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Exploring the role of phytohormones in modulating in vitro orchid growth

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Abstract

Orchids have a high ornamental potential due to their diversity of colors, shapes, and sizes. However, improper extraction by traders and collectors can lead to the risk of these species' extinction. Therefore, this research aimed to evaluate the use of naphthaleneacetic acid (ANA) and 6-benzylaminopurine (BAP) in the *in vitro* growth of *Pseudolaelia corcovadensis*, *Macradenia paraensis*, and *Maxillaria brasiliensis*. The experiment was carried out in the Plant Tissue Culture Laboratory with endemic orchids: *Pseudolaelia corcovadensis*, *Macradenia paraensis*, and *Maxillaria brasiliensis*. The experiment was set up in a completely randomized design in a 4 x 3 factorial scheme, with a total of 12 treatments and 10 replications each: T0 - MS medium (100%); T1 - MS medium (100%) + 0.5 mg $L^{-1}$of ANA; T2 - MS medium (100%) + 0.5 mg L^{-1}) of ANA and 0.5 mg L^{-1}) of BAP; and T4 - MS medium (100%) + 0.5 mg L^{-1} of BAP. The treatments were performed using MS medium, and the plants were kept in laboratory conditions until 120 days of age, when contamination and growth were assessed. The statistical analysis highlighted a high rate of contamination during the repotting cycles. Even with contamination, the orchids showed vegetative growth using phytohormones. This observation allows us to conclude that 100% MS medium should be used for Maxillaria *brasiliensis*, and 100% MS medium + 0.5 mg L^{-1}ANA proved to be the most satisfactory for *Macradenia paraensis* and *Pseudolaelia corcovadensis*.

Keywords: *Macradenia paraensis, Maxillaria brasiliensis,* micropropagation, *Pseudolaelia corcovadensis*.

Introduction

The Orchidaceae family has a high ornamental value due to its infinity of colors, sizes and shapes, and is of significant economic importance in the world market. However, commercialization has led to improper specimen extraction from their habitat, posing several species at risk (Soares et al., 2020).

Among these at-risk species, three shall be highlighted: *Maxillaria brasiliensis* has a small, isolated lemon-yellow inflorescence with an orange lip, occurring in Brazil's north, northeast, south, and southeast (Meneguzzo, 2024). The *Macradenia paraensis* orchid produces rachis-shaped inflorescences containing up to 30 brownish-green to red flowers, with confirmed occurrences in the north, midwest, and southeast (Koch, 2024). Another relevant species is *Pseudolaelia corcovadensis*, which produces clusters of pink flowers and only occurs in the southeast, in the states of Minas Gerais and Rio de Janeiro (Menini Neto, 2024).

Orchids have a low germination rate in the wild, with only 5% of seeds germinating under natural conditions (Soares et al., 2020). *In vitro* multiplication by seed germination has been widely used in the large-scale production of orchid seedlings: a rapid process with minimal losses while maintaining the genetic variability of the species (Mengarda et al., 2017; Nascimento et al., 2022).

However, orchids are slow-growing, so seedling production is time-consuming (Patavardhan et al., 2022). Therefore, applying phytohormones has proved to be a promising strategy for improving these plants' development, growth, and propagation in laboratory environments (Suzuki et al., 2004; Suzuki et al., 2010).

In *in vitro* cultivation, the precise use of phytohormones is crucial during the multiplication phase, stimulating the shoots proliferation of shoots (Oliveira,

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2024). Auxins, such as naphthaleneacetic acid (ANA), are often used to form shoots and adventitious roots in orchids, promoting cell division and tissue growth (Patavardhan et al., 2022).

In addition, cytokinins, such as 6-benzylaminopurine (BAP), play a prominent role in the *in vitro* cultivation of orchids. They act synergistically with auxins, stimulating the differentiation and elongation of shoots, promoting the formation of multiple shoots, and contributing to rapid multiplication (Patavardhan et al., 2022; Talukdar et al., 2022; Mubarok et al., 2024).

In short, phytohormones play a fundamental role in the *in vitro* cultivation of orchids, significantly influencing the growth of healthy and vigorous shoots. However, it is crucial to find the right combination of phytohormones and their concentrations for *in vitro* orchid multiplication (Cruz et al., 2024). This research aimed to evaluate the use of naphthaleneacetic acid (ANA) and 6-benzylaminopurine (BAP) in the *in vitro* growth of *Pseudolaelia corcovadensis*, *Macradenia paraensis* and *Maxillaria brasiliensis*.

Material and Methods

The experiment was carried out at the Plant Tissue Culture Laboratory (LCTV) using three species of orchids endemic to Brazil: *Pseudolaelia corcovadensis (PC)*, *Macradenia paraensis (MP)*, and *Maxillaria brasiliensis (MB)* (**Figure 1**).

The study was performed using explants from plants grown in MS culture medium (Murashige; Skoog, 1962) with macronutrients and micronutrients at a concentration of 50%, plus 2g L^{-1} of activated charcoal and 30g L^{-1} of sucrose. After preparing the solution, the pH was adjusted to 5.7±0.3, and the media was gelled with 3.5g L-1 of agar before autoclaving.

The experiment was set up in a completely randomized design in a 4 x 3 factorial scheme, with a total of 12 treatments and 10 replicates each: T0 - MS medium (100%); T1 - MS medium (100%) + 0.5 mg L^{-1} of naphthaleneacetic acid (ANA); T2 - MS medium (100%) + 0.5 mg L^{-1} of ANA and 0.5 mg L^{-1} of 6-benzylaminopurine (BAP); and T4 - MS medium (100%) + 0.5 mg L^{-1} of BAP.

The experimental plot consisted of one explant per 100 mL flask, sealed with a polypropylene lid, containing 20 mL of culture medium.

The explants were immersed in 70% alcohol (v:v) for 1 minute, then in a 1% active chlorine solution for 30 minutes, along with three drops of neutral detergent (Tween 20®). The triple wash was done in a laminar flow chamber using distilled and autoclaved water.

The experiment was maintained under a 16-hour photoperiod with a temperature of 25±3°C, relative humidity of 45 to 46% and photosynthetic active radiation of 45-55 mol m^{-2} s^{-1}.

The explants were cultivated for 120 days assessing the following growth variables: plant height (ALT), obtained by measuring from the base of the pseudobulb to the largest leaf, using a ruler graduated in cm; number of leaves (NF) obtained directly counting all the fully opened leaves; and number of roots (NR) obtained directly counting all the roots present in the plant.

The data was analyzed using Sisvar 5.8 software (Ferreira, 2011). The hypothesis of normality was tested using the Shapiro-Wilk test, analysis of variance (ANOVA) was carried out, and the F test was applied at 5% probability to detect differences between factors and, when found, the Scott-Knott test at 5% probability compared the differences between the means of the treatments.

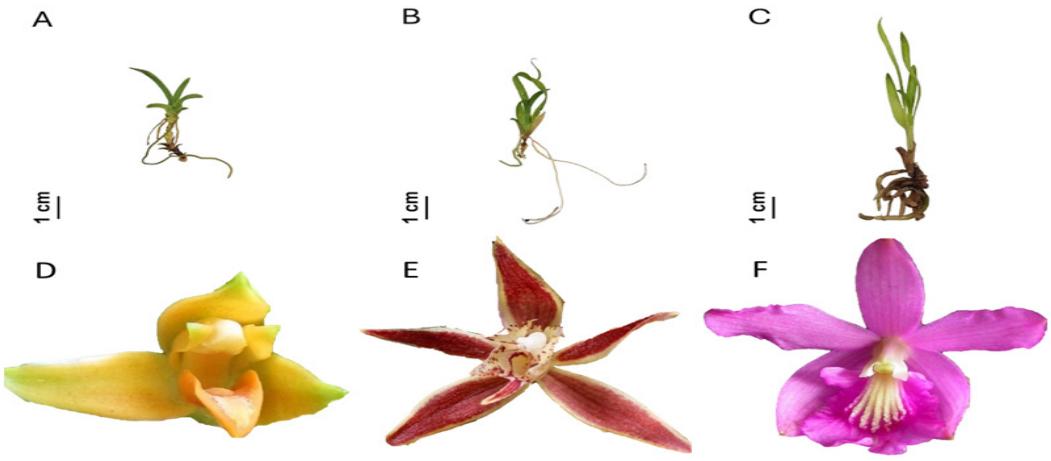

Figure 1. Explants and flowering of *Maxillaria brasiliensis* (A and D), *Macradenia paraensis* (B and E) and *Pseudolaelia corcovadensis* (C and F).

Results and Discussion

The experiment highlighted a high contamination rate of the inoculated plants during the repotting cycles for *in vitro* multiplication of the orchids (**Figure 2**A, B, and C).

The contaminations occurring during orchid multiplication were mainly by endophytic fungi and bacteria (**Figure 3**), which led to partial or total necrosis of the explants. After two repotting cycles (240 days), which was a short time, the researchers produced a sufficient quantity of explants to perform the experiment.

Contamination of the culture medium of plants inoculated *in vitro* can be the result of various factors, such as the use of contaminated explants, incorrect tissue culture techniques, poor hygiene in the laboratory environments, or the use of contaminated instruments when handling the cultures (Thomas et al., 2011; Thomas; Aswath, 2014; Esposito-Polesi, 2020). It is crucial to note that the contamination intensity varies according to the origin and type of explant used, the collection site, and the time of year (Dutra et al., 2009).

There is a need to carry out more studies and research to avoid major *in vitro* contamination of these species or genera used in the study since no articles address this for these species. Based on new alternatives, such as using fungicides and antibiotics in the composition of the culture medium or the application after inoculation. These are less complex strategies that can reduce the number of contaminations in an *in vitro* study.

The *Maxillaria brasiliensis* species showed statistically similar results for the variable number of leaves when analyzing the treatments within each species, ranging from 2.47 to 2.54, with an overall average of 2.52 leaves (**Table 1**). In the case of *Macradenia paraensis*, treatments T1, T2, and T3, with 2.76, 2.43, and 2.24, respectively, were superior to T0, with 0.71 leaves (Table I). In *Pseudolaelia corcovadensis*, the T1 result (3.54) showed a statistically significant difference to the others (Table I).

When looking at the different treatments for the three species used, in T0, the *Maxillaria brasiliensis* and *Pseudolaelia corcovadensis* species showed statistically significant values (Table I). As for T1, only the result for the orchid *Pseudolaelia corcovadensis* (3.54) was superior to the others and considered the best treatment (Table I). When analyzing treatments T2 and T3, the results obtained for MB and MP were statistically similar and significant, with the highest values for both treatments (Table I).

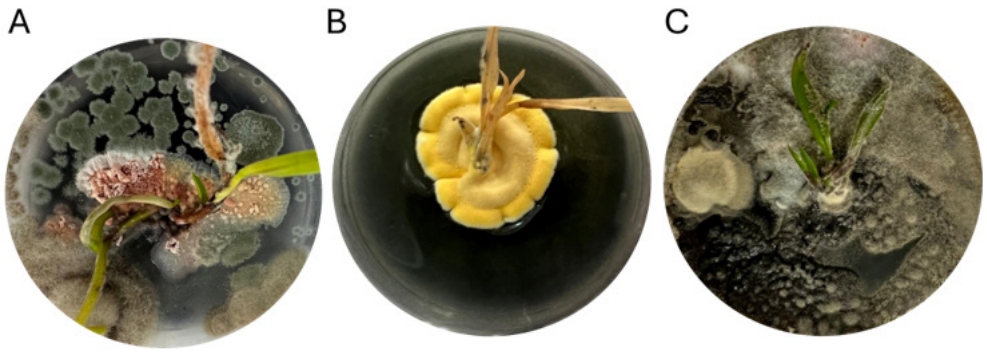

Figure 2. Contamination by exogenous endophytic fungi and bacteria in the *in vitro* multiplication of the orchid species studied

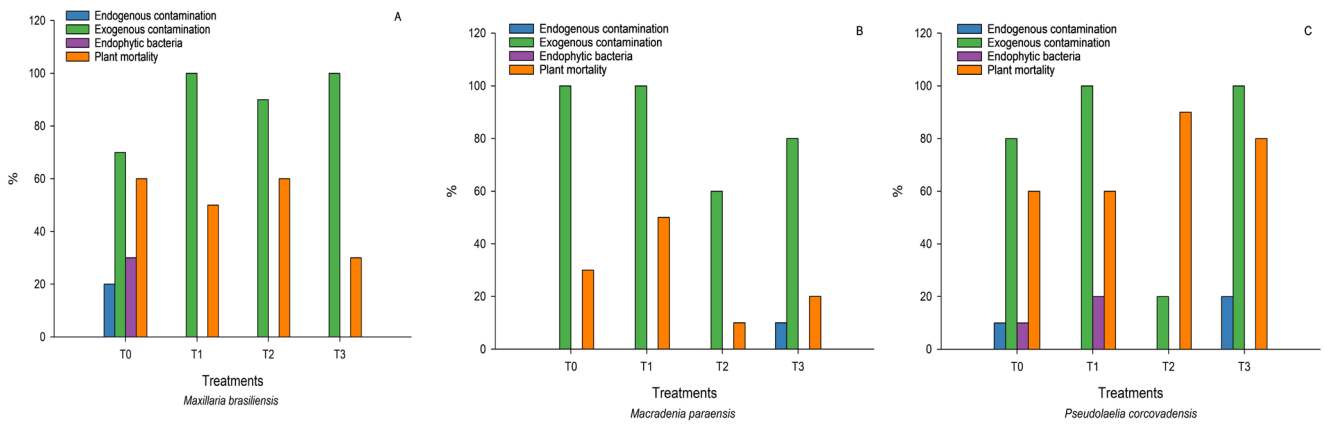

Figure 3. Contamination of the culture medium in the species *Maxillaria brasiliensis* (A), *Macradenia paraensis* (B), and *Pseudolaelia corcovadensis* (C).

Table 1. Number of leaves, roots, and height of *in vitro* plants of *Maxillaria brasiliensis* (MB), *Macradenia paraensis* (MP), and *Pseudolaelia corcovadensis* (PC) according to treatments

Treatments	Number of Leaves			Overall Average
	MB	MP	PC	
T0**	2.47 Aa*	0.71 Bb	1.96 Ba	1.77 B
T1	2.54 Ab	2.76 Ab	3.54 Aa	2.89 A
T2	2.52 Aa	2.43 Aa	0.71 Cb	2.07 B
T3	2.53 Aa	2.24 Aa	0.71 Cb	1.98 B
Overall Average	2.52 a	2.05 b	1.73 c	2.17

Treatments	Number of Roots			Overall Average
	MB	MP	PC	
T0**	2.22 Aa*	0.71 Cb	1.22 Bb	1.45 C
T1	2.26 Ab	2.68 Aa	3.09 Aa	2.62 A
T2	2.29 Aa	2.00 Ba	0.71 Bb	1.85 B
T3	2.32 Aa	2.43 Aa	0.71 Bb	1.95 B
Overall Average	2.28 a	1.98 b	1.43 c	1.97

Treatments	Plant height (cm)			Overall Average
	MB	MP	PC	
T0**	2.76 Ab*	0.00 Bc	6.75 Aa	3.14 B
T1	2.60 Ab	4.00 Ab	6.30 Aa	4.06 A
T2	2.11 Ab	3.85 Aa	0.00 Bc	2.02 C
T3	2.61 Ab	4.66 Aa	0.00 Bc	2.60 C
Overall Average	2.47 b	3.22 a	3.26 a	2.89

*Equal capital letters between rows in the column and equal lower case letters between columns in the row do not differ by the Scott-Knott test at 5% probability.**T0: MS treatment (control); T1: MS + ANA treatment; T2: MS + ANA + BAP treatment; T3: MS + BAP treatment.

Looking at the results of the treatment split within the *Maxillaria brasiliensis* species, it can be seen that there is statistical similarity between the values, which range from 2.22 to 2.32, with an overall average of 2.28 roots (Table 1). For the MP species, treatment T0 showed 0.71 roots, a much lower value statistically compared to the others (Table 1). The results of 0.71 for treatments T2 and T3 for the PC orchid were the lowest, with a discrepant difference with T1, which had 3.09 roots (Table 1).

When unfolding the T0 treatment on the MB species, it can be seen that the result of 2.22 was the highest value obtained (Table 1). For T1, the species *Macradenia paraensis* and *Pseudolaelia corcovadensis* showed statistically significant results, with the PC value (3.09) being the highest (Table 1). In treatments T2 and T3, the MB and MP orchids were also found to have significant numbers, showing that the *Pseudolaelia corcovadensis* species had a value (0.71) different from the other two species.

As in the other analyses of variance, the *Maxillaria brasiliensis* species showed significantly similar results analyzing plant height, with an overall average of 2.47 (Table 1). The treatment T0 of the MP orchid was the worst, with a zero result, showing no plant growth (Table 1). In the results for CP, the treatments T0 and T1 showed values of 6.75 and 6.30, respectively, with a difference from T2 and T3, which were zero (Table 1).

When considering the breakdown of the T0 treatment by species, there were statistically significant differences between the values of PC (6.75), MB (2.76), and MP (0.00) (Table 1). In T1, the result of 6.30 obtained for *Pseudolaelia corcovadensis* is considered the highest (Table 1), unlike T2, where its result was zero and the species *Macradenia paraensis* stood out with a value of 3.85 (Table 1). The same occurred in T3, where the MP value (4.66) is the highest compared to the others, and the PC value is zero (Table 1).

Adding plant growth regulators to the *in vitro* culture media can compensate for possible low levels of endogenous hormones in the explants and stimulate the multiplication or elongation of the aerial part (Nascimento, 2007).

Adding ANA to the growing medium had the opposite effect to BAP, fostering the development of the plant's apical meristem. Suzuki et al. (2004) assessed the stioling of *Catassetum fimbriatum*. The authors found that the height of the apical meristem was higher when auxin was added *to in vitro* cultivation, as observed in this experiment for *Macradenia paraensis* and *Pseudolaelia corcovadensis.*

The MS culture medium (Murashige; Skoog, 1962) is the standard in *in vitro* plant cultivation. Its composition includes macronutrients, micronutrients, vitamins, Fe-EDTA, sucrose, and agar necessary for their development and growth, and activated charcoal is added when working with orchids (Miyata et al., 2014).

As obtained by Miyata et al. (2014), the MS culture medium showed promising results in the cultivation of hybrids of *Cattleya walkeriana, Cattleya intermedia*, and *Brassolaelio cattleya*, where the medium increased the number of plant sprouts and contributed to greater root length. In the MB species, the MS 100% culture medium proved to be the most advantageous for the species, being highly concentrated, with high concentrations of nitrogen (840.90 mg L^{-1}), calcium (119.98 mg L^{-1}), zinc (1.96 mg L^{-1}), and manganese (1.08 mg L^{-1}).

According to Taiz et al. (2021), naphthaleneacetic acid (ANA) is the most commonly used auxin to stimulate the rooting of the explant, the growth of the aerial parts, and the maintenance of a plant with apical dominance. Auxins are responsible for promoting cell division, unlike the effect of cytokinins, stimulating the cell differentiation (Cruz et al., 2024).

Supplementing an *in vitro* growing medium for most orchid species can stimulate rooting and forming good-quality roots. Rooting is crucial: a healthy root system is essential for adapting and surviving plants subjected to *ex vitro* conditions (Liu et al., 2013). Several studies demonstrate the importance of auxins *in the in vitro* rooting of orchids, as observed by Souto et al. (2010), in which adding 0.5 and 1.0 mg $L^{-1}$of ANA to Knudson C medium significantly stimulated the formation of roots and leaves of *Cattleya bicolor*, while the absence of ANA was significantly inhibitory to root development.

Xiang *et al.* (2003) observed that a higher survival rate was obtained for the species *Cymbidium sinenis* acclimatizing plants after induction *of in vitro* rooting adding 1 mg L^{-1} of ANA to the growing medium.

Conclusion

For the *Maxillaria brasiliensis* species, 100% MS medium is recommended for better plant growth. For *Macradenia paraensis* and *Pseudolaelia corcovadensis*, MS medium (100%) + 0.5 mg L^{-1} of naphthaleneacetic acid (ANA) (T1) proved to be the most satisfactory compared to the others. Studies on contamination control in orchid inoculation should be encouraged to ensure good *in vitro* multiplication of these species.

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