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MORPHOGENIC RESPONSE OF LEAF EXPLANTS OF *Pleroma heteromallum* UNDER DIFFERENT COMBINATIONS OF *IN VITRO* GROWTH REGULATORS

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ABSTRACT

Objective: The study aimed to evaluate the morphogenic responses of leaf explants of *Pleroma heteromallum* cultured *in vitro*, with the goal of expanding the knowledge on micropropagation strategies for the species.

Theoretical Framework: *Pleroma heteromallum* is an endemic species of Brazil with ornamental, medicinal, and phytoremediation potential. Its sexual propagation presents limitations, and the species is generally multiplied through stem cuttings. Micropropagation represents a promising alternative for large-scale production of seedlings with phytosanitary quality and continuous availability.

Method: Five experiments were carried out testing MS media supplemented with different combinations of BAP, IBA, 2,4-D, PVP, and activated charcoal. The morphogenic responses of the leaf explants were evaluated throughout the culture period, with records of the percentages of callogenesis, rhizogenesis, and possible shoot formation.

Results and Discussion: It was observed that PVP and activated charcoal did not inhibit oxidation and impaired callus formation. MS medium without growth regulators did not induce callogenesis, resulting in explant death. Treatments containing BAP alone showed inferior performance compared with those combined with IBA or 2,4-D. The peak of callogenesis and rhizogenesis occurred on the 20th day, followed by progressive callus oxidation. Only one explant, cultivated in BAP:IBA, formed callus, roots, and shoots, indicating potential for indirect organogenesis.

Research Implications: The results support future micropropagation protocols for the species and contribute to its management and conservation.

Originality/Value: This study provides unprecedented evidence on the *in vitro* morphogenesis of *P. heteromallum*, highlighting promising hormonal combinations for its propagation.

Keywords: *Tibouchina heteromalla* (syn.), Melastomataceae, Micropropagation, Indirect Organogenesis, Plant Tissue Culture, *Ex situ* Conservation.

RESPOSTA MORFOGÊNICA DE EXPLANTES FOLIARES DE *Pleroma heteromallum* SOB DIFERENTES COMBINAÇÕES DE REGULADORES DE CRESCIMENTO *IN VITRO*

RESUMO

Objetivo: O estudo teve como objetivo avaliar as respostas morfogênicas de explantes foliares de *Pleroma heteromallum*, cultivados *in vitro*, visando ampliar o conhecimento sobre estratégias de micropropagação para a espécie.

Referencial Teórico: *P. heteromallum* é uma espécie endêmica do Brasil com potencial ornamental, medicinal e fitorremediador. Sua propagação sexuada apresenta entraves, sendo geralmente multiplicada por estaquia caulinar. A micropropagação representa uma alternativa promissora para produção massal de mudas com qualidade fitossanitária e disponibilidade contínua.

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Método: Foram conduzidos cinco experimentos testando meios MS suplementados com diferentes combinações de BAP, AIB, 2,4-D, PVP e carvão ativado. As respostas morfogênicas dos explantes foliares foram avaliadas ao longo do cultivo, com registro dos percentuais de calogênese, rizogênese e possíveis brotações.

Resultados e Discussão: Observou-se que PVP e carvão ativado não inibiram a oxidação e prejudicaram a formação de calos. O meio MS isento de reguladores não induziu calogênese, resultando em morte dos explantes. Tratamentos contendo BAP isolado apresentaram desempenho inferior aos combinados com AIB ou 2,4-D. O pico de calogênese e rizogênese ocorreu no 20º dia, seguido de progressiva oxidação dos calos. Apenas um explante, cultivado em BAP:AIB, formou calos, raízes e brotos, indicando potencial para organogênese indireta.

Implicações da Pesquisa: Os resultados subsidiam protocolos futuros de micropropagação da espécie e contribuem para seu manejo e conservação.

Originalidade/Valor: Este estudo reúne evidências inéditas sobre a morfogênese *in vitro* de *P. heteromallum*, destacando combinações hormonais promissoras para sua propagação**.**

Palavras-chave: *Tibouchina heteromalla* (sin.), Melastomataceae, Micropropagação, Organogênese Indireta, Cultura de Tecidos Vegetais, Conservação *Ex situ*.

RESPUESTA MORFOGÉNICA DE EXPLANTES FOLIARES DE *Pleroma heteromallum* BAJO DIFERENTES COMBINACIONES DE REGULADORES DE CRECIMIENTO *IN VITRO*

RESUMEN

Objetivo: El estudio tuvo como objetivo evaluar las respuestas morfogénicas de explantes foliares de *Pleroma heteromallum* cultivados *in vitro*, con el fin de ampliar el conocimiento sobre las estrategias de micropropagación para la especie.

Marco Teórico: *Pleroma heteromallum* es una especie endémica de Brasil con potencial ornamental, medicinal y fitorremediador. Su propagación sexual presenta limitaciones, y generalmente se multiplica por estacas caulinares. La micropropagación representa una alternativa prometedora para la producción masiva de plántulas con calidad fitosanitaria y disponibilidad continua.

Método: Se realizaron cinco experimentos probando medios MS suplementados con diferentes combinaciones de BAP, AIB, 2,4-D, PVP y carbón activado. Las respuestas morfogénicas de los explantes foliares fueron evaluadas a lo largo del cultivo, registrándose los porcentajes de calogénesis, rizogénesis y posibles brotaciones.

Resultados y Discusión: Se observó que el PVP y el carbón activado no inhibieron la oxidación y perjudicaron la formación de callos. El medio MS sin reguladores de crecimiento no indujo calogénesis, resultando en la muerte de los explantes. Los tratamientos con BAP aislado mostraron un rendimiento inferior en comparación con aquellos combinados con AIB o 2,4-D. El pico de calogénesis y rizogénesis ocurrió en el día 20, seguido de oxidación progresiva de los callos. Solo un explante, cultivado en BAP:AIB, formó callos, raíces y brotes, indicando potencial para organogénesis indirecta.

Implicaciones de la investigación: Los resultados respaldan futuros protocolos de micropropagación de la especie y contribuyen a su manejo y conservación.

Originalidad/Valor: Este estudio reúne evidencia inédita sobre la morfogénesis *in vitro* de *P. heteromallum*, destacando combinaciones hormonales prometedoras para su propagación.

Palabras clave: *Tibouchina heteromalla* (sin.), Melastomataceae, Micropropagación, Organogénesis Indirecta, Cultivo de Tejidos Vegetales, Conservación *Ex situ*.

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1 INTRODUCTION

Pleroma heteromallum (D.Don) D.Don, known as jaguar ear, is a shrub species endemic to Brazil, found in the Cerrado and Atlantic Forest biomes (Guimarães, 2024) and characterized by naturally isolated populations (Campos *et al*., 2009). Its ornamental value stems from its purple flowers and grayish-white hairy leaves (Lorenzi, 2015), in addition to its ecological importance as a pioneer species tolerant to soils with heavy metals, relevant in environmental recovery actions (Cuevas-Reyes *et al*., 2018). It also has medicinal uses (Kuster *et al*., 2009) and landscaping potential (Lorenzi, 2015; Latoh *et al*., 2019).

The use of native species in landscaping has intensified with the aim of enhancing local flora and reducing maintenance requirements (Granemann *et al*., 2017; Gomes *et al*., 2020; Correa *et al*., 2023; Prestes *et al*., 2020). However, the sexual propagation of *Pleroma heteromallum* is hampered by the small size of the seeds, low germination, and unevenness of the seedlings (Nienow *et al*., 2010; Fragoso *et al*., 2017). Cuttings are possible (Latoh *et al*., 2018, 2019), but limited by seasonality, availability of material, and risk of pathogen spread (Silva *et al*., 2019).

In this context, micropropagation emerges as an alternative for producing seedlings on a large scale and with high phytosanitary standards (Dalpiaz *et al*., 2019; Faria *et al*., 2020; Soares *et al*., 2023). *In vitro* plant regeneration can occur through different morphogenic pathways, with organogenesis being one of the most commonly used. In direct organogenesis, new organs originate from previously differentiated tissues, while in indirect organogenesis, regeneration occurs from undifferentiated cells organized into callus (Hasnain *et al*., 2022).

Given these limitations, this study aimed to evaluate the morphogenic response of *P. heteromallum* leaf explants grown *in vitro* in MS medium supplemented with different combinations of BAP, AIB, 2,4-D, PVP, and activated carbon, in order to identify conditions favorable to callogenesis and indirect morphogenesis.

2 METHODOLOGY

2.1 PLANT MATERIAL AND GENERAL CULTIVATION CONDITIONS

Leaves measuring approximately 1 cm², from *Pleroma heteromallum* seedlings cultivated *in vitro*, were used as explants for five experiments. Each explant was inoculated in test tubes containing approximately 10 mL of culture medium.

The basal medium used was Murashige and Skoog (MS basal) (Murashige and Skoog, 1962), supplemented with vitamins (Phygenera®), 30 g L^{-1} of sucrose, and 7 g L^{-1} of agar. The pH was adjusted to 5.8 ± 0.1 before adding the agar, followed by autoclaving for 20 min at 121 °C and 1.2 atm.

The tubes were kept in a B.O.D. incubator (EletroLab, EL 212) at 25 ± 1 °C, with a photoperiod of 16 h and approximate irradiance of 25 µmol m^{-2} s^{-1}, provided by fluorescent lamps (Philips TL-D 15W/75-6). The experimental unit consisted of one explant per tube.

The data were submitted to the Shapiro–Wilk normality test (5%) and transformed when necessary using the equation $[(x + 1)^{0.5}]$. Analysis of variance (ANOVA) was conducted at 5% probability and, when significant, the means were compared using Tukey's test (5%). Statistical analyses were performed using SISVAR 5.8 software (Ferreira, 2011).

2.2 EXPERIMENT I: PVP, ACTIVATED CARBON, AIB, AND BAP IN INDIRECT MORPHOGENESIS

The first experiment evaluated the effect of PVP, activated carbon, and the plant growth regulators AIB and BAP on indirect morphogenesis from leaf explants of *P. heteromallum*. Five treatments were tested: T1: basal MS medium (control); T2: basal MS medium + 4.9 µM AIB + 4.4 µM BAP + 1 g L^{-1} PVP; T3: basal MS medium + 4.9 µM AIB + 4.4 µM BAP + 1 g L^{-1} activated carbon; T4: basal MS medium + 4.9 µM AIB + 4.4 µM BAP; and T5: basal MS medium + 4.4 µM BAP.

The experiment was conducted in a completely randomized design (CRD) with seven replicates. After 42 days, the survival percentage (SP), oxidation percentage (OP), callus formation percentage (CP), shoot induction percentage (SI), and root induction percentage (RI) of the explants were evaluated.

2.3 EXPERIMENT II: AIB AND 2,4-D IN INDIRECT MORPHOGENESIS

This test evaluated the effect of the plant growth regulators AIB and 2,4-D on indirect morphogenesis from leaf explants of *P. heteromallum*. Two treatments were applied: T1: Basal MS medium + 4.4 µM BAP + 4.9 µM AIB and T2: Basal MS medium + 4.4 µM BAP + 4.5 µM 2,4-D.

The experiment was conducted in DIC with ten replicates. After 30 days, all explants were transferred to media with the same treatments and remained there for another 30 days,

totaling 60 days of culture. After this period, the survival percentage (SP), oxidation percentage (OP), callus formation percentage (CP), shoot induction percentage (SI), and root induction percentage (RI) of the explants were evaluated.

2.4 EXPERIMENT III: BAP AND AIB IN INDIRECT MORPHOGENESIS (WITHOUT TRANSPLANTING)

The third experiment evaluated the effect of different combinations of BAP and AIB on indirect morphogenesis without replanting the explants. The following were tested: T1: Basal MS medium (control); T2: Basal MS medium + 2.2 μM BAP; T3: Basal MS medium + 4.4 μM BAP; T4: Basal MS medium + 2.2 μM BAP + 4.9 μM AIB; and T5: Basal MS medium + 4.4 μM BAP + 4.9 μM AIB.

The experiment was organized in DIC, with ten replicates, and conducted for 40 days. At the end of the experiment, the survival percentage (SP), oxidation percentage (OP), callus formation percentage (CP), shoot induction percentage (SI), and root induction percentage (RI) of the explants were evaluated.

2.5 EXPERIMENT IV: BAP AND AIB IN INDIRECT MORPHOGENESIS (WITH REPICAGEM)

This experiment evaluated the effect of different combinations of the plant growth regulators BAP and AIB on the indirect morphogenesis of leaf explants of *Pleroma heteromallum*. After 30 days of cultivation, the explants were repotted into tubes containing the same treatment, but with freshly prepared culture medium, in order to verify whether possible residues or exudates accumulated in the initial medium could interfere with the morphogenic process.

The treatments used were: T1: basal MS (control); T2: basal MS + 2.2 μM BAP; T3: basal MS + 4.4 μM BAP; T4: basal MS + 2.2 μM BAP + 4.9 μM AIB; T5: basal MS + 4.4 μM BAP + 4.9 μM AIB; and T6: basal MS + 6.6 μM BAP + 4.9 μM AIB.

The experimental design was completely randomized (CRD), with ten replicates per treatment, totaling 60 experimental units. After replanting on the 30th day, the explants remained in culture for another 15 days, totaling 45 days of culture.

The percentage of oxidized explants (PO), the percentage of induced explants (curved leaves) (PI), the percentage of callus-bearing explants (PC), the percentage of root-bearing explants

(PR), and the percentage of sprouting explants (PB) were evaluated. The data ting to the 30th day were submitted to the statistical analyses provided for in the study.

2.6 EXPERIMENT V: INDUCTION OF SPROUTING FROM CALLUS MASS

This experiment evaluated the effect of different concentrations of BAP on the induction of sprouting from callus masses originating from Experiment IV. The callus masses were inoculated in basal MS medium containing different concentrations of BAP: T1: 1.1 μM; T2: 2.2 μM; T3: 4.4 μM; T4: 6.6 μM; and T5: 8.8 μM.

The experimental design was completely randomized (CRD), with four replicates per treatment, totaling 20 experimental units. Evaluations were performed after 30 days of cultivation, recording the survival percentage (SP) and sprout induction percentage (SI) from the callus masses.

3 RESULTS AND DISCUSSIONS

3.1 EXPERIMENT I: PVP, ACTIVATED CARBON, AIB, AND BAP IN INDIRECT MORPHOGENESIS

There was no sprouting or rooting induction in any of the treatments, which is why these variables were disregarded in the statistical analyses. For the other variables, the normality test and analysis of variance were significant. The mean comparison test indicated that treatment T4 had the highest survival rate (SR) and the lowest oxidation rate (OR) among all treatments, which, in turn, did not differ from each other. For the percentage of callogenesis (PC), the control treatment (T1) showed the lowest mean, while the other treatments did not differ statistically from each other (Table 1).

Table 1

Means of survival percentage (SP), percentage of oxidized explants (PO), and percentage of explants with callus (PC) of Pleroma heteromallum (D.Don) D.Don 42 days after inoculation of leaf explants in different culture media.

Treatment	SP	PO (	PC
Baseline MS (Control) (T1)	0.00b	100.00b	0.00c
MS + 4.9 µM AIB + 4.4 µM BAP + PVP (T2)	28.57b	71.43	71.43ab
MS + 4.9 µM AIB + 4.4 µM BAP + activated charcoal (T3)	14.29b	85.71b	42.86abc
MS + 4.9 µM AIB + 4.4 µM BAP (T4)	85.71a	14.29a	100.00a
MS + 4.4 µM BAP (T5)	0.00b	100.00b	28.57ab
Overall Average (%)	25.71	74.29	48.57
Coefficient of variation (%)	12.12	10.25	13.46

Averages followed by the same letter in the column do not differ from each other according to Tukey's test at a 5% probability level. Source: Authors, 2022.

Total oxidation of explants in the control treatment (T1) resulted in 0% survival and no callus induction. In T2, 71.43% of explants oxidized completely; however, two explants retained about 10% of their intact area, resulting in 28.57% survival. A similar situation occurred in T3, which showed 85.72% oxidation, with only one explant preserving approximately 60% of its area, which corresponded to 14.28% survival.

The T4 treatment stood out for promoting callogenesis in 100% of the explants, in addition to presenting 85.72% survival and only 14.28% oxidation. A slight darkening of the tissues was observed around the 30th day in this treatment, but without compromising callus development. In T5, total oxidation of the explants was observed; although 28.57% of them began callus formation, all subsequently oxidized.

Given the results, especially the high survival rate observed in T4, this treatment demonstrated superior performance compared to the others evaluated.

PVP and activated carbon are known for their ability to adsorb inhibitory and toxic substances released by explants from the culture medium, preventing oxidation and eventual tissue death (Rondon *et al*., 2019; Verde *et al*., 2021), but the presence of these compounds can inhibit *in vitro* growth, depending on the species and tissue used (Meneguzzi *et al*., 2020).

A negative effect of adding PVP and activated carbon to the culture medium was observed. These compounds, in addition to not inhibiting the oxidation of explants, seem to impede the h y process of callus induction. Miranda *et al*. (2019) also found that PVP and activated carbon were ineffective in inhibiting the oxidation of *Eremanthus incanus* (Asteraceae) explants.

Werner *et al*. (2009) found that the best treatment for controlling oxidation of *Caesalpinia echinata* (Fabaceae) explants was activated carbon, in which 40% of the explants showed low to moderate oxidation, but without callus formation, due to the adsorbent effect of this compound, which immobilizes part of the elements that make up the medium, including phytohormones.

Schwalbert *et al*. (2015) found an inhibitory effect on the growth of *Desmodium incanum* (Fabaceae) by adding activated charcoal to the culture medium. The authors suggested that this effect occurs due to the adsorbent property of this compound; thus, while activated charcoal adsorbs compounds that are toxic to plant growth, it also adsorbs nutrients that are essential for plant growth.

The initial responses of callus induction are marked by curvature and then by intumescence of the explants until callus formation. Based on the results observed in the present study, it was noted that the absence and/or decrease in the concentration of the plant growth regulators BAP and AIB in the culture medium, either due to their non-addition or their adsorption by PVP or activated charcoal, is the factor that suppresses the callogenesis process and, consequently, causes the oxidation and death of *P. heteromallum* leaf explants.

3.2 EXPERIMENT II: AIB AND 2,4-D IN INDIRECT MORPHOGENESIS

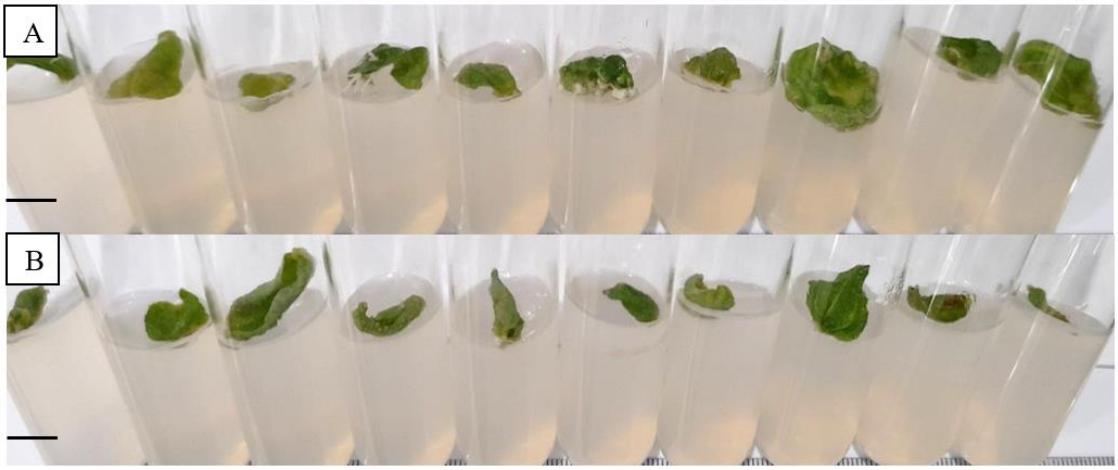
Twenty days after the start of the experiment, 100% survival of the explants and a uniform response to induction were observed in both treatments, as evidenced by the curvature of the leaves (Figure 1).

There was no sprouting induction in any of the treatments, which is why this variable was disregarded from the statistical analyses. The normality test was significant for all variables, leading to the need to transform the data before performing the analysis of variance.

With the exception of root induction percentage (PR), the analysis of variance did not indicate a significant difference between treatments. According to the mean comparison test, T1 presented a higher percentage of rhizogenesis, surpassing T2.

Figure 1

Leaf explant of Pleroma heteromallum after 20 days of in vitro culture in MS medium supplemented with 4.4 µM BAP and 4.9 µM AIB – T1 (A) and 4.4 µM BAP and 4.5 µM 2,4-D – T2 (B). Bars: 1.0 cm.

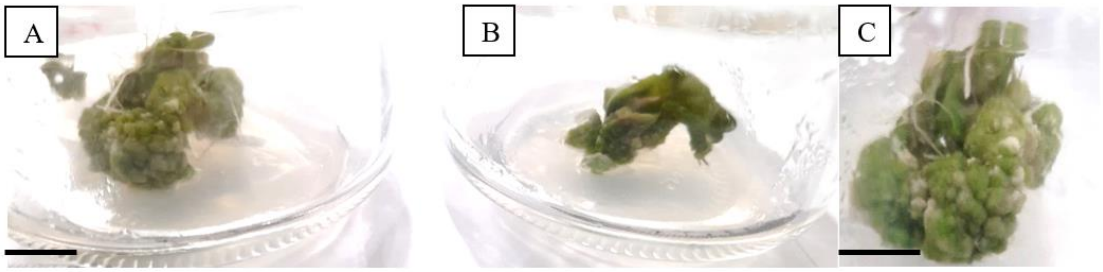


Source: Authors, 2022.

In both treatments, total oxidation was observed in 10% of the explants, resulting in 90% survival. Callogenesis occurred in all surviving explants; however, it was more vigorous in T1, which had greater callus mass, with the formation of compact, green or white calluses (Figure 2). In addition, root induction was observed in 70% of the explants that formed callus in T1, while in T2 this percentage was only 10%. These results demonstrate the superiority of the BAP + AIB combination over 2,4-D in promoting rhizogenesis in *P. heteromallum* from leaf explants.

Figure 2

Appearance of callus mass originating from Pleroma heteromallum leaf explants grown in MS medium supplemented with 4.4 µM BAP and 4.9 µM AIB (A) and 4.4 µM BAP and 4.5 µM 2,4-D (B) after 45 days of culture. Note the compact green or white callus mass and the presence of roots in T1 (C). Bars: 1.0 cm.



Source: Authors, 2022.

The results indicate the need to add the plant growth regulators BAP and AIB or BAP and 2,4-D to the culture medium for the process of callogenesis to occur via leaf explants of *P. heteromallum*, since in the absence of these plant growth regulators there is no induction of the callogenesis process, resulting in rapid oxidation and death of the explants. When the culture medium is supplemented with BAP alone, although the process of callogenesis induction (leaf curvature) is observed in some explants, over time, oxidation and death of the explants occur.

3.3 EXPERIMENT III: BAP AND AIB IN INDIRECT MORPHOGENESIS (WITHOUT TRANSPLANTING)

Microbial contamination was observed in 100% of the repetitions of treatment T2, which led to its exclusion from the statistical analyses. The normality test was significant for all variables, so the data were transformed before performing the analysis of variance. Table 2 shows the results obtained after 40 days of cultivation of *Pleroma heteromallum* leaf explants in the different treatments.

Table 2

Means of survival percentage (SP), oxidation percentage (OP), callus induction percentage (CP), shoot induction percentage (SP), and root induction percentage (RP) at 40 days of in vitro culture of P. heteromallum.

Treatment	SP	PO	CI	PB	PR
			(%)		
Baseline MS - control (T1)	30ab	70ab	0b	0ns	30a
MS + 4.4 µM BAP (T3)	0b	100b	0b	0ns	0b
MS + 2.2 µM BAP + 4.9 µM AIB (T4)	60a	40a	60a	0ns	0b
MS + 4.4 µM BAP + 4.9 µM AIB (T5)	70a	30a	70a	10ns	0b
Overall Average (%)	40	60	32.5	2.5	7.5
Coefficient of variation (%)	15.21	14.21	12.91	6.48	9.70

Source: Authors, 2022.

After 40 days of cultivation, it was found that 70% of the explants in the control treatment (T1) showed total oxidation. The remaining 30% of explants emitted roots, indicating the possible presence of endogenous auxin concentrations capable of inducing rhizogenesis even without exogenous supplementation.

In treatment T3, all explants showed complete oxidation during the same period. Despite this, curvature of the explants was observed throughout cultivation, suggesting the onset of callogenesis, although the process did not progress. It is likely that the concentration of BAP and/or the endogenous hormone levels of the explants were insufficient to sustain the complete development of callus.

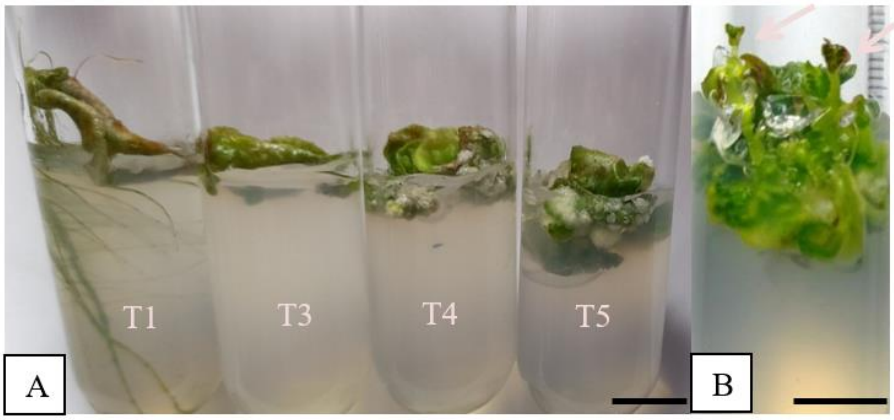
For the variable percentage of shoot induction, there was no significant difference between treatments. However, in T5, 10% of the explants emitted shoots.

Treatments T4 and T5 did not differ statistically from each other for any of the variables analyzed. However, visually, the callus mass formed in T5 was more vigorous, presenting greater volume and better morphological appearance when compared to that observed in T4.

Figure 3 shows details of the curvature and root emission observed in T1 (control); the leaf curvature associated with the onset of callogenesis in T3; and callus formation in T4 and T5 (Figure 3A), in addition to shoot emission in the only responsive explant in T5 (Figure 3B).

Figure 3

Details of P. heteromallum leaf explants in different in vitro culture media: curvature and root emission at T1; leaf curvature indicating the onset of callogenesis at T3; callogenesis at T4 and T5 (A); and sprouting in explants cultured at T5 (B). Bars: 1.0 cm.

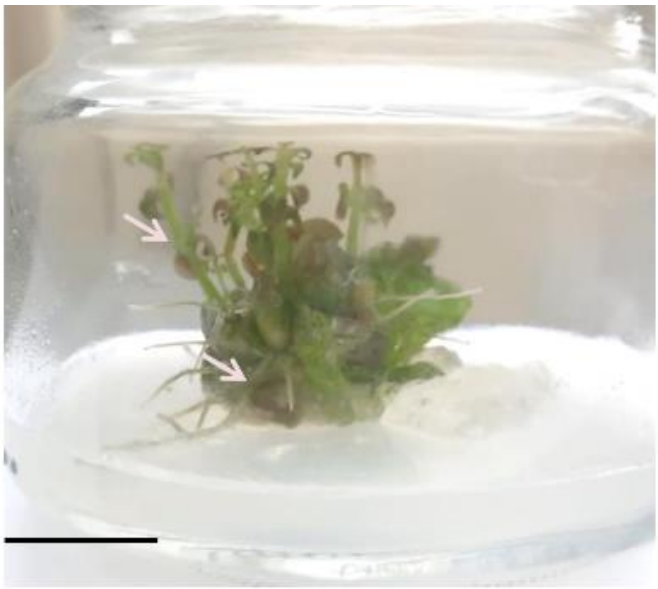


Legend: Basal MS – control (T1); MS + 4.4 µM BAP (T3); MS + 2.2 µM BAP + 4.9 µM AIB (T4); MS + 4.4 µM BAP + 4.9 µM AIB (T5). Source: Authors, 2022.

In the callus masses obtained, which were transferred to flasks containing MS medium supplemented with 8.8 µM BAP, shoot development and root emission were observed in only one of the replicates from T5 (Figure 4). In the other replicates of T4 and T5, the callus masses progressively oxidized until death, without organogenesis.

Figure 4

Indirect organogenesis from callus mass originating from P. heteromallum leaf explants. Arrows indicate shoots and roots developed after subculture in MS medium supplemented with 8.8 µM BAP. Bar: 1.0 cm.



Source: Authors, 2022.

The process of *in vitro* organogenesis is complex and influenced by external and internal factors, including the source of the explant, the culture medium, the action of phytohormones, especially auxin and cytokinin, and the ability of the tissue to respond to hormonal changes and environmental factors during cultivation (Oliveira *et al*., 2013).

Prudente *et al*. (2016) observed callus formation and subsequent shoot regeneration in leaf explants of *Miconia ligustroides* (Melastomataceae) cultured in 54 µM BAP and 13.5 µM 2,4-D, respectively.

Lawarence and Murugan (2017) inferred that in the *in vitro* propagation of *Osbeckia aspera* (Melastomataceae), compact and friable green calluses are induced using MS medium supplemented with the hormonal combinations ANA (0.5 mg $^{L^{-1}}$) + BAP (0.5 mg $^{L^{-1}}$) and 2,4-D (0.5 mg L^{-1}) + BAP (0.5 mg L^{-1}), respectively. Plant regeneration was possible using the combinations of ANA and BAP, however, the maximum number of shoots was produced by combining ANA (0.5 mg $^{L^{-1}}$) + BAP (2.5 mg $^{L^{-1}}$), where an average of 21.28 shoots per explant were obtained.

Nauli *et al*. (2020) in studies with *Melastoma malabathricum* (Melastomataceae) found that the medium containing ANA (1.0 mg $^{L^{-1}}$) and TDZ (0.1 mg $^{L^{-1}}$) promoted the best results in the callogenesis process (100%). Both callus and adventitious roots formed in a medium

containing only ANA (1.0 mg L^{-11}). These researchers also found that a single explant formed callus and shoots in a medium containing TDZ (2 mg L^{-1} 1) + ANA (0.1 mg L^{-1}).

3.4 EXPERIMENT IV: BAP AND AIB IN INDIRECT MORPHOGENESIS (WITH REPLICATION)

Callus induction from leaf explants of *Pleroma heteromallum* varied according to the combinations of plant growth regulators added to the culture medium and the duration of *in vitro* culture.

It was observed that the peak oxidation of the explants generally occurred after the 20th day of culture. The control treatment (T1) showed total oxidation (100%) on the 20th day, while in the other treatments this process gradually intensified, reaching 100% in T2, 90% in T3, 50% in T4 and T6, and 40% in T5 on the 45th day.

Regarding the percentage of induced explants (leaf curvature), there was no induction in the control treatment. In the others, the peak occurred on the 15th day after inoculation, with T2 and T3 presenting 80% induced explants; T4 and T5, 90%; and T6, 70%. These values remained until the 20th day, when the death of part of the explants due to oxidation was observed.

The percentage of explants with callus varied according to the culture medium used, peaking between the 20th and 30th day after inoculation. In treatments T2 and T3, the maximum value was 10%, while T4, T5, and T6 showed higher rates, with 80%, 90%, and 70%, respectively.

Similarly, the percentage of explants with roots was also influenced by the growth regulators present in the medium. Treatments T1 and T2 reached 10% rooted explants on the 15th day; T3 showed no root formation; T4 reached 20% on the 20th day; T5 reached 40% on the 20th day; and T6 reached 20% on the 30th day.

The normality test was significant for all variables, requiring data transformation prior to analysis of variance. ANOVA indicated significant differences between treatments for the variables percentage of oxidized, induced, and callus explants. The mean comparison test, considering the results on the 30th day after inoculation, is shown in Table 3.

Table 3

Means for percentage of oxidized explants (PO), percentage of induced explants (PI), percentage of callus explants (PC), and percentage of root explants (PR) on the 30th day after inoculation of Pleroma heteromallum leaf explants in the different treatments.

Treatment	PO	II	PC	PR
	%			
Baseline MS – control (T1)	100b	0b	0b	0ns
MS + 2.2 µM BAP (T2)	90b	10b	10b	10ns
MS + 4.4 µM BAP (T3)	60ab	40ab	10b	0ns
MS + 2.2 µM BAP + 4.9 µM AIB (T4)	20a	80a	80a	20ns
MS + 4.4 µM BAP + 4.9 µM AIB (T5)	30a	70a	70a	40ns
MS + 6.6 µM BAP + 4.9 µM AIB (T6)	30a	70a	70a	20ns
Overall Average (%)	55	45	40	15
Coefficient of variation (%)	13.85	14.33	13.33	13.53

Averages followed by the same letter in the column do not differ from each other according to Tukey's test at 5% probability; ns: not significant according to the f test at 5%. Source: Authors, 2022.

For the variables percentage of oxidized explants (PO), percentage of induced explants (PI), and percentage of explants with callus (PC), treatments T1 and T2 were statistically equal to each other and inferior to treatments T4, T5, and T6, which did not differ from each other.

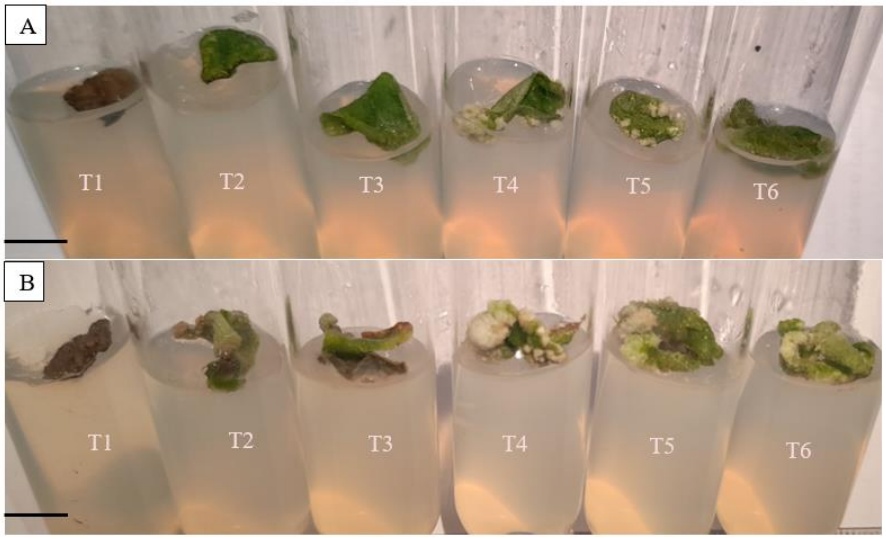
T3 was statistically equal to the other treatments for the percentage of oxidized explants (PO) and percentage of induced explants (PI). For the percentage of explants with callus (PC), T3 was statistically equal to treatments T1 and T2 and lower than treatments T4, T5, and T6. The percentage of explants with roots (PR) was statistically equal for all treatments.

The calluses formed tend to be friable or compact green. Darkening indicating oxidation tends to occur in explants grown in MS medium free of plant growth regulators or supplemented only with BAP.

The evolution of callogenesis of explants on the 15th and 20th days, proving the need to add BAP and AIB plant growth regulators to the culture medium in the callogenesis process of *P. heteromallum* leaf explants, can be seen in Figure 5.

Figure 5

Callogenesis from leaf explants of Pleroma heteromallum on the 15th (A) and 20th (B) day after inoculation in MS medium supplemented with different concentrations of BAP and AIB. Bars: 1.0 cm.



Legend: basal MS medium - control (T1); MS medium supplemented with 2.2 µM BAP (T2); MS medium supplemented with 4.4 µM BAP (T3); MS medium supplemented with 2.2 µM BAP and 4.9 µM AIB (T4); MS medium supplemented with 4.4 µM BAP and 4.9 µM AIB (T5); and MS medium supplemented with 6.6 µM BAP and 4.9 µM AIB (T6). Source: Authors, 2022.

The occurrence of phenolic oxidation in explants is one of the greatest challenges to the application of *in vitro* culture techniques, especially for woody species, hindering the culture establishment process (Santos *et al*., 2019).

Karimah *et al*. (2020) observed callogenesis in *Melastoma malabathricum* (Melastomataceae) explants cultured in MS medium free of phytohormones, suggesting that concentrations of endogenous hormones in the explants are adequate for callus induction and formation in the species.

The occurrence of embryogenic and non-embryogenic callus was observed within the same treatment, provided that BAP and AIB were combined in the culture medium. Similar results were observed by Costa *et al*. (2015). These authors considered that this variation in callus formation within the same treatment may be due to the hormonal gradients present in the explant.

In general, auxin and cytokinin are two groups of plant growth regulators most commonly used in plant tissue culture. Their addition to the culture medium is useful for regulating the cell during division, elongation, differentiation, and organ formation (Neta *et al*., 2022). The auxin/cytokinin ratio in the culture medium is decisive for the organogenic response. Generally, higher concentrations of auxin induce root formation, while higher concentrations

of cytokinin induce adventitious bud formation, and intermediate auxin/cytokinin ratios induce disorganized cell proliferation (callus) (Jardim *et al*., 2010).

Calluses can harbor cells with active centers responsible for cell division. Under adequate concentrations of cytokinin and auxin, these centers are stimulated and differentiate into organs. Competent cells, capable of responding to specific stimuli, have the potential to differentiate and give rise to shoots or roots (Santos *et al*., 2022).

The darkening of callus may indicate the onset of explant death, caused by a decrease in nutrients in the medium or by chlorophyll degradation (*Karimah* et al., 2020). The transfer of callus mass on the 30th day of cultivation to the same culture medium, i.e., containing the same concentrations of BAP + AIB plant growth regulators, did not have the expected effect. The oxidation process of the callus mass continued even after transfer to a new culture medium.

3.5 EXPERIMENT V: INDUCTION OF SHOOTS FROM CALLUS MASS

The callus masses transferred to a culture medium containing only the cytokinin BAP did not regenerate sprouts, undergoing oxidation and death over time. After 30 days of *in vitro* culture, it was found that all explants showed total oxidation, regardless of the treatment used.

The results obtained indicate that the concentrations of BAP+IBA tested in previous experiments, as well as the isolated use of BAP in MS medium, were not sufficient to sustain tissue viability or promote shoot formation from *P. heteromallum* callus mass. Thus, widespread oxidation compromised the continuity of morphogenic development, making it impossible to obtain shoots.

4 CONCLUSION

Pleroma heteromallum leaf explants responded to growth regulators applied under controlled cultivation conditions. However, recurrent oxidation of callus remained a significant obstacle, suggesting that endogenous physiological factors, physicochemical characteristics of the culture medium, and environmental conditions of cultivation, such as light intensity, photoperiod, and temperature, may be negatively modulating the morphogenic response.

Given this scenario, there is a need for additional experiments exploring new arrangements of plant growth regulators, different concentrations of antioxidants, adjustments in transplanting intervals, and modifications in general cultivation conditions, with the aim of improving the stability and efficiency of organogenic responses.

In summary, the results obtained reinforce that regeneration via indirect organogenesis in *P. heteromallum* is feasible and represents a promising alternative for both seedling production and *ex situ* conservation strategies for the species.

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