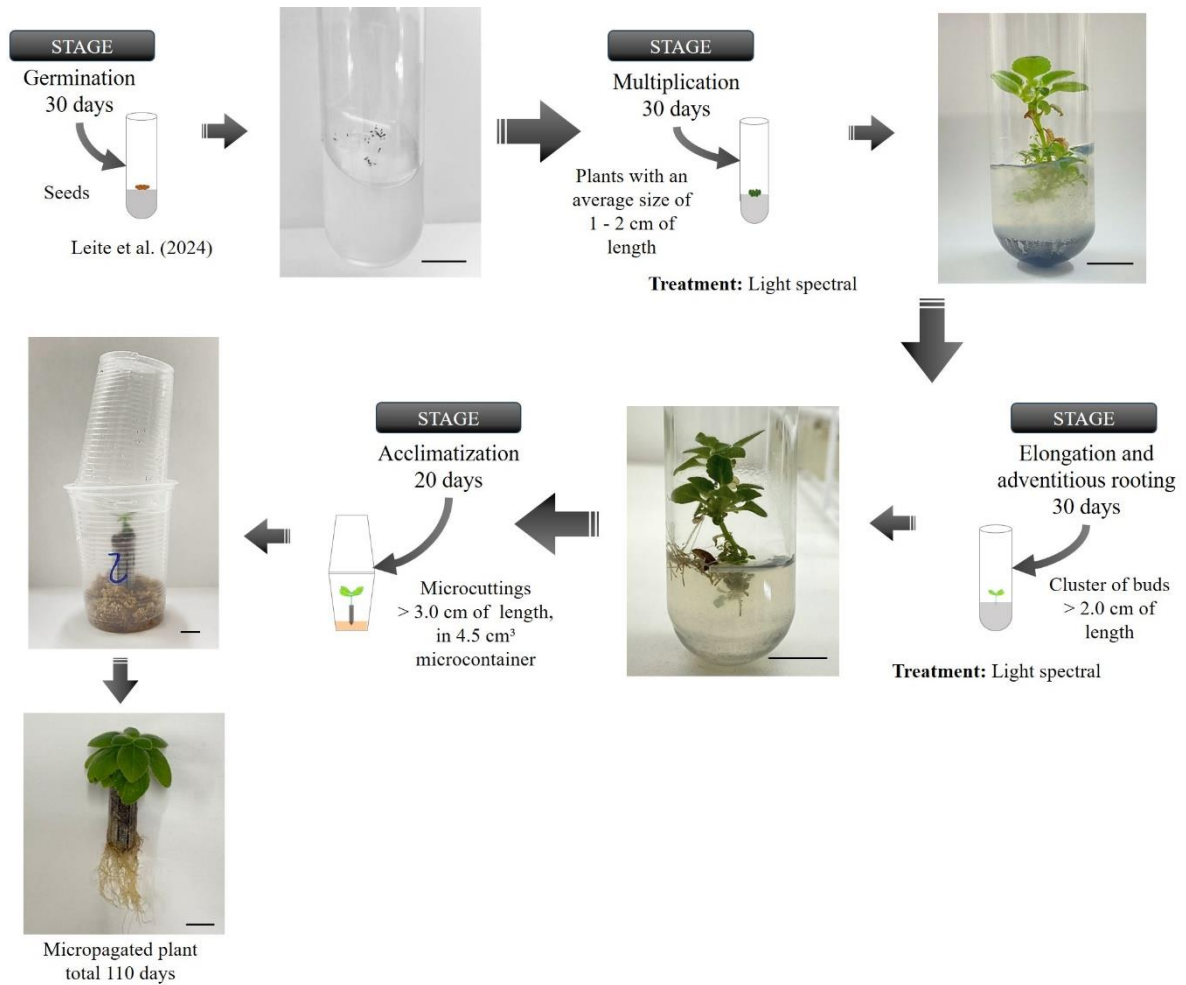


Figure 1. Schematic representation of the stages performed in the *in vitro* culture of *Paliavana sericiflora*. Bar: 1 cm. National Genetic Heritage Management System (SisGen): ACF88E2.

Effect of the light spectrum on *in vitro* multiplication

After asepsis and germination of the seeds, the explants were isolated and standardized to a size of 1-2 cm only aerial part (Leite *et al.* 2024). These explants were then inoculated into test tubes (25 x 150 mm) containing 10 mL of basal MS medium (Murashige and Skoog, 1962) supplemented with 0.4 g L⁻¹ activated charcoal, 30 g L⁻¹ sucrose, 0.50 mg L⁻¹ 6-benzylaminopurine (BAP, Sigma®), 0.05 mg L⁻¹ α -naphthaleneacetic acid (NAA, Sigma®), and 6 g L⁻¹ agar. The medium pH was adjusted to 5.8 ± 0.05 prior to agar addition. Sterilization was achieved by autoclaving the medium at 121 °C and 1.0 kgf cm⁻² for 20 min.

After inoculation, the explants were kept in a growth room for 30 days at a temperature of 24 ± 1 °C and a photoperiod of 16 hours, evaluated in 30 days. Four light spectra were tested according to the treatments.

A completely randomized design with 20 replicates per treatment was employed. Following the study period, evaluations were conducted for: vigor (Leite *et al.* 2024), number of buds, number of leaves, number of roots, shoot length (cm), and photosynthetic pigments content [chlorophyll *a*, *b*, *a/b*, total (*a+b*), and carotenoids].

Effect of light spectrum on *in vitro* elongation and rooting

During the multiplication stage, shoots exceeding 2 cm in height and free of visible nutrient deficiencies were isolated to form bud clusters. These shoots were then subcultured into test tubes (25 x 150 mm) containing 10 mL of basal MS medium (Murashige and Skoog, 1962) supplemented with 0.4 g L⁻¹ activated charcoal, 30 g L⁻¹ sucrose, 0.05 mg L⁻¹ BAP, 0.1 mg L⁻¹

each of NAA and indole-3-butyric acid (IBA, Sigma®), and 6 g L⁻¹ agar. The conditions for preparing the cultivation medium were the same as the item effect of the light spectrum on *in vitro* multiplication.

After inoculation, the explants were kept in a growth room for 30 days at a temperature of 24 ± 1 °C and a 16-hour photoperiod. Four light spectra were tested according to the treatments: white LED, red LED, blue LED and red and blue LED lamp 1:1 combination (purple).

A completely randomized design with 20 replicates per treatment was employed. Following the study period, the following parameters were assessed: vigor (Leite *et al.* 2024), number of shoots, number of leaves, number of roots, shoot length (cm), photosynthetic pigment content (chlorophyll *a*, *b*, *a/b*, total (*a+b*), and carotenoids), stomatal density (µm²), and leaf area (cm²).

Acclimatization and obtaining micropropagated seedlings

Following *in vitro* development, complete plantlets with shoots and roots were transferred to 4.5 cm³ microcontainer (patent application BR 10 2023 007189 9, Leite *et al.* 2024) placed within polypropylene cups in a mini-incubator system (Brondani *et al.* 2018). The substrate for acclimatization comprised a mixture of decomposed pine bark and vermiculite, 60 plants being acclimatized. Plants were cultivated under a 16-hour photoperiod with a light intensity of 40 µmol m⁻² s⁻¹ for 20 days to evaluate survival.

Light spectrum range

The explants were kept in a growth room and four light spectra with different intensities were tested: white LED lamp/37 µmol m⁻² s⁻¹, red LED lamp/9 µmol m⁻² s⁻¹, blue LED lamp/14 µmol m⁻² s⁻¹ and, red and blue LED lamp 1:1 combination (purple) / 14 µmol m⁻² s⁻¹. The spectral quality (Fig. 2) was obtained by normalizing the intensity via a USB-650 RED TIDE radiometer spectrum (Ocean Optics Inc.)

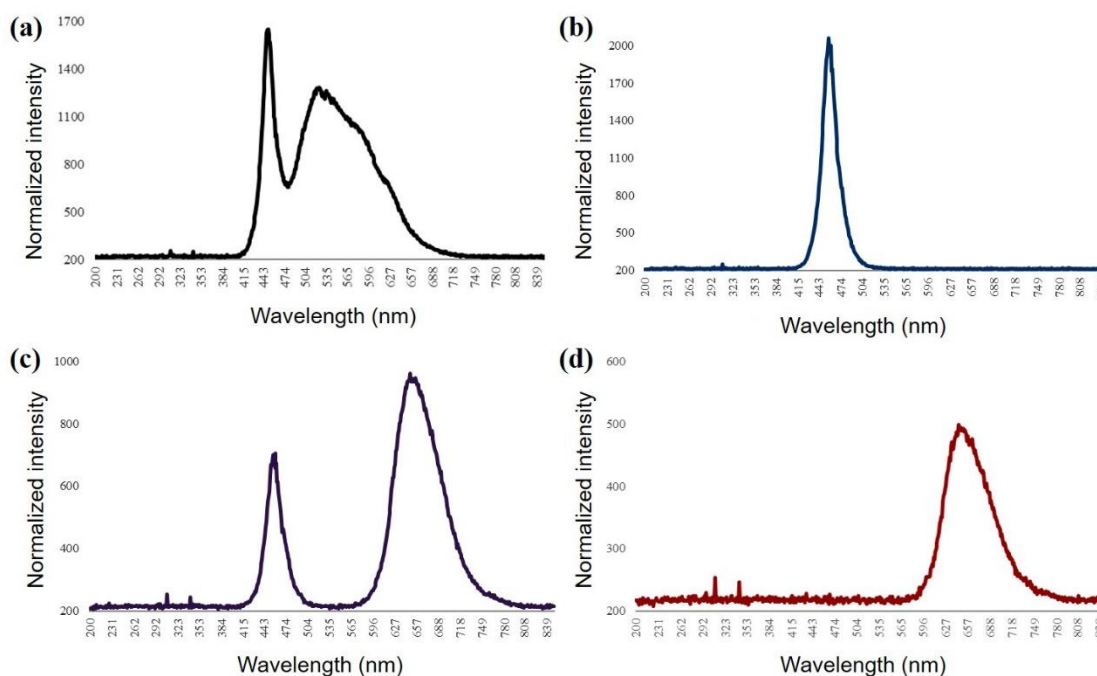


Figure 2. Graphs of the normalized intensity as a function of the wavelength (nm) of the light emitted during the *in vitro* culture of *Paliavana sericiflora*. (a) White LED lamp, (b) blue LED lamp, (c) red and blue LED lamp 1:1 combination (purple) and (d) red LED lamp.

Leaf area, photosynthetic pigments and stomatal density

Leaf area was determined using ImageJ *software*, with an HP Deskjet Ink Advantage 1516 scanner. Twenty leaves were assessed per treatment, collected from the 3rd and 4th paired leaves, from the apex towards the base.

The methodology adopted was in accordance with Gonçalves *et al.* (2024).

Stomata were analysed under a microscope, using the middle third, without the main vein, of the first three fully expanded leaves from the apex to the base of the explants. Each leaf or fragment was dehydrated using 70% alcohol and then stored on slides.

To make the slides, the material was washed in distilled water, and then the abaxial side of the leaf was lightly scraped, and digested with 0.5% sodium hypochlorite for 12 hours. Then, the leaves were immersed in safranin, and washed to remove excess dye, and 1:1 glycerine water was used to make the slides. The slides were analysed and photographed using a light microscope (ZEISS-JENEMED2®), with all images captured at the same scale using a Premiere® MA88-300 camera.

To determine the stomatal density (DES) of the leaves, three fields with a leaf area of 10.28 μm^2 were randomly photographed, within which the number of stomata was counted and the number of stomata per 1 μm^2 was then calculated.

Statistical analysis

Normality of residuals and homoscedasticity of data were assessed using the Shapiro-Wilk ($P > 0.05$) and Hartley tests ($P > 0.05$), respectively. Subsequently, one-way analysis of variance (ANOVA) was performed ($P < 0.05$) to compare treatment means, followed by Tukey's test ($P < 0.05$) for multiple comparisons. Principal component analysis (PCA) was employed to explore data patterns. The R software (version 4.0.3, 2020) with packages ExpDes.pt (Ferreira *et al.* 2021; Arnhold 2013), Factorextra, and FactoMineR (Kassambara and Mund 2020) was used for statistical analyses.

Results

Effect of light spectrum on *in vitro* multiplication

Only four variables significantly differed between the spectra tested. Similarly, vigour (Fig. 3a) and shoot length (Fig. 3c) showed similar results, with the red spectrum showing higher averages. For vigour, the higher the average was, the lower the vigour of the shoots. The red spectrum had an average close to two, where it was possible to see the induction of shoots but with reduced leaf growth.

Plants grown under red light exhibited slight etiolation and longer shoots compared to other treatments (Fig. 3b). However, this light spectrum also resulted in a lower number of roots. White and blue light spectra yielded the highest number of roots, although not different from the red and blue combination (Fig. 3b). The chlorophyll *a/b* ratio (Fig. 3d) was significantly higher under white, red, and blue light compared to other treatments. No significant differences were observed in the number of shoots per explant across all light spectra, with an average of approximately three shoots per explant.

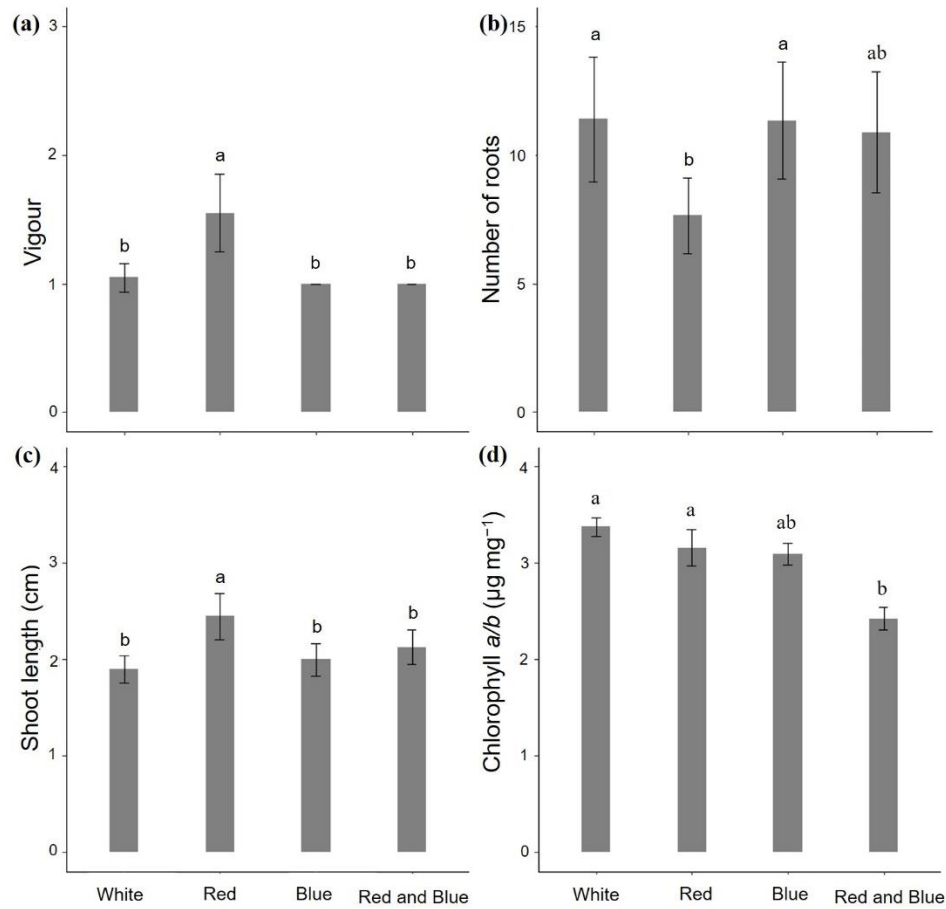


Figure 3. Features observed at 30 days of *in vitro* multiplication of *Paliavana sericiflora* regarding the spectrum used (white, red, blue, and, red and blue combination, 1:1): (a) vigour, (b) number of roots, (c) Shoot length (cm) and (d) chlorophyll *a/b*. Averages followed by the same letter do not differ significantly according to Tukey's test. Bars represent the standard error of the sample.

Principal component analysis (PCA) revealed the strongest correlations between red light treatments and the variables for vigor and shoot length (Fig. 4). White and blue light spectra were positioned near the variables associated with photosynthetic pigments, while the red and blue combination spectrum aligned closely with the number of leaves.

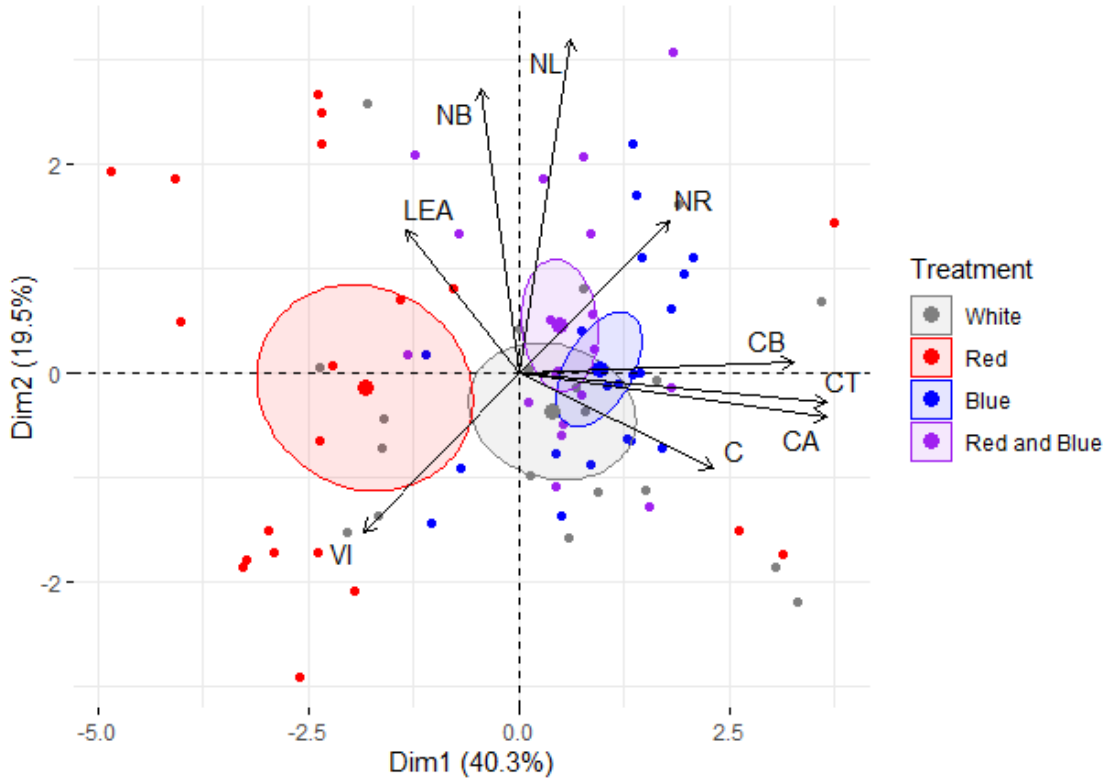


Figure 4. Principal component analysis (PCA) of *Paliavana sericiflora* explants at the *in vitro* multiplication stage after 30 days of culture. Vigour (VI), number of roots (NR), number of buds (NB), number of leaves (NL), length of the aerial part (LEA), total chlorophyll *a+b* (CT), chlorophyll *a* (CA), chlorophyll *b* (CB) and carotenoids (C). Dim1 = principal component 1 (PC1), Dim2 = principal component 2 (PC2).

During multiplication, the white and blue spectral bands (Figs. 5a and 5c) produced the greatest number of shoots suitable for the next stages of cultivation, with underdevelopment and etiolation observed in the red spectral band (Fig. 5b) and little bud formation in the red and blue bands (Fig. 5d).

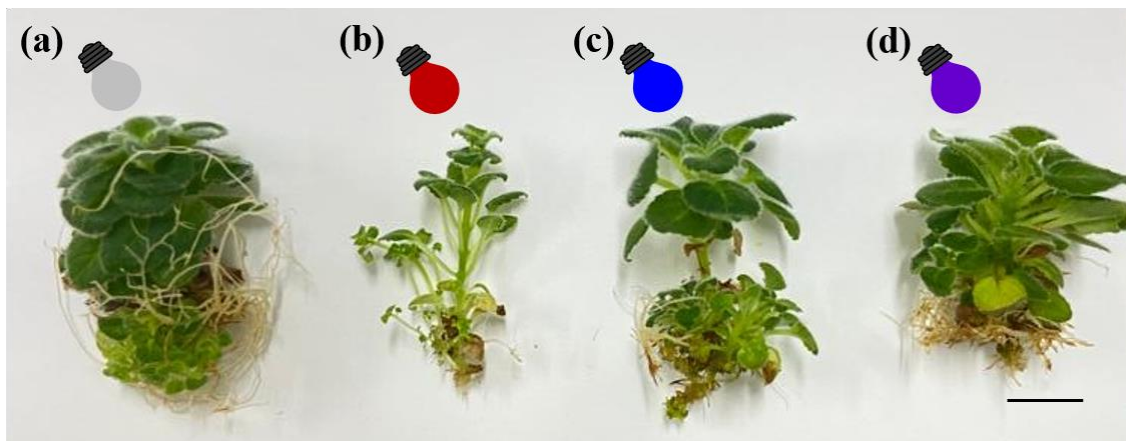


Figure 5. Features observed at 30 days of *in vitro* multiplication of *Paliavana sericiflora* regarding the spectrum band used a: white, b: red, c: blue and d: red and blue combination (1:1). Bar: 1 cm.

Effect of light spectrum on *in vitro* elongation and adventitious rooting

Only four variables showed significant differences among the light spectra used. Shoot length (Fig. 6a) showed opposite results to leaf area (Fig. 6d), where the tallest plants had the smallest leaf area. The red spectrum was responsible for producing the largest plants, reaching 3 cm of the aerial part, however with a leaf area of less than 0.25 mm². Similar to the leaf area,

the stomatal density was directly correlated, where the largest leaf areas had a greater number of stomata per area (Fig. 6e).

The red and blue combinations and the white and blue spectra were the ones that produced plants with the largest leaf area, which reached 0.65 mm² when the white one was used. For chlorophyll *a* and carotenoids (Figs. 6b and 6c), the blue spectrum had the highest content of these photosynthesizing pigments.

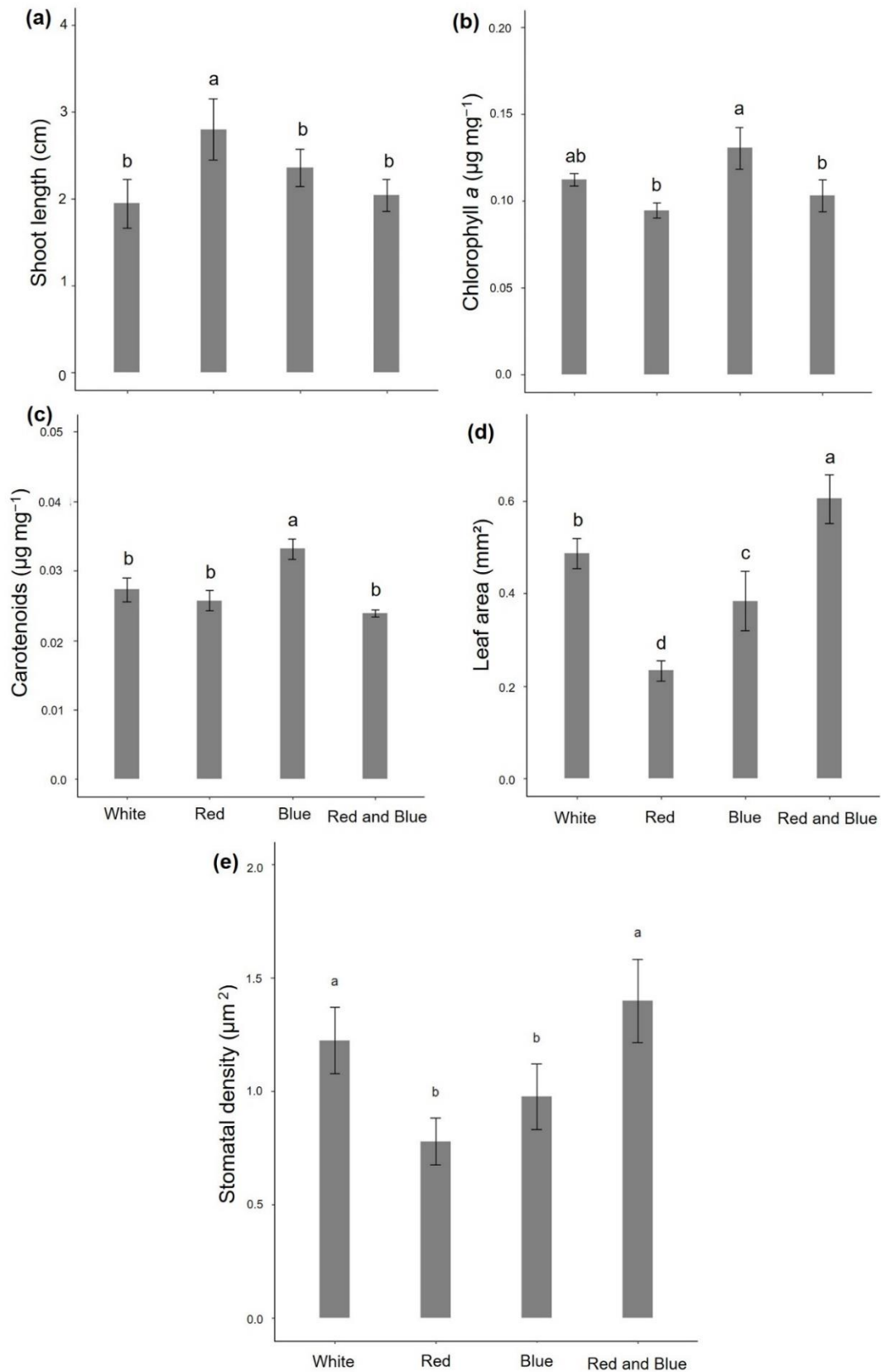


Figure 6. Features observed at 30 days of *in vitro* elongation and rooting of *Paliavana sericiflora* regarding the spectrum band used (white, red, blue, and red and blue combination, 1:1). (a) Shoot length (cm), (b) chlorophyll a content ($\mu\text{g mg}^{-1}$), (c) carotenoid content ($\mu\text{g mg}^{-1}$), (d) leaf area (mm^2) and (d) stomatal density (μm^{-2}). Means followed by the same letter do not differ significantly according to Tukey's test. Bars represent the standard error of the sample.

The PCA showed a strong correlation between the red spectrum and the elongation of shoots and between the blue spectrum and both carotenoids and plant vigour. The white, red and blue combination spectra were the most responsive when the leaf area, stomatal density, and number of leaves and roots were considered (Fig. 7).

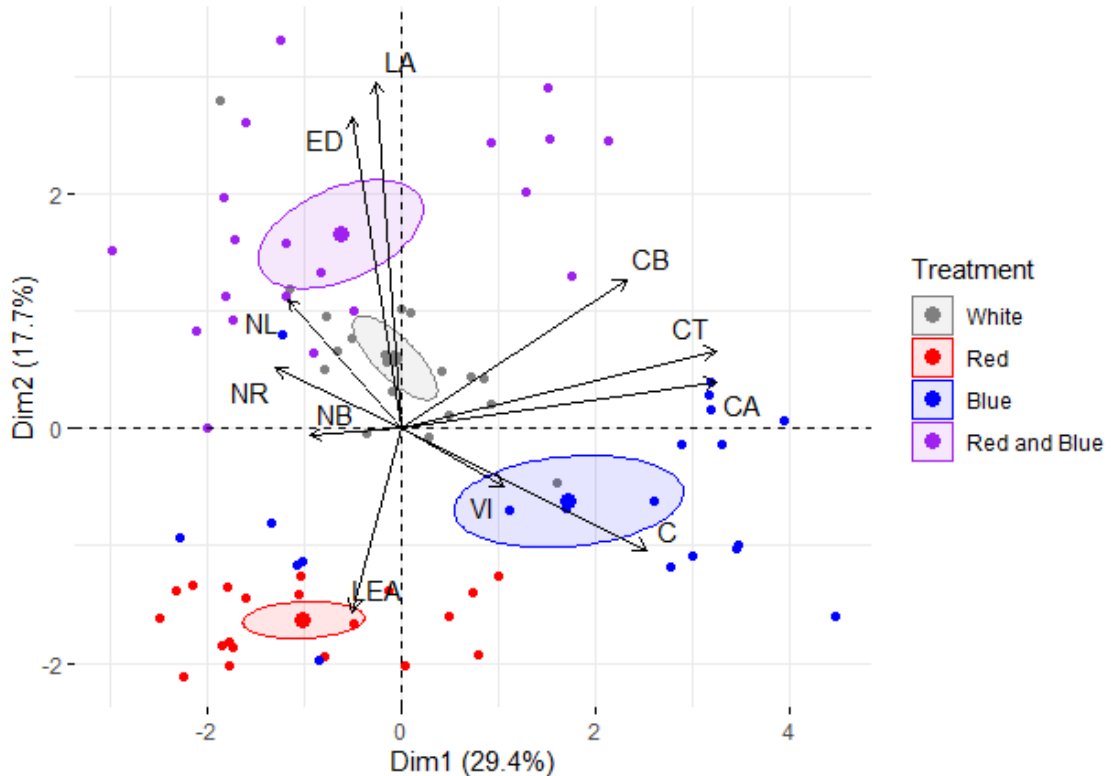


Figure 7. Principal component analysis (PCA) of *Paliavana sericiflora* explants at the *in vitro* elongation and rooting stages after 30 days of culture. Vigour (VI), number of roots (NR), number of buds (NB), number of leaves (NL), length of the aerial part (LEA), leaf area (LA), stomatal density (ED), total chlorophyll *a+b* (CT), chlorophyll *a* (CA), chlorophyll *b* (CB) and carotenoids (C). Dim1 = principal component 1 (PC1), Dim2 = principal component 2 (PC2).

In terms of elongation/rooting, the white and red and blue spectral bands (Figs. 8a and 8d) produced the greatest number of roots, followed by the blue band (Fig. 8c) and, finally, the red band (Fig. 8b), which produced the most elongated plants with the fewest roots but were successfully acclimatized.

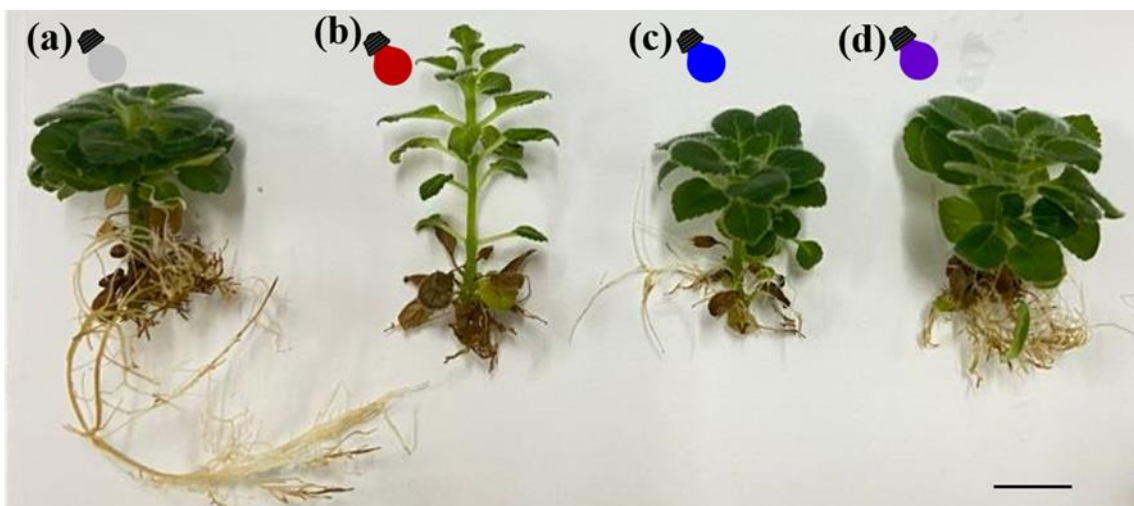


Figure 8. Features observed at 30 days of *in vitro* elongation and rooting of *Paliavana sericiflora* regarding the

spectrum band used a: white, b: red, c: blue and d: red and blue combination (1:1). Bar: 1 cm.

In the elongation and rooting stages, all spectral bands promoted the formation of complete plants suitable for acclimatization. Stomata were observed only on the abaxial side of the leaves up to the leaf edge (Fig. 9a). On the leaves of plants in all treatments, numerous trichomes were observed on both sides of the leaf and all over the limb (Fig. 9b), at the end of acclimatization, which reinforces fidelity between the clones produced.

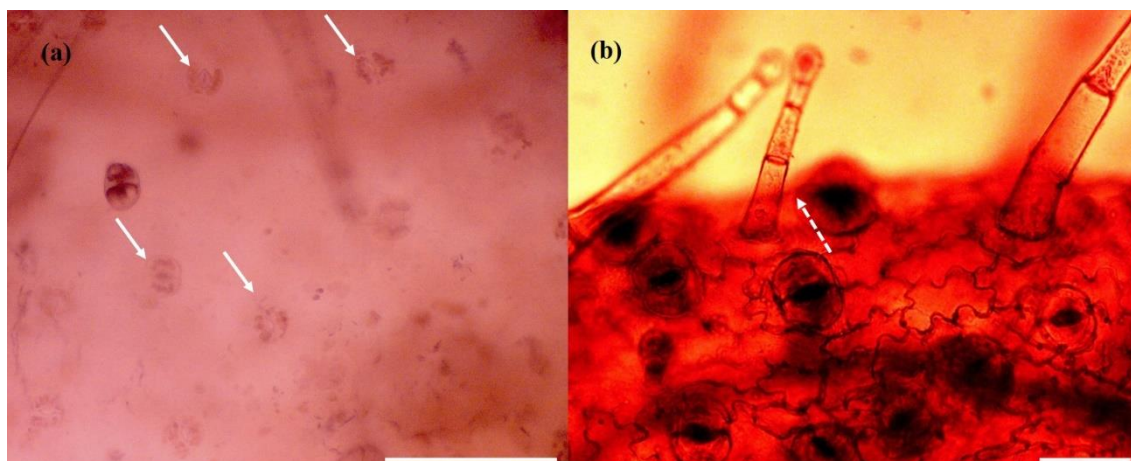


Figure 9. Characteristics of *Paliavana sericiflora* at 30 days of *in vitro* elongation and rooting under white light. (a) Stomata visualized in the middle third of the second fully expanded leaf, with continuous arrows highlighting the stomata. (b) trichomes found on the leaf edge, with the serrated arrow highlighting the trichome. Bar: (a) 1 μm and (b) 5 μm .

All bands of the light spectrum generated complete plants, with stem, leaf and root, suitable for acclimatization. Acclimatization in a biodegradable microcontainer in the mini-incubator system was effective, with 100% survival of the transplanted plants from adventitious rooting.

Discussion

Improving micropropagation protocols plays a crucial role since they can reduce cultivation time and maximize gains or products of interest. Among the points that can be adjusted, the quality of light presents various responses. The replacement of fluorescent lamps with LED lamps has been used mainly to stimulate the production of secondary metabolites, which are important in plant cultivation processes (Hashim *et al.* 2021).

In vitro multiplication aims to rapidly increase the number of shoots while maintaining optimal vigor. While test tubes facilitated the production of vigorous plantlets, the number of new shoots generated within the evaluated period was lower compared to studies using larger container volumes. An average of four shoots were obtained using 250 mL flasks containing 40 mL of medium (Leite *et al.* 2024). However, this method offers a potential alternative for mass production of shoots for subsequent cultivation stages, as it allows for the growth of multiple bud clusters per flask.

The white, blue, and red and blue combination spectral bands promoted the number of roots when the number of shoots increased. However, since energy was spent on root formation, the growth of the aerial part of the plant decreased, with red producing the largest plants. The prominence of red over white and blue was also observed in mulberry studies (Pasa *et al.* 2012), where the shoots in multiplication grew more when grown in red light or in the dark for the black mulberry cultivar (*Rupus* sp.) Xavante variety.

Light spectra act directly on the content of photosynthesizing pigments. In some cases, this effect is only observed when the proportion of some of these pigments is evaluated, such as the

ratio between chlorophyll *a* and chlorophyll *b*, which was greater for white, red, and blue pigments. In many cases, chlorophyll *a* is always two or three times greater than chlorophyll *b*, regardless of the range, and the difference between treatments will be low (Gonçalves *et al.* 2023; Souza *et al.* 2022).

Many plants grow *in vitro* and need to progress through all stages, depending on their features. In some cases, during the multiplication and elongation stages, it is possible to see the presence of roots. As seen by Leite *et al.* (2024), for the same species under study, it is possible to see that root production is facilitated. In this way, the elongation and rooting stages can occur simultaneously with a few adjustments to the growing medium, thus reducing the growth time.

In elongation/rooting, only the variables shoot length, chlorophyll *a* content, carotenoid content and leaf area showed significant differences between the spectrum bands tested. Shoot length is shown in red. In studies on *Myrtus communis* L., red light also stimulated the growth of shoots in the medium, and the authors highlighted that this occurred independently of the cytokinin content in the medium culture (Cioć *et al.* 2018). Some studies have reported that red light promotes shoot elongation on *Dendranthema grandiflorum* (Braga *et al.* 2009). The species under study can also increase the production of phenolics, flavonoids, antioxidants, and other compounds (Hashim *et al.* 2021; Younas *et al.* 2018).

The highest levels of chlorophyll *a* and carotenoids were detected in the blue spectral range. Similar results were found for *Eucalyptus dunnii* Maiden and the hybrid *Eucalyptus urophylla* (Frade *et al.* 2023), where blue light produced higher levels of these pigments, followed by yellow light. Blue light can stimulate the production of other secondary metabolites, some of which are even pharmacologically active, as in studies with cherry tomato seedlings grown in a growth chamber (Kim *et al.* 2014).

Various factors influence leaf area, which can increase or decrease in width and length, depending on the environment and nutritional conditions. In the literature, there are reports of individuals with reduced leaf area when exposed to high irradiance, as well as increased leaf area when exposed to low irradiance (Lavanhole *et al.* 2018). This can lead to differences in the content of photosynthesizing pigments present in each of them, or low intensities can reduce the production of photoassimilates (George *et al.* 2008). However, this study did not reveal any significant differences in total chlorophyll content. The combination of red and blue light was one of the least effective at producing these pigments, as it resulted in twice the leaf area of the red treatment with the same amount of pigments. Stomatal density did not differ between the light spectra studied, which allows us to infer that there was no somaclonal variation.

The use of microcontainer increased survival during acclimatization, superior to data seen in previous studies (Leite *et al.* 2024). The protocol used made it possible to optimize the *in vitro* culture of the species, reducing cultivation days and accelerating the process of producing seedlings, which is extremely important for the species, which is vulnerable to extinction.

In conclusion, light spectrum had no significant effect on bud multiplication during the *in vitro* culture. Elongation and rooting stages could be performed concurrently. The red and blue light combination and white light spectrum yielded the best results for plant growth and vigor during these combined stages, promoting the development of more vigorous plantlets for acclimatization. Acclimatization was efficient with complete plants, achieving a 100% survival rate. Approximately 110 days are required to produce seedlings ready for hardening off.

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**ARTIGO 3 - EFFECT OF ZnO-NPs-NPS IN MICROCONTAINER ON THE
ACCLIMATIZATION AND DEVELOPMENT OF MICROPROPAGATED
SEEDLINGS OF *Paliavana sericiflora* Benth.**

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EFFECT OF ZnO-NPs-NPS IN MICROCONTAINER ON THE ACLIMATIZATION AND DEVELOPMENT OF MICROPROPAGATED SEEDLINGS OF *Paliavana sericiflora* Benth.¹⁶

Abstract

This study investigated the effect of incorporating zinc oxide nanoparticles (ZnO-NPs-NPs) on the acclimatization and growth of micropropagated seedlings of *Paliavana sericiflora* Benth. The seedlings were grown in 4.5 cm³ microcontainer in polypropylene cups using a mini-tubing system. The experimental factor was the concentration of ZnO-NPs-NPs added to the polymer in the tubes (PLA), with the following levels: 0, 0.025, 0.05, 0.075, 0.1 and 1 g kg⁻¹. After the acclimatization period, the plants were transplanted into 250 cm³ tubes. The results indicated that the ideal concentration of ZnO-NPs-NPs was 0.1 g kg⁻¹, promoting greater vigor in the plants, greater accumulation of dry matter and superior development in terms of height, diameter and leaf area. This concentration resulted in 100% seedling survival. In contrast, the concentration of 1 g kg⁻¹ of ZnO-NPs-NPs had a phytotoxic effect, highlighting the need for more in-depth studies into the relationship between the concentration of ZnO-NPs-NPs and the specific characteristics of each plant species. The high correlation between vegetative variables and the ideal concentration of ZnO-NPs-NPs suggests that incorporating ZnO-NPs-NPs into microcontainer could be a promising strategy for optimizing the acclimatization and growth of micropropagated seedlings of *Paliavana sericiflora* and other plant species.

Keywords: Zinc oxide, Rupestrian field, Vulnerable to extinction, *In vitro* culture, Biodegradable microcontainer.

Introduction

In vitro cultivation, also known as micropropagation, offers a wide range of benefits for various sectors. This technique makes it possible to obtain plants with high genetic and health quality on a large scale, boosting crop productivity and profitability. In addition, micropropagation is an important tool for the preservation of endangered species, enabling their recovery and reintroduction into the natural environment. Currently, India stands out as the main producer and exporter of micropropagated plants in general, with an estimated annual production of 5 million units (Shukla and Shukla, 2024).

Micropropagation consists of the multiplication of plants from explants of organs, tissues or cells, cultivated *in vitro* under aseptic conditions in suitable culture media, with strict control of environmental and nutritional variables (George *et al.* 2008; Thorpe, 2007). This tissue culture technique stands out due to its many applications in the forestry sector (Alfenas *et al.* 2004), above all by minimizing genetic variability in relation to the original material (Nehra *et al.* 2005), inducing rejuvenation of plant tissue and increased physiological vigour (Dutra *et al.* 2009), and the possibility of creating germplasm banks (Leite *et al.* 2024).

One of the main challenges of growing plants *in vitro* is the high mortality rate during the acclimatization phase. This phenomenon stems from the inadequate control of transpiration, since, *in general*, plants grown *in vitro* do not have functional stomata, which are responsible for regulating water loss to the external environment (Manokari *et al.* 2021; Miranda *et al.* 2024). To minimize transpiration and ensure effective acclimatization, a commonly used

¹⁶ O artigo será submetido na revista *3 Biotech*.

technique is the mini-stem system (Brondani *et al.* 2018). This approach optimizes the survival of plants in the *ex vitro* environment.

The way the nutrients are made available and the period of application can significantly influence future results. In this context, nanofertilizers enable the efficient delivery of nutrients and optimizing their use by crops (Jakhar *et al.* 2022; Haydar *et al.* 2024). The application of fertilizers not only helps with *ex vitro* rooting and the consequent survival of the plants, but also contributes to enhancing their physiological vigor. Several studies have already explored the use of nanofertilizers in different plant species, and many of them are obtained through green synthesis (Haydar *et al.* 2024), featuring slow and controlled release mechanisms, which favor the gradual absorption of nutrients by plants.

Among the essential nutrients for plants, zinc (Zn) stands out as a micronutrient of crucial importance, although its availability in tropical soils is often limited. Its high mobility within the plant organism enables action in various redox reactions (Araujo *et al.* 2016). Zinc plays a fundamental role in electron transfer and energy transformation, and may even be involved in chlorophyll synthesis in some plant species (Taiz *et al.* 2017). In addition, this micronutrient is essential for the synthesis of tryptophan, a precursor of auxins, plant hormones that regulate plant growth and development (Brondani *et al.* 2012).

Adequate zinc supply can therefore encourage new shoots and stimulate root growth, optimizing seedling development. On the other hand, a deficiency of this element can cause disturbances in auxin production, leading to inhibition of stem elongation and a reduction in leaf size (Natarelli *et al.* 2021). Given the importance of zinc for plant growth, the incorporation of nanoparticles containing this micronutrient into acclimatization is a promising alternative for boosting the production of high-quality clonal seedlings.

Zinc (Zn) can be made available in the form of zinc oxide nanoparticles (ZnO-NPs-NPs), with diameters between 1 and 100 nanometers (Rai, 2013). An recent strategy for delivering ZnO-NPs-NPs to plants consists of directly incorporating these nanoparticles into the polymer of the growing container, such as polylactic acid (PLA). PLA stands out as a biodegradable bioplastic, with wide availability and the ability to decompose without causing damage to the environment (Taib *et al.* 2022).

The objective of this study was to optimize acclimatization and maximize the survival rate of micropropagated seedlings of *Paliavana sericiflora* Benth. by investigating the effect of incorporating zinc oxide nanoparticles (ZnO-NPs-NPs) into the formulation of the microcontainer used in the acclimatization phase.

Materials and Methods

Study site and plant material

The studies were carried out at the Laboratory for the *In Vitro* Cultivation of Forest Species and the Forest Nursery, located in the Department of Forestry Sciences at the Lavras School of Agricultural Sciences (ESAL/UFLA), Federal University of Lavras (UFLA), in Lavras, Minas Gerais, Brazil. The plants used came from the plant bank cultivated *in vitro* (Leite *et al.* 2024; code ACF88E2 in SisGen)

Obtaining and evaluating nanoparticles

The production of ZnO-NPs-NPs followed the methodology described by Alves *et al.* (2012). Two aqueous solutions were prepared, one containing zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$) and the other containing sodium hydroxide (NaOH), both at a concentration

of 1 mol L^{-1} . The solutions were mixed and homogenized under moderate stirring and heating at 80°C for two hours. After this period, the white precipitate obtained was collected by filtering through filter paper and washed successively with ethanol and plenty of deionized water. The precipitate was then dried at 60°C for 48 hours and stored under vacuum. The dried material was macerated and analyzed by scanning electron microscopy (SEM) (Fig. 1).

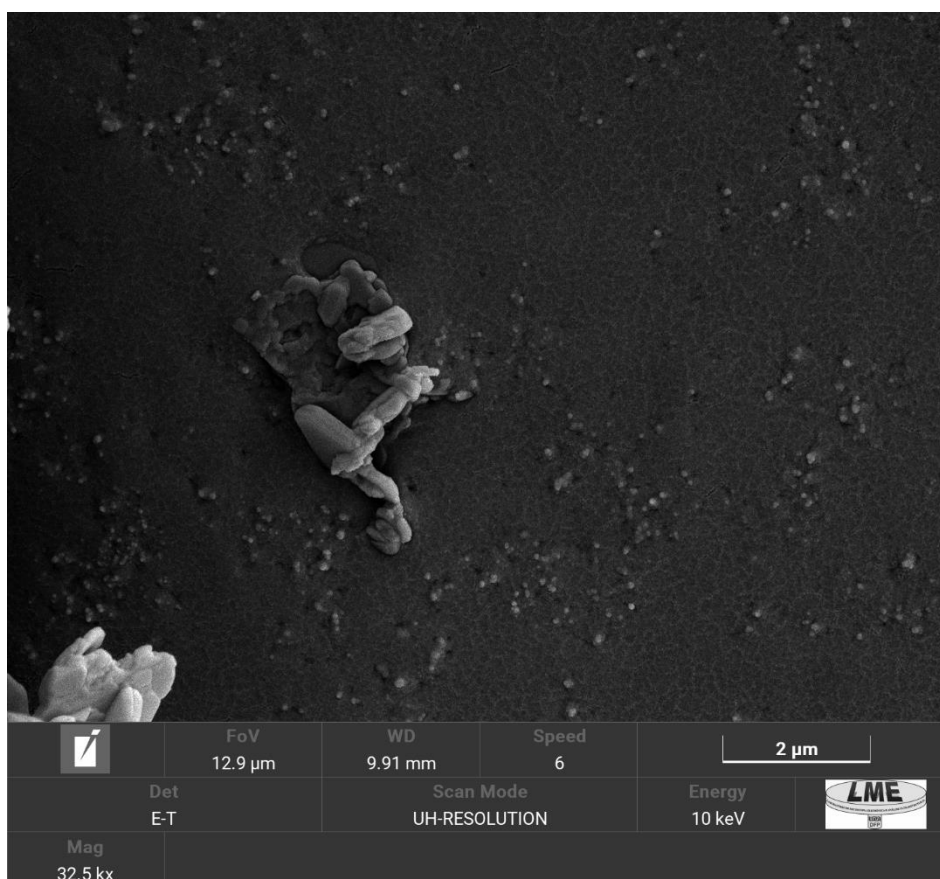


Figure 1 - Zinc oxide (ZNO-NPS) nanoparticles after the maceration process. Bar: $2 \mu\text{m}$.

Filament and microcontainer production

The PLA filament was enriched with ZnO-NPs in proportions of 0.025, 0.050, 0.075, 0.1 and 1 g kg^{-1} of PLA. To do this, the ZnO-NPs was dispersed in chloroform and added to the PLA granules under constant stirring. The mixture was then dried in an oven at 60°C for 48 hours. After drying, the granules were added to the FILMAQ3D CV extruder to make the filament at a temperature of 100°C . On leaving the extruder, the filament was pulled by the FTR1, which was responsible for cooling the filament and measuring its diameter.

The 4.5 cm^3 microcontainers (protected by patent application BR 10 2023 007189 9), 4 cm high and 1.2 cm in diameter, were produced by a 3D printer (Orion Delta) using zinc-enriched filament according to each treatment.

Acclimatization and hardening off

Complete plants produced *in vitro* (aerial part and root) were used in acclimatization treatments. The seedlings, were transplanted into 4.5 cm^3 and about 1g, microcontainer in transparent polypropylene cups using a mini-incubator system (Figure 2). The substrate used was decomposed pine bark. The plants were grown under a 16-hour photoperiod and an

irradiance of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 30 days. After this period, plant survival rates were assessed and the formation of adventitious roots and plant tissues was visualized.

After the acclimatization period in microcontainer, the plants were transplanted into 250 cm^3 tubes. The substrate used consisted of decomposed pine bark and medium vermiculite in a ratio of 1:1 (v/v). The tubes were kept in a greenhouse with irrigation every other day for 60 days. The experiment was set up in a completely randomized design with 20 replications per treatment. The experimental factor was the concentration of ZnO-NPs added to the polymer in the tubes (PLA), with the following levels: 0, 0.025, 0.05, 0.075, 0.1 and 1 g kg^{-1} . The following were assessed: vigor (grading scale), number of leaves, number of shoots, height (cm), neck diameter (cm), shoot dry mass (g), root dry mass (g), total dry mass (g), Dickson quality index (IQD), leaf width (cm), leaf length (cm), leaf area (cm^2), photosynthetic pigment content [chlorophyll *a*, *b*, total (*a+b*) and carotenoids].

To evaluate vigour in a comprehensive and objective way, a predefined grading scale from 1 to 5 was developed: 1 - excellent, vigorous shoot induction, active growth and no signs of nutritional deficiency, 2 - good, shoot induction (or not), reduced growth compared to the ideal, no visible symptoms of nutritional deficiency, 3 - fair: no induction of shoots, reduced growth, leaves with necrotic or yellowed spots, 4 - bad: appearance of visual symptoms of nutritional deficiency, and necrosis of plant tissues, 5 - very bad: death of more than 75% of leaf and shoot tissues.

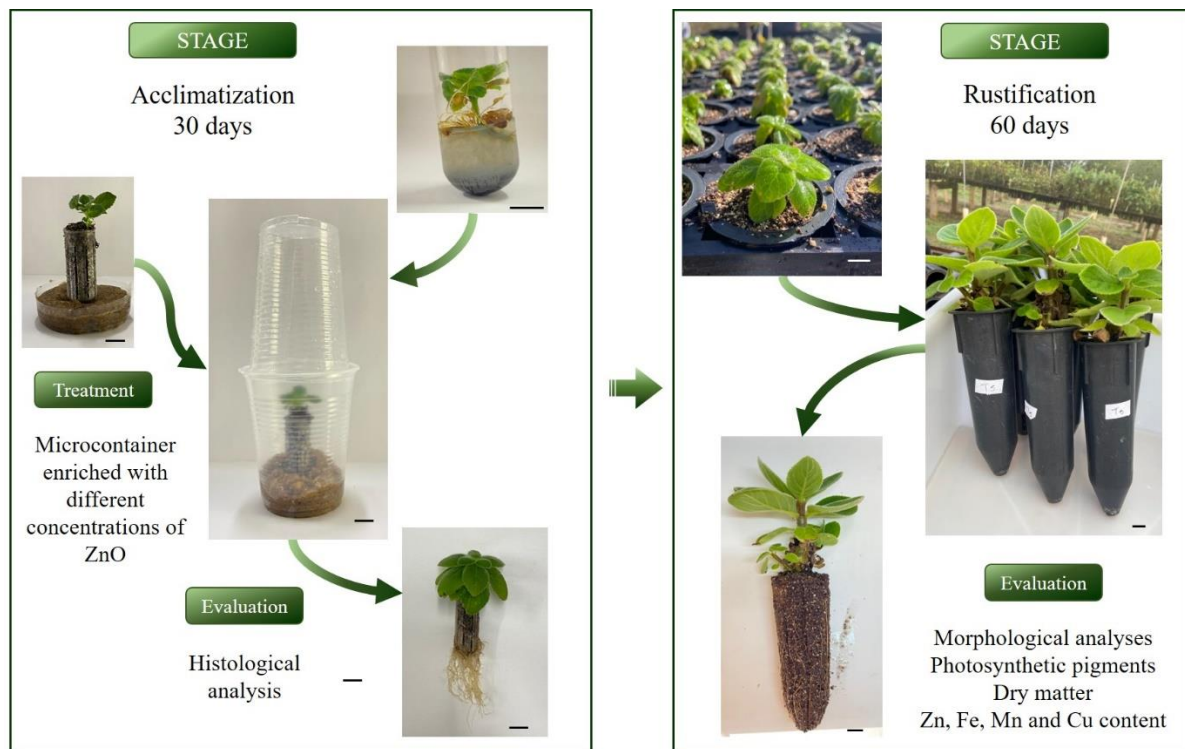


Figure 2 - Schematic representation of the research carried out on the acclimatization and hardening off of *Paliavana sericiflora*. Bar: 1cm.

Leaf analysis, photosynthetic pigments and histological analysis

The area, length and width of the leaves were measured using image J software and the images were obtained using an HP Deskjet Ink Advantage 1516 scanner. Twenty leaves per

treatment were evaluated, taken from the 3rd and 4th pair of young leaves, the aerial part towards the coleus.

To quantify the photosynthetic pigments (chlorophylls *a* and *b*, carotenoids) in the plant leaves, a method based on Lichtenthaler (1987) and Wellburn (1994) was used. Leaf discs of 25 mg were removed and submerged in 5 mL of dimethyl sulfoxide (DMSO) solution for 48 hours in a dark environment, ensuring the preservation of the pigments. The absorbance of the solutions was measured in triplicate on a Genesys 10UV spectrophotometer (ThermoScientific, USA) in quartz cuvettes with an optical path of 10 mm, using specific wavelengths: 665 nm (chlorophyll *a*), 649 nm (chlorophyll *b*) and 480 nm (carotenoids). The concentrations of chlorophylls *a*, *b*, total and carotenoids were calculated using the equations of Wellburn (1994).

To make the slides, fragments of approximately 1cm² were taken from the median regions of the leaves and the base of the stem of the treatment with the best morphological responses. The material was subjected to ethanolic dehydration (70, 80, 90, 100%) at room temperature (Johansen, 1940). It was then immersed in a pre-infiltration solution consisting of a solution of 100% ethanol and base resin, in a 1:1 ratio, according to the manufacturer's instructions (Leica HistoResin Kit) for 24 hours. After this process, the base resin was infiltrated for 15 days at 4°C.

The HistoResin Kit (hydroxyethyl methacrylate, Leica, Heidelberg) was used for polymerization. The sections were taken on a semi-automatic rotating microtome and then stained in 1% Toluidine Blue, pH 6.7 (Feder; O'brien, 1968). The images were obtained using a Nikon Eclipse E100 light microscope coupled with an Infinity capture camera.

Determining the elements

To investigate the presence of specific chemical elements in the plant leaves (iron, zinc, manganese and copper), a detailed elemental analysis was carried out using a Shimadzu EDX-900 energy dispersive X-ray fluorescence (EDX) spectrometer. Leaf samples were taken from three plants per treatment and dried at 45°C until they reached a constant weight. After drying, the leaves were ground in liquid nitrogen and sieved through a 150 nm mesh.

With the material dried and sieved, 200mg of powder per repetition was standardized and placed in the sample holders covered with a 5µm thick film. The exposure time for the reading was 100s. The operating conditions selected were collimator - 10 mm, atmosphere - air, tube voltage - 50 kV, tube current - 1000 µA, irradiation time - 100 s, acquisition mode - Quantitative/FP, analytical line - Kα.

Statistical analysis

Nanoparticle size will be analyzed descriptively, while the other variables were checked for homoscedasticity and normal distribution of residues using the Hartley and Shapiro-Wilk tests, respectively, with a 5% probability of error. The results obtained were analyzed by analysis of variance (ANOVA), followed by Tukey's test to compare the means. The program R version 4.0.3 (2020) and the functions contained in the packages ExpDes.pt (2021) and Anova (2013) were used, and principal component analysis was carried out using the packages Factorextra, and FactoMineR (Kassambara & Mundt, 2020).

Results

At 30 days of acclimatization, the seedlings produced *in vitro* showed 100% survival, in all treatments tested, demonstrating a successful acclimatization process. Qualitative analysis of the root anatomy revealed good formation and connection of the adventitious roots with the

vascular cambium in all treatments (Fig. 3a arrows), indicating efficient root development. In addition, continuous cell formation was observed good connection with the vascular exchange.

The leaf mesophyll showed differences between the treatments, with greater structural complexity in the treatments with ZnO-NPs nanoparticles compared to the treatment without ZnO-NPs (Fig. 3b and Fig. 3c).

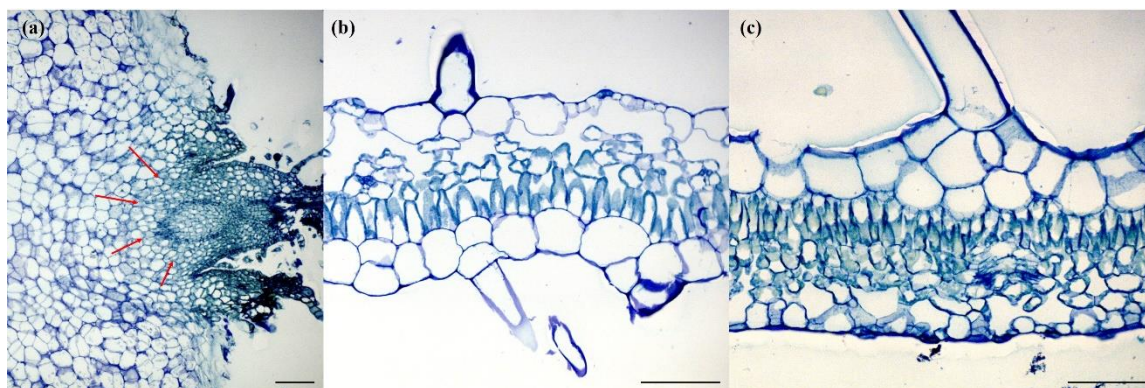


Figure 3 - Cross-sections of the stem (a) and mesophyll (b without ZnO-NPs and c with ZnO-NPs), observed during the acclimatization of *Paliavana sericiflora*. Arrows show the connection between the root and the stem. Bars: 1 μm .

The vigor of the seedlings, assessed on a scale of scores from 1 to 5 (where 5 represented underdeveloped plants), was significantly lower at the lower doses of ZnO-NPs in the soil, resulting in plants with underdevelopment, chlorosis and leaf fall. In contrast, the concentration of 0.1 g kg^{-1} of ZnO-NPs provided the most vigorous seedlings (Fig. 4a) with the best growth. In association with vigor, the accumulation of aerial and total dry mass (Figs. 4b and 4c) showed a similar trend, with the treatment with the most vigorous plants (0.1 g kg^{-1} of ZnO-NPs) showing the greatest accumulation of dry matter.

Initially, the tubes (Fig. 4d) weighed approximately 1.5 g. After 90 days of cultivation, the treatments without the addition of ZnO-NPs, with 0.025 and 0.050 g kg^{-1} and 1.0 g kg^{-1} , showed a change in the mass of the container. This reduction may be associated with the good biodegradability of the material. On the other hand, the container with the best response in terms of clone growth showed an increase in mass. This increase can be explained by the greater absorption of water, as well as the association with fungi and bacteria that act in the biodegradation process, helping to release the ZnO-NPs nanoparticles.

The biodegradation of the tubes may have influenced the release of the ZnO-NPs nanoparticles, promoting the absorption of the nutrient by the seedlings. The association with fungi and bacteria is one of the possibilities of interaction with the environment that can accelerate the decomposition of the material and the release of nanoparticles. The increase in mass in the container with better seedling growth suggests that the microorganisms helped absorb water and nutrients, as well as releasing the ZnO-NPs, favoring plant development.

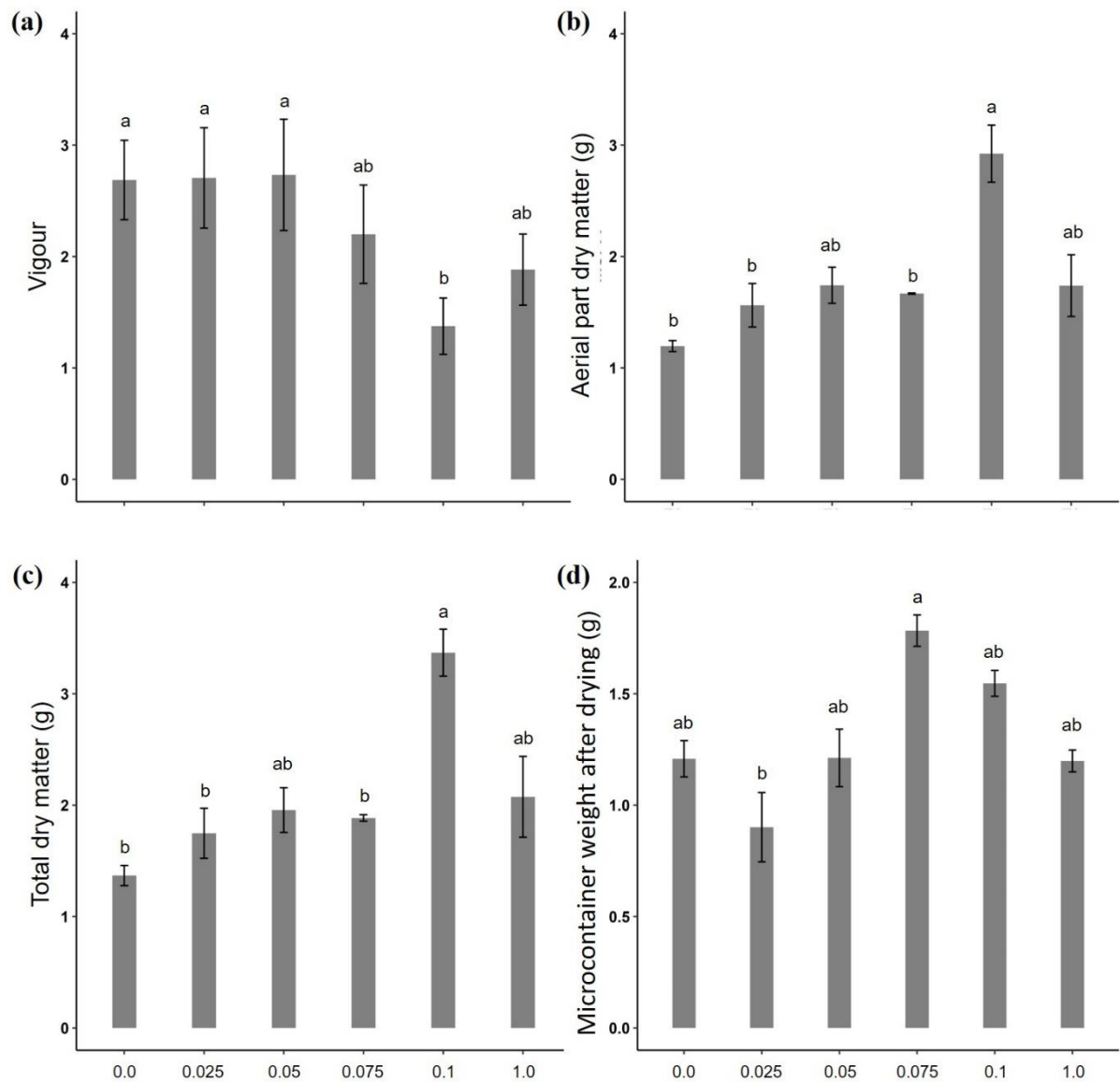


Figure 4 - Characteristics assessed at 60 days of hardening off *Paliavana sericiflora* as a function of the concentrations of ZnO-NPs used in the microcontainer (0, 0.025, 0.05, 0.075, 0.1 and 1.0 g kg⁻¹). (a) vigor, (b) aerial dry matter (g), (c) total dry matter (g), (d) Microcontainer weight after drying (g). Means followed by the same letter do not differ significantly according to Tukey's test. Bars represent the standard error of the sample.

The number of shoots and, subsequently, stems can provide a greater number of flowers, fruits and seeds, increasing the chances of maintaining the species in the field. From this perspective, the number of shoots (Fig. 5a) was higher in the treatment without ZnO-NPs. This increase, however, resulted in lower growth in height (Fig. 5c). Stem diameter and plant height (Fig. 5b and 5c) showed similar responses. The treatment with 0.1 g kg⁻¹ resulted in plants with the largest stem diameter at ground level, as well as being the tallest in the experiment.

Leaf size is crucial for the survival of *P. sericiflora* of hardening off. Larger leaf areas, after hardening off, maximize the production of photoassimilates, favoring the plant's growth and development. In line with the results for height and diameter, the area, width and length of the leaves (Figs. 5d, 5e and 5f) showed higher values in the treatment with 0.1 g kg⁻¹, showing the positive effect of this concentration of ZnO-NPs on the leaf development of the species.

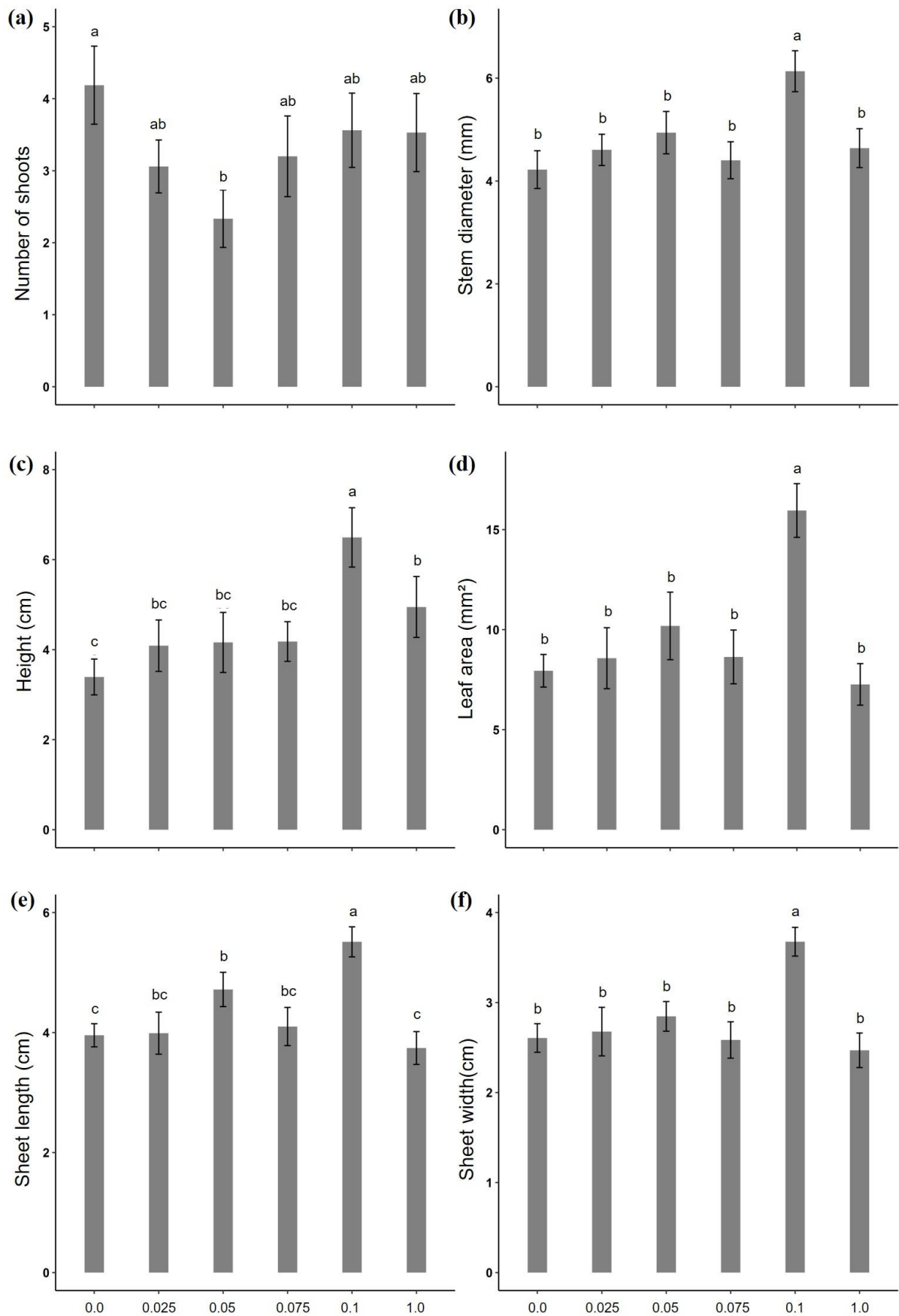


Figure 5 - Characteristics assessed at 60 days of hardening off *Paliavana sericiflora* as a function of the concentrations of ZnO-NPs used in the microcontainer (0, 0.025, 0.05, 0.075, 0.1 and 1 g kg⁻¹). (a)

number of shoots, (b) stem diameter (cm), (c) height (cm), (d) leaf area (mm²), (e) leaf length (cm) and (f) leaf width (cm). Averages followed by the same letter do not differ significantly according to Tukey's test. Bars represent the standard error of the sample.

The photosynthetic pigments responsible for converting light into chemical energy showed different responses to the concentrations of ZnO-NPs tested. The treatment with 1 g kg⁻¹ resulted in the highest content of chlorophyll *a*, carotenoids and total chlorophyll (Figs. 6a, 6c and 6d), not differing from 0.05 for total chlorophyll and carotenoids, suggesting that these treatments present the best establishment responses. For chlorophyll *b*, the concentration of 0.05 g kg⁻¹ provided the highest content, with the other pigments also standing out. These results indicate that the application of ZnO-NPs in appropriate concentrations can optimize the content of photosynthetic pigments in *P. sericiflora*, favouring photosynthesis and plant development.

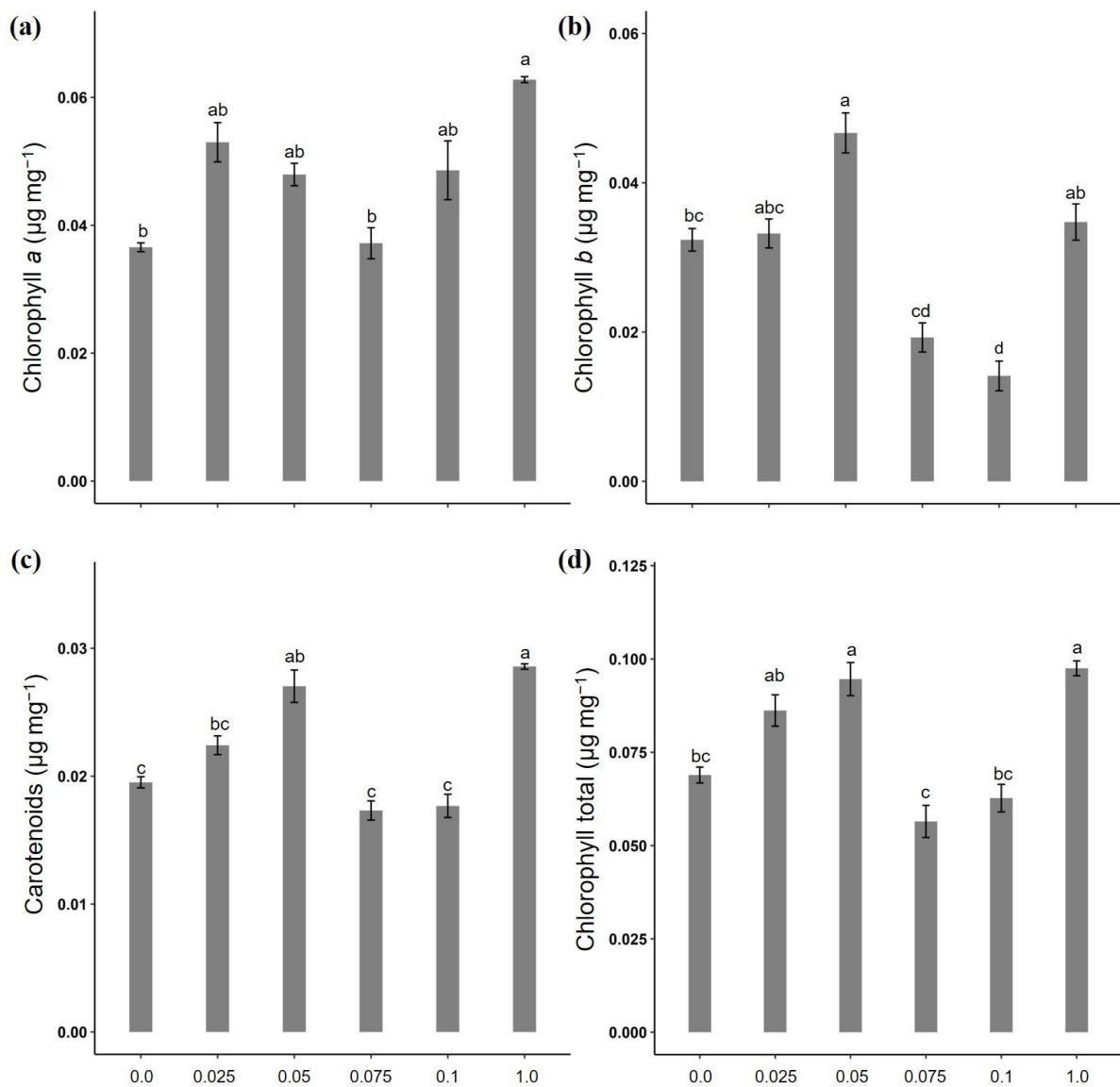


Figure 6 - Characteristics evaluated at 60 days of hardening off of *Paliavana sericiflora* as a function of the concentrations of ZnO-NPs used in the microcontainer (0, 0.025, 0.05, 0.075, 0.1 and 1 g kg⁻¹). (a) chlorophyll *a* (µg mg⁻¹), (b) chlorophyll *b* (µg mg⁻¹), (c) carotenoids (µg mg⁻¹) and (d) total chlorophyll (µg mg⁻¹). Averages followed by the same letter do not differ significantly according to Tukey's test. Bars represent the standard error of the sample.

Principal component analysis (PCA) (Fig. 7) explained more than 50% of the variability in the data. In Dimension 1, the variables total dry matter, shoot dry matter and height captured the greatest variability in the data. In Dimension 2, photosynthetic pigments covered the variability of the residual data. The treatment with 0.1 g kg⁻¹ showed the greatest dissimilarity from the other treatments tested and the greatest correlation with the morphological and dry matter variables. The treatments with 0.025, 0.05 and 1 g kg⁻¹, on the other hand, showed a higher correlation with photosynthetic pigments. Vigor showed a high correlation with the treatment without added zinc, indicating that higher values for this variable represent worse conditions for the plants.

The use of 0.075 g kg⁻¹ showed a high correlation for the number of leaves and number of shoots, and differed from the other ZnO-NPs concentrations. These results suggest that the application of ZnO-NPs can influence the morphological development, physiology and vigor of *P. sericiflora*, optimizing the growth and productivity of the species.

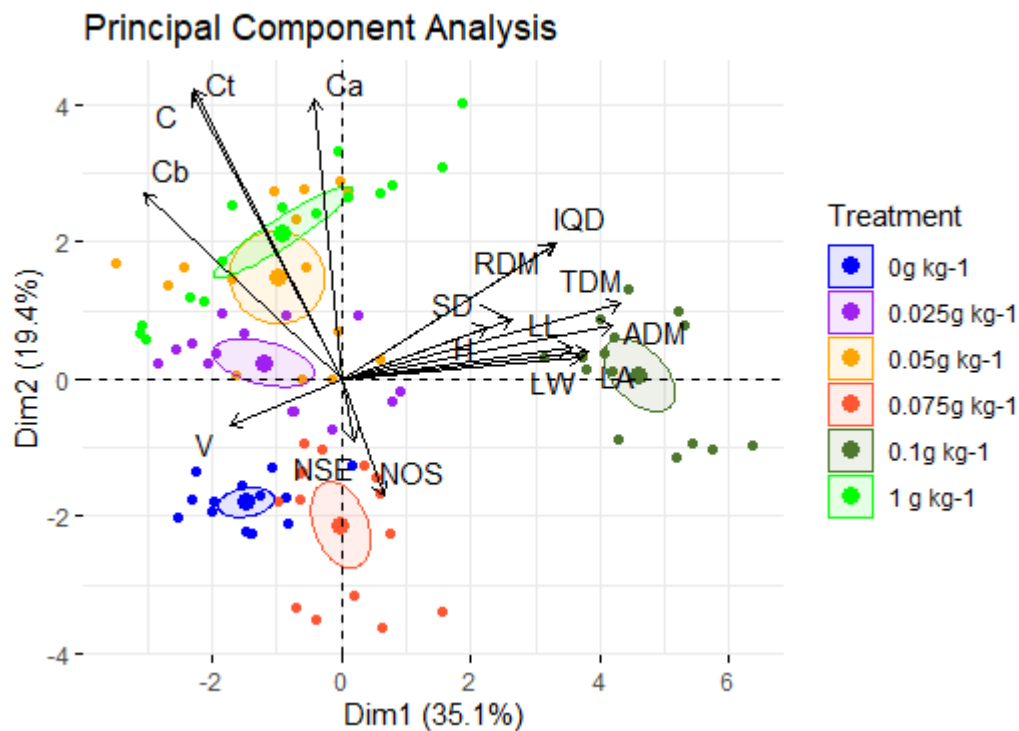


Figure 7 - Principal component analysis (PCA) of *P. sericiflora* hardening off at 60 days. Dim1 = principal component 1 (PC1), Dim2 = principal component 2 (PC2). V - Vigor, NSE - number of leaves, NOS - number of shoots, H - height, SD - stem diameter, ADM - aerial dry matter, RDM - root dry matter, TDM - total dry matter, IQD - dickson quality index, LA - leaf area, LL - leaf width, LW - leaf length, Ca - chlorophyll a, Cb - chlorophyll b, C - carotenoids, Ct - total chlorophyll.

X-ray fluorescence spectrometer analysis (Fig. 8) revealed that the zinc, copper, iron and manganese levels in the leaves were higher in the 0.1 g kg⁻¹ treatment, followed by the 0.05 g kg⁻¹ treatment. This higher concentration of zinc in the leaves may be directly related to the greater availability of zinc in the substrate, provided by the growing container, inducing the bioavailability of other elements such as iron, attention should be paid to the amount of iron absorbed since zinc and iron compete for the same chelating agent, zinc can reduce iron concentration.

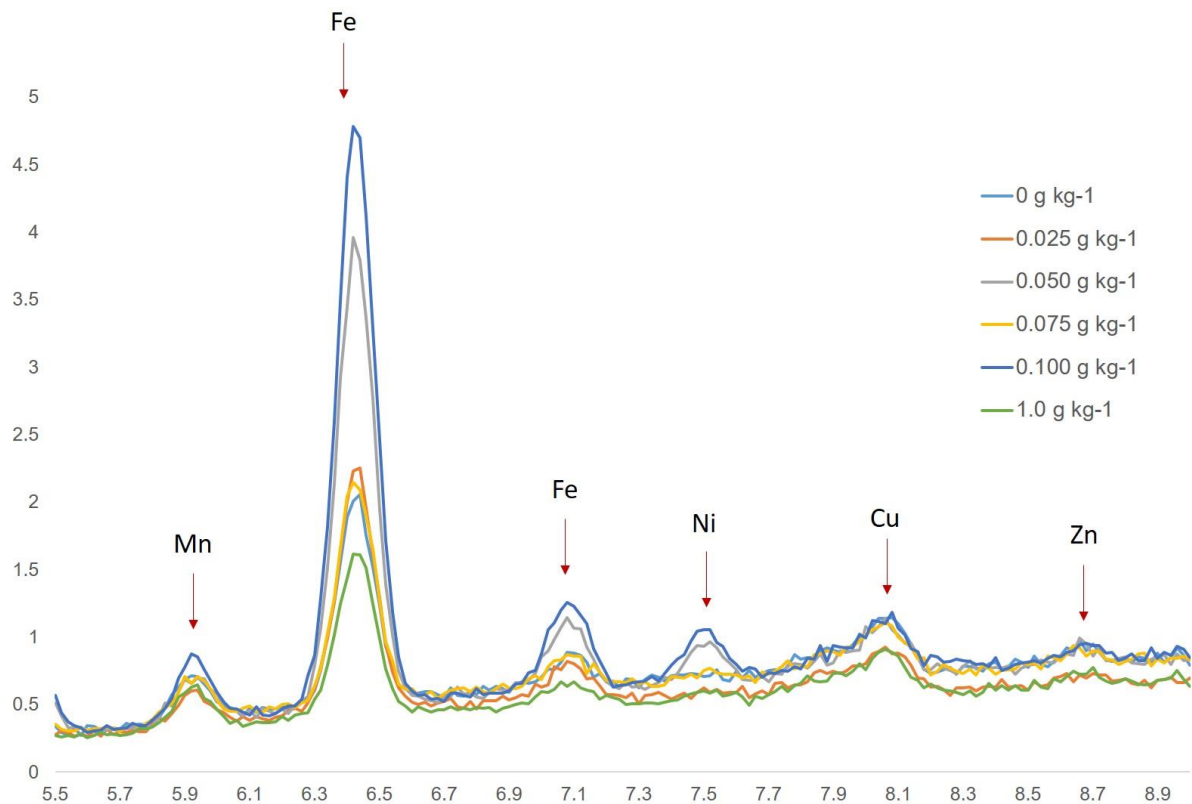


Figure 8 - Graph of the X-ray fluorescence of the light emitted from the dry matter of *Paliavana sericiflora* leaves as a function of the ZnO-NPs concentrations used in the microcontainer (0, 0.025, 0.05, 0.075, 0.1 and 1 g kg⁻¹).

Discussion

Determining the ideal dose of zinc nanoparticles (ZnO-NPs NPs) varies according to the plant species in question. Studies show that the appropriate dose can promote photosynthesis, enzyme activity and the absorption of other nutrients essential for plant growth (Lv *et al.* 2022), This optimization of nutrition can be reflected in the vigor of the seedlings, as evidenced in a study where the highest doses within a certain limit of ZnO-NPs NPs resulted in greater plant vigor (Burman *et al.* 2012).

However, it is crucial to consider that excessive use of ZnO-NPs NPs can lead to toxicity. A study with chickpea seedlings showed that the application of 1.5 mg L⁻¹ of ZnO-NPs NPs resulted in a significant increase in the root system, while the application of 10 mg L⁻¹ significantly reduced root growth (Burman *et al.* 2012). The same study showed toxic effects at even higher concentrations.

The increase in plant dry matter varies according to the dose of ZnO-NPs NPs applied. Studies show that lower doses generally result in a greater increase in the dry matter content of the aerial part and total plant (Saleem *et al.* 2023). In a study with nine different crops (wheat, maize, radish, beans, lettuce, tomatoes, peas, cucumbers and beet), the application of ZnO-NPs in two types of soil, at concentrations ranging from 20 to 200 mg kg⁻¹, resulted in the greatest increase in crop biomass and the yield of some grains (Garcia-Gomez *et al.* 2018). On the other hand, higher concentrations, between 225 and 1000 mg kg⁻¹, reduced the increase and yield of some crops.

Specific studies with the species in question have shown that the application of 100 mg kg⁻¹ of ZnO-NPs NPs gave the best results in terms of growth and productivity, while treatment with higher doses resulted in a reduction in yield. This reduction may be related to phytotoxicity, a harmful effect caused by excess ZnO-NPs NPs in the plant.

The positive effect of zinc nanoparticles (ZnO-NPs-NPs) on plant growth has been corroborated in several studies. A notable example is the case of guinea grass (*Panicum maximum*), where the application of 100 mg kg⁻¹ of ZnO-NPs NPs resulted in a significant increase in the length of the aerial part, leaf area and number of leaves, compared to the dose of 500 mg kg⁻¹ which already showed a reduction compared to the control, suggesting possible phytotoxicity (Gomes *et al.* 2023). In addition, the 100 mg kg⁻¹ dose stood out in relation to the 500 mg kg⁻¹ dose for variables such as stalk thickness, plant length, productivity, total levels of carbohydrates and proteins, and photosynthesizing pigments.

This complex structure of the mesophyll, seen in, may contribute to better responses in the hardening off of the seedlings, as it increases the photosynthetic leaf area and the efficiency of light absorption. These results suggest that the application of ZnO-NPs in adequate concentrations can influence the growth and development of *P. sericiflora*, optimizing the plant's characteristics for propagation and field management purposes.

The application of zinc nanoparticles (ZnO-NPs-NPs) in low concentrations proves to be more efficient than the use of zinc sulphate (ZnSO₄ in conventional use). Studies show that it is possible to achieve maximum productivity or similar effects with the application of ZnO-NPs NPs in 70% lower quantities compared to conventional inputs (Du *et al.* 2019; Sun *et al.* 2020).

Several studies have shown that the application of zinc nanoparticles (ZnO-NPs NPs) induces an increase in the content of chlorophylls and carotenoids in plants, resulting in improvements in their growth species (Faizan *et al.* 2018; Kolencik *et al.* 2020; Saleem *et al.* 2023). In a study with soybean plants, the application of 10, 100 and 1000 mg L⁻¹ of ZnO-NPs-NPs resulted in a significant increase in the production of chlorophylls a and b, with an increase in the ratio between them of 21%, 29% and 23%, respectively (Leopold *et al.* 2022). It is important to note that this study differs from the one presented, as the concentrations (75 and 100 mg L⁻¹) of ZnO-NPs NPs showed the lowest chlorophyll levels compared to the other treatments.

Carotenoids act photoprotection of plant cells, fighting oxidative damage and acting as adjuncts in light capture. In stressful situations, as demonstrated in soybean plants that showed signs of stress when ZnO-NPs NPs were applied (Leopold *et al.* 2022), carotenoids act antioxidant. This intense antioxidant function may explain the paradox observed in *Paliavana* seedlings, where the treatments with the highest carotenoid content showed lower increases in mass and lower vigor. It is believed that the excessive allocation of resources to carotenoid production to the detriment of vegetative growth may be linked to some kind of stress.

The application of nanoparticles (NPs) to supply nutrients to plants is gaining prominence. In addition to zinc, ZnO-NPs (zinc oxide) NPs can stimulate the absorption of other essential nutrients, such as iron and manganese (Sun *et al.* 2020; Lv *et al.* 2022). When doped with copper, ZnO-NPs NPs can also have a nematicidal, antifungal (Trifão *et al.* 2022) and even antibacterial action (Lv *et al.* 2022). In seedling production, it is crucial to pay attention to the correct concentration of NPs to avoid phytotoxicity effects, which can result in low plant vigor.

Conclusion

This research successfully demonstrated the synthesis and incorporation of zinc oxide nanoparticles (ZnO-NPs NPs) into filaments for making microcontainer. This strategy resulted em 100% survival of acclimatized plants.

The results indicated a possible phytotoxicity effect at a concentration of 1 g of ZnO-NPs NPs, highlighting the need for further research into this relationship, taking into account the specific characteristics of each plant species. On the other hand, the concentration of 0.1 g of ZnO-NPs NPs stood out as the most promising, promoting greater vigor in the plants, greater accumulation of dry matter and superior development in terms of height, diameter and leaf area. This high correlation between vegetative variables and the concentration of ZnO-NPs NPs is a crucial factor for the survival of the species when transplanted to the field.

The lower level of stress observed in plants grown with lower carotenoid levels suggests that this strategy may contribute to improving plant performance when transplanted to the field. Future studies should further investigate this relationship in order to elucidate the underlying physiological mechanisms and optimize the use of ZnO-NPs NPs for growing different plant species in field conditions.

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TERCEIRA PARTE

CONSIDERAÇÕES FINAIS

Este estudo apresentou protocolos otimizados de micropropagação para *P. sericiflora*, uma espécie de grande importância ecológica para os campos rupestres. A pesquisa explorou a influência de fatores interferentes no cultivo *in vitro* da espécie, tais como carboidratos, trocas gasosas e espectro luminoso, buscando com isso condições ideais para o crescimento e desenvolvimento das mudas.

Os resultados demonstraram que a micropropagação se configura como uma ferramenta robusta para a produção em larga escala de mudas de *P. sericiflora*, sendo pioneira, abrindo lacunas para a aplicação dessa técnica em outras espécies do campo rupestre ferruginoso. Essa abordagem pode contribuir significativamente para a conservação da biodiversidade dessa fitofisionomia.

A pesquisa comprova a efetividade dos microtubetes enriquecidos com nanopartículas de ZnO-NPs na aclimatização de mudas micropropagadas de *P. sericiflora*. Auxiliando na taxa de sobrevivência das mudas após o transplante para o ambiente *ex vitro*, demonstrando seu potencial para otimizar o processo de aclimatização, podendo ser aplicado a diversas espécies.

Os resultados deste estudo abrem um leque de possibilidades para a produção de mudas de alta qualidade de espécies ameaçadas de extinção, contribuindo para a conservação da biodiversidade e o manejo sustentável de áreas degradadas. Sugerimos pesquisas futuras que explorem a aplicação da micropropagação e do uso de microtubetes enriquecidos não somente com zinco, mas com as nanopartículas disponíveis no mercado, em outras espécies do campo rupestre ferruginoso, expandindo o conhecimento e as ferramentas disponíveis para a preservação da biodiversidade.

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