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REGULAR ARTICLE

Oxidation and viability of *Vanilla phaeantha* explants exposed to ethylene during *in vitro* cultivation.

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TFC: Experimental data collection; Data storage; Data analysis; Literature review; Manuscript writing. LGRP: Experimental data collection; Data storage; Data analysis. ESO: Experimental data collection; Data storage; Data analysis. ASA: Experimental data collection; Literature review; Manuscript writing. ARCN: Conceptualization; Experimental data collection; Data storage; Data analysis; Literature review; Manuscript writing. MT: Conceptualization; Experimental data collection. MVR: Conceptualization; Manuscript revision; Supervision; Funding acquisition

Abstract

Vanilla phaeantha is a native species of Brazil with great potential for both the fragrance industry and ornamental cultivation. However, the propagation of this orchid through traditional methods is limited, making in vitro micropropagation a promising alternative. This study aims to evaluate the in vitro micropropagation of *Vanilla phaeantha*, with a focus on the effects of exogenous ethylene application on the viability and development of leaf and root explants. This research was carried out in June 2024, utilizing explants obtained from plants grown in the Federal University of Lavras' Botanical Garden. The explants underwent a disinfection process and were inoculated in Murashige and Skoog (MS) medium, supplemented with different ethylene concentrations (0, 15, 20, 25, and 30 ppm). The explants were cultivated under controlled temperature and continuous light. The results showed that, regardless of the explant type or ethylene concentration, all explants exhibited complete oxidation, with no callus formation, shoots, or roots. This suggests that the MS medium, although suitable for many plant species, may not be ideal for *Vanilla phaeantha* without modifications, such as the addition of antioxidants or other growth regulators. The study indicates that ethylene may not be beneficial for the in vitro propagation of *Vanilla phaeantha*, as its exogenous application seems to induce oxidative stress, hindering the regeneration of plant structures. In conclusion, further research is needed to optimize in vitro culture conditions, possibly focusing on alternative hormonal treatments and adjustments to the culture medium to improve the propagation success of this orchid species.

Keywords

Orchid; MS culture medium; Floriculture; Conservation.

Introduction

The genus *Vanilla* stands out not only for its unique botanical characteristics but also for its vast, yet unexplored, potential. Comprising over 120 species, many of which remain poorly studied or are not even included in commercial chains, this group of perennial vines features succulent stems, aerial and subterranean roots, as well as a unique floral structure (Karremans et al., 2023). The richness of the Orchidaceae family, exemplified by species such as those in the Vanilla genus, highlights its biological and economic potential, which is still underexplored but full of possibilities for research and innovation (Oliveira et al., 2022).

Understanding the genotypic and phenotypic diversity of the genus Vanilla is essential for developing strategies that promote the sustainable production of vanilla. Wild species of Vanilla, which have not yet been commercially exploited, represent a significant potential for responsible and environmentally balanced market introduction (Barragán-Ocaña et al., 2024). The success of new crops will largely depend on the training of producers to properly manage the cultivation and processing of vanilla beans, ensuring quality. This progress is already observed in some regions of Brazil, particularly in the Midwest, where certain species of Vanilla are gaining recognition for their commercial value (Silva Oliveira et al., 2022).

Among the Vanilla species native to Brazil, Vanilla phaeantha stands out for possessing an enzymatic structure favorable to the production of vanillin and other phenolic compounds essential for the characteristic flavor of vanilla. Studies indicate that the vanillin content of this species is comparable to that of Vanilla planifolia, which is widely cultivated for commercial purposes (Oliveira et al., 2022; Barragán-Ocaña et al., 2024). In addition to its potential for the fragrance and flavor industry, Vanilla phaeantha also attracts ornamental interest, being an orchid found in the phytogeographical domains of the Caatinga, Cerrado, and Atlantic Forest, with a predominant occurrence in resting areas (Barberena et al., 2021).

In this context, in vitro cultivation techniques have been consolidating as an important biotechnological tool for both species' conservation and increased vanillin production. This method enables the production of plants with broad genetic

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diversity in a relatively short period, while also ensuring high phytosanitary quality (Soares et al., 2020). Among the various molecules used in in vitro cultivation, ethylene plays a key role as a plant growth regulator, potentially exerting both positive and negative effects depending on its concentration and the developmental stage of the culture (Neves et al., 2021).

This phytohormone plays a crucial role in the regulation of various physiological processes, such as germination, cellular differentiation, senescence, and stress response (Jangra et al., 2023). In the in vitro environment, where conditions are highly controlled and ventilation is often limited, the accumulation of ethylene can become an adverse factor, compromising plant development (Pasternak & Steinmacher, 2024).

At low concentrations, ethylene can stimulate processes such as organogenesis and somatic embryogenesis, promoting the formation of shoots and adventitious roots, as well as contributing to the adaptation of plants to osmotic and oxidative stress. However, at elevated levels, this phytohormone can inhibit growth, induce hyperhydricity, and cause premature abscission of vegetative structures, compromising both regeneration and acclimatization of seedlings (Polivanova & Bedarev, 2022; Pasternak & Steinmacher, 2024). In this context, this study aims to evaluate the in vitro micropropagation of *Vanilla phaeantha*, with a focus on the effects of exogenous ethylene application on the viability and development of leaf and root explants.

Materials and methods

Plants of *Vanilla phaeantha* cultivated in the crowded orchidarium at the Botanical Garden of the Federal University of Lavras were identified and selected (Figure 1). From these plants, leaf and root explants were collected and subjected to micropropagation tests to evaluate their in vitro viability.

Figure 1. Mother plant of *Vanilla phaeantha* cultivated at the Botanical Garden of the Federal University of Lavras, from which the explants were obtained.

Leaf explants were obtained from the median portion of each leaf, with the central vein removed within a 1 cm radius. For root explants, selection occurred in regions close to the meristematic portion, with a length of 1 cm (Figures 2 and 3).

After selection, the explants were subjected to a decontamination process using 2% sodium hypochlorite for 20 minutes, followed by immersion in 70% ethanol for 10 minutes. They were then washed three times with autoclaved distilled water and subsequently inoculated into the *in vitro* culture medium.

For the composition of the *in vitro* culture medium, the MS medium (Murashige & Skoog, 1962) was used, supplemented with 6 g L^{-1} of agar and 30 g L^{-1} of sucrose. The pH of the medium was adjusted to 5.8, and the medium was then autoclaved to ensure sterility before the inoculation of the explants. After the medium solidified, different concentrations of ETHREL® were added through a sterile filter, characterizing the ethylene variation treatments (0, 15, 20, 25, and 30 ppm).

After inoculation, the bottles were transferred to a growth room with a controlled temperature of 25 ºC and continuous light. The development of the explants was evaluated based on their oxidation after 30 days of in vitro cultivation.

The experimental design was completely randomized with a 2 x 5 factorial. Two types of explants (root and leaf) and four ethylene concentrations, along with the absence of this hormone in the medium as a negative control, were tested, with 30 repetitions per treatment. The data obtained were subjected to analysis of variance and the Skott-Knott test for mean comparison at a 5% significance level using the statistical software SISVAR (Ferreira, 2019).

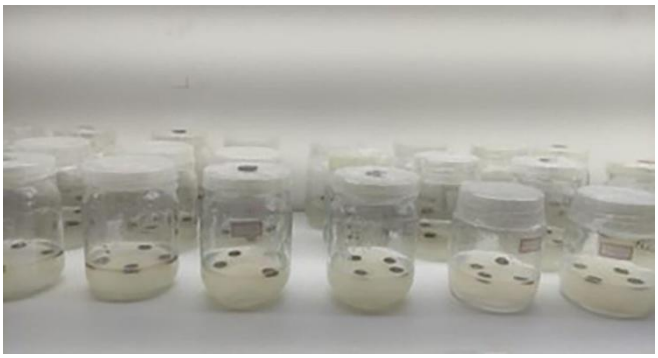

Figure 2. Leaf explants of *Vanilla phaeantha* with a 1 cm radius inoculated in MS medium with different concentrations of ethylene.

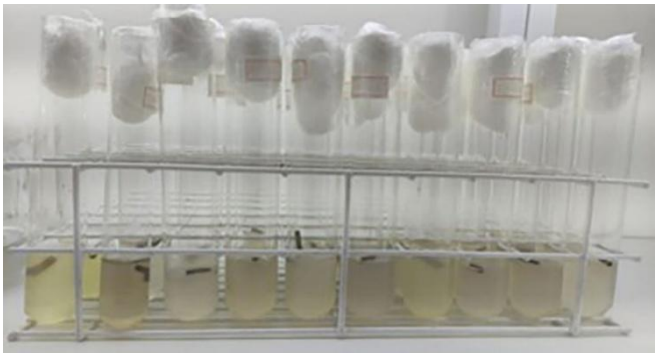

Figure 3. Root explants of *Vanilla phaeantha* with a length of 1 cm that were inoculated in MS medium with different concentrations of ethylene.

Results and discussion

After 30 days of cultivation, oxidation was observed in 100% of the plant material in all explants, regardless of the type or concentration of ethylene tested (Figure 4). Therefore, no callus, shoot, root, or other morphological structures, which are typically a result of in vitro cultivation, were formed. The oxidation of leaf and root explants of orchids cultivated in MS medium in vitro may be associated with a combination of endogenous and exogenous factors, which directly influence the viability and morphogenetic response of the tissues (Rathore et al., 2013).

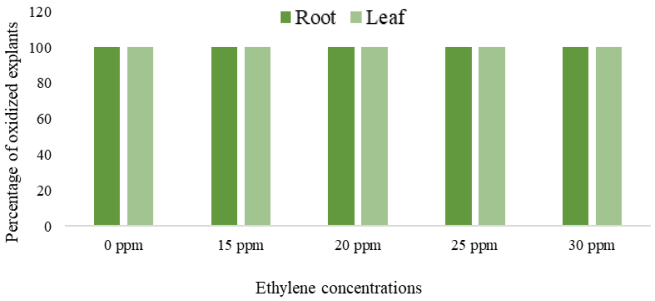

Figure 4. Percentage of oxidized root and leaf explants from *Vanilla phaeantha* orchids.

Ethylene has various physiological functions in plants, such as the development of shoots, roots, caulies, and other morphological structures, acting even before the formation of the plant (Ahammed et al., 2020). The induction of root formation through the use of ethylene has been tested in different species, as the importance of roots in plants is to absorb nutrients and water, aiding in their growth and development. Hormones such as ethylene and auxin have proven effective in root growth through the interaction between both and other hormones involved in this process (Hu et al., 2017). However, in some species such as Arabidopsis and rice species, the characteristics of ethylene or its precursors, such as 1-aminocyclopropane-1-carboxylic acid (ACC), act as inhibitors of primary root elongation when applied exogenously (Ruzicka et al., 2007). Similarly, in monocot plants such as maize, wheat, and sorghum, ethylene also inhibits the formation and development of primary roots (Yang et al., 2015). These studies have shown that ethylene can affect root development both by inhibiting the cells of the apical meristem and the cells in the root elongation zone (Qin & Huang, 2019), concluding that the cellular division of root morphological structures may be controversial depending on the species studied, as in some cases ethylene may be beneficial, while in others it can inhibit root growth or even cause oxidation of plant material, as was the case with the orchids in the present study.

Ethylene is naturally synthesized by plants and is known to cause senescence and abscission of leaves and flowers. It is involved in fruit ripening, contributes to both biotic and abiotic stress factors (Khan et al., 2017), and regulates the expression of various genes responsible for the development of morphological tissues. With regard to leaves, ethylene induces chlorophyll loss (Koukounaras et al., 2006), causing the leaves to yellow. As observed in *Vanilla phaeantha* orchids, the use of this hormone on in vitro leaf explants resulted in leaf oxidation, indicating that the role of exogenous ethylene in this plant structure is similar to the function of ethylene naturally produced by the plant.

Ethylene plays roles in stress signaling and the activation of metabolic pathways related to senescence and plant defense. Under normal conditions, oxidation in plant tissues occurs due to the accumulation of reactive oxygen species (ROS), often associated with lipid peroxidation and the degradation of phenolic compounds (Arumugam & Panneerselvam, 2012). Exposure to ethylene may have stimulated excessive ROS production through the activation of enzymes such as peroxidase and polyphenol oxidase, which are common in tissue browning processes. Another relevant factor is the possible inhibition of endogenous antioxidants, such as catalase and superoxide dismutase, which would normally neutralize free radicals. This antioxidant system dysregulation may occur due to the sensitivity of orchid explants, which have specialized structures with low cell division rates in vitro, making them more susceptible to oxidative stress (Juras et al., 2020). Oxidation may also have been caused by a secondary signaling cascade, such as the interaction between ethylene and other hormones like cytokinins, which also modulate stress responses (Cunha Neto et al., 2025).

Initially, oxidation is often caused by the presence of phenolic compounds, which are released by plant tissues when exposed to stress or mechanical damage during the in vitro establishment phase. These compounds can be rapidly oxidized, resulting in the formation of products that cause the darkening of explants, which can be toxic to the cells and inhibit the formation of new structures. Furthermore, the composition of the MS culture medium, while generally favorable for the growth of many plant species, may not be ideal for orchids, which often require specific adjustments in the concentration of mineral salts, growth regulators, and antioxidant agents to prevent oxidation and promote tissue regeneration (Arumugam & Panneerselvam, 2012).

The absence of formed structures in the explants may also result from the lack of specific morphogenic factors, such as an adequate concentration of auxins and cytokinins, which are crucial for the induction of organogenesis or somatic embryogenesis in orchids. Therefore, oxidation and failure in the formation of structures may indicate the need to optimize the culture medium and include antioxidant agents or adjust the concentration of growth regulators to improve the in vitro response of *Vanilla phaeantha* explants (Chang, 2007).

The inefficiency of ethylene in root explants showed that the response of this hormone in vitro can be controversial depending on the species studied, as in some species it participates in signaling pathways involving metabolism, leading to the development of primary and lateral roots (Neves et al., 2024), but not in the case of *Vanilla phaeantha* orchids. This occurred because ethylene stimulates the accumulation of auxins in the meristematic zone as well as in the root elongation zone, inhibiting cell proliferation in this region, thereby impairing root formation. In the leaf explant, ethylene showed the same response as known in other species (Gepstein & Thimann, 1981), participating in senescence, chlorophyll loss, and toxicity, leading to the complete oxidation of the plant material. In species such as mustard, for example, the use of Ethephon, an ethylene-donor compound, at low concentrations induced an increase in leaf area, while at high concentrations it inhibited it (Khan, 2005; Khan et al., 2008).

In other words, the response to the use of this hormone depends on the concentration used, the species studied, and whether it is applied exogenously or not, as it can result in the development of plant morphological structures, senescence, or even toxicity, causing oxidation, depending on the factors mentioned.

To minimize oxidation in vitro orchid cultures, strategies such as adding antioxidants (ascorbic acid or activated charcoal) to the culture medium can be employed to neutralize free radicals and absorb phenolic compounds. Adjusting cultivation conditions, including reduced light intensity, moderate temperature, and a slightly acidic pH (5.6–5.8), may also help mitigate oxidative stress. Furthermore, the selection of young explants and pretreatment with antioxidant solutions can reduce oxidative enzyme activity (Juras et al., 2020).

Conclusions

The present study on the micropropagation of *Vanilla phaeantha* highlighted the difficulty of *in vitro* cultivation due to the oxidation of explants, regardless of their leaf or root morphogenesis and independent of the ethylene concentration tested. The results suggest that the MS culture medium, although widely used, may not be suitable for the cultivation of *Vanilla phaeantha* without additional modifications, such as the addition of antioxidants or other growth regulators.

Future testing possibilities could include alternative culture medium formulations for *Vanilla phaeantha*, incorporating different antioxidant agents to reduce explant oxidation. Additionally, investigating the synergistic effects of growth regulators with ethylene application may yield significant insights into morphogenetic control.

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