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# Effect of different sources and concentrations of carbohydrates on the morphological and physiological characteristics of *Vanilla planifolia* Jacks. $RH_{350}$

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**ABSTRACT.** Veracruz is the leading vanilla-producing state in Mexico; however, its plantations face significant challenges from pathogens like *Fusarium oxysporum* f. sp. *vanillae* (*Fov*). Therefore, it is crucial to develop an effective protocol to ensure the availability of *in vitro* plants of the $RH_{350}$ genotype, which exhibits 50% resistance to *Fov*. This genotype was developed through *in vitro* selection against *Fov* filtrates using the pathogenic strain *Fov* $H_3$. This study evaluated the effects of two concentrations (15 and 30 g $L^{-1}$) and five different carbon sources (maltose, fructose, sucrose, galactose, and glucose) on the morphological (number of shoots, leaves, and roots, shoot, leaf, and root length) and physiological (photosynthetic pigment content) characteristics of *in vitro* plants. This study was conducted to meet the growing demand for high-quality $RH_{350}$ plants. The results revealed significant differences among the carbohydrates and their respective concentrations; however, the 30 g $L^{-1}$ concentration of fructose yielded the greatest number of shoots and leaves. A similar concentration of maltose positively impacted shoot length, while 15 g $L^{-1}$ of this carbohydrate yielded the best results in terms of chlorophyll content. These findings emphasize the importance of both the concentration and the type of carbon source in micropropagating plants such as *V. planifolia*, $RH_{350}$.

**Keywords:** Sources of carbon; micropropagation; tissue culture; *in vitro,* photosynthetic pigments; orchids.

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## Introduction

*Vanilla planifolia* Jacks. is a highly sought-after orchid species used as a source of natural vanilla flavoring in various products, including food, beverages, pharmaceuticals, cosmetics, tobacco, and handicrafts (Velázquez-Rosas et al., 2025). However, the traditional propagation method for *V. planifolia*, stem cuttings, is inefficient, slow, and uneconomical. Thus, a faster propagation method, such as micropropagation, is needed to meet the growing demand for vanilla pods. While many *in vitro* culture protocols for *V. planifolia* micropropagation are currently available, more research is needed to optimize the components of the culture medium and increase the quantity and quality of plantlets produced (Ramos-Castella et al., 2014).

Carbohydrates are crucial for the growth and development of plants *in vitro* because of their essential composition and content. Plants grown *in vitro* lose some of their autotrophic characteristics because they cannot fully produce their own energy source and must rely on an external source of carbohydrates (Martins et al., 2019). Carbohydrates are also essential for intermediary and respiratory metabolism. Through various protein receptors, carbohydrates communicate with plants, alter gene expression that influences growth (Hernández-Bernal et al., 2022), and impact the quality of micropropagated plants (Chen et al., 2017).

Sucrose is the most widely used carbon source due to its beneficial effects in tissue culture (Flores-Hernández et al., 2017; Carrión-Pereira et al., 2019). It acts as a food source that sustains metabolism and provides carbon precursors for plant structural and functional components (Marino et al., 1993) as well as for optimal plant development (Muller et al., 2011). However, more recent studies have reported that plant cell cultures can utilize various mono- and disaccharides, including glucose, sucrose, and maltose (Fowler, 1982; Pérez et al., 2019). In some species, maltose has been found to be a superior carbohydrate source than sucrose

because it increases the morphology of dicotyledons, monocotyledons, and polycotyledons and promotes the development of apical and radical meristems (Pérez et al., 2019).

The *in vitro* response of crops to different types and concentrations of carbohydrates appears to depend on the genotype to a certain extent. Several studies have been conducted to define the most appropriate carbon source and concentration to meet the plant's energy demands for growth and development (Akyüz, 2025). However, there is currently insufficient information on the effect of carbohydrate doses and sources on optimizing the micropropagation process of *Vanilla planifolia* Jacks.

Currently, there is not enough information about the effects of different carbohydrate concentrations and sources to optimize the micropropagation process of *V. planifolia* (Cuenca & Vleitez, 2000). Most of the established protocols are based on the use of the Murashige & Skoog (1962) (MS medium), which considers appropriate the addition of 30 g $L^{-1}$ sucrose to the culture medium. However, previous studies have shown that lower doses of sucrose (15 and 20 g $L^{-1}$) can stimulate the number and quality of plantlets in this crop (Córdoba-Nieto et al., unpublished data). Therefore, the present study aimed to optimize the concentration and source of carbon given the $RH_{350}$ genotype's potential utility due to its 50% resistance to fungal filtrates of the virulent *Fov* $H_3$ strain. The goal is to produce high-quality $RH_{350}$ seedlings in large quantities. This will help meet the significant demand for this valuable species for commercial exploitation.

## Materials and methods

### Plant material, propagation, and culture conditions

For this study, four-month-old *in vitro* plants of *V. planifolia* $RH_{350}$ (Figure 1) were selected from the Plant Tissue Culture Laboratory at the Institute of Biotechnology and Applied Ecology (INBIOTECA) at the University of Veracruz, Mexico. This genotype was obtained by *in vitro* selection against filtrates of the virulent $H_3$ strain of *Fov*, demonstrating 50% resistance (Ramírez-Mosqueda & Iglesias-Andreu, 2018). For mass multiplication of this genotype, ten nodal segments of the selected plant material (Figure 1) were grown in a temporary immersion system (RITA® Vitropic), with an immersion frequency of five minutes every 24 hours, following the protocol proposed by Ramos-Castellá et al. (2014).

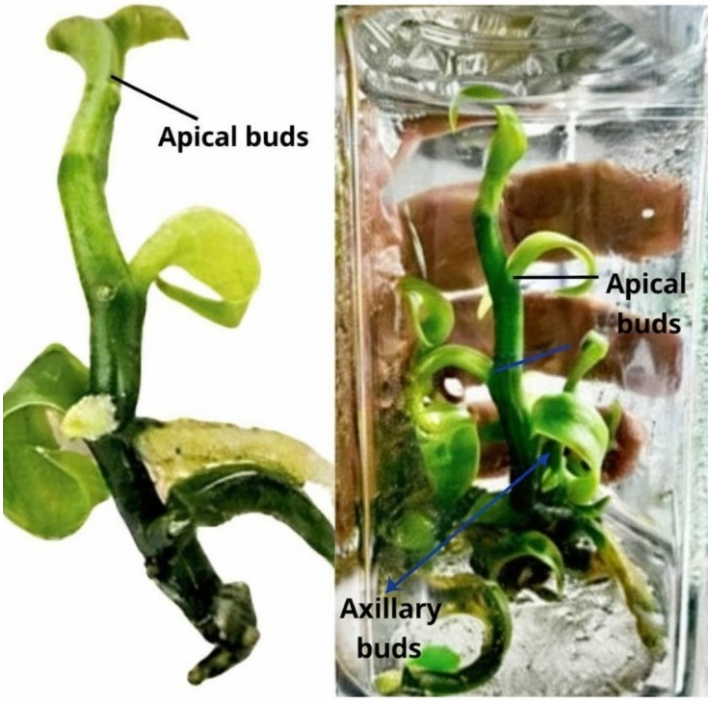


**Figure 1.** Mother plant of *Vanilla planifolia* $RH_{350}$ used in the propagation of the study material. Arrows indicate axillary buds.

The effects of two concentrations (15 and 30 g $L^{-1}$) of five distinct carbon sources—maltose, fructose, glucose, and galactose—were assessed using a completely randomized experimental design (Table 1). A total of 180 samples were obtained by using five flasks for each treatment, with three explants in each flask, for a total of 20 shoots per treatment.

**Table 1**. Concentrations and carbon sources studied in *Vanilla planifolia* $RH_{350}$.

| Treatments (T) | Carbon sources | Concentration (g $L^{-1}$) |
|---|---|---|
| T1 | Fructose | 30 |
| T2 | Fructose | 15 |
| T3 | Glucose | 30 |
| T4 | Glucose | 15 |
| T5 | Maltose | 30 |
| T6 | Maltose | 15 |
| T7 | Galactose | 30 |
| T8 | Galactose | 15 |
| T9 | Sucrose | 30 |
| T10 | Sucrose | 15 |

Using the Murashige & Skoog (1962; Sigma-Aldrich, MS) basal medium, 15 or 30 g $L^{-1}$ of carbohydrate treatment, 50 mg $L^{-1}$ of cysteine hydrochloride (Sigma-Aldrich), and 2.1 mg $L^{-1}$ of 6-benzylaminopurine (BAP) (Sigma-Aldrich) were used (Table 1). After adjusting the pH to 5.8 ± 0.01, the culture medium was autoclaved at 1.5 kg $m^{-2}$ at 121°C for 15 minutes. The cultures were then incubated for 50 days at 25 ± 2°C under a 16/8 hours light/dark photoperiod with a fluorescent light intensity of 80 µmol $m^{-2}$ $s^{-1}$.

After 30 days of cultivation, the number of shoots generated per explant in each treatment was counted. The length of each developed shoot was measured using a millimeter-sized leaf positioned at the bottom of a Petri dish. Following five weeks of cultivation, the number, length, number of leaves, length of the leaves, number of roots, and length of the roots of the formed shoots were assessed.

Similarly, photosynthetic pigment content was evaluated using the methodology of Porra et al. (1989). A 0.2 g sample of fresh leaf tissue was used for this purpose. After macerating the tissue in a sterile mortar cooled with 20 mL of a 1:1 v /$v^{-1}$ solution of 80% acetone and 20% ethanol, it was centrifuged for 12 minutes at 6,000 rpm. The amounts of carotenes at 441 nm, chlorophyll B at 645 nm, and chlorophyll A at 663 nm were measured in the obtained supernatant. Lastly, photosynthetic pigment content was calculated using the following formulas:

$$\text{Chlorophyll A} = [(12.25 \times A_{663} - 2.25 \times A_{645})] \times V/100 \times W$$

$$\text{Chlorophyll B} = [(20.30 \times A_{645} - 4.91 \times A_{663})] \times V/100 \times W$$

$$\text{Chlorophyll A + B} = [(7.34 \times A_{663} + 17.76 \times A_{645})] \times V/100 \times W$$

$$\text{Carotenes} = [(4.46 \times A441 - (\text{Chlorophyll A} + \text{Chlorophyll B})] \times V/100 \times W$$

where: V is the total volume of the acetone and ethanol extract (mL), and W is the total fresh mass (g) of the plant tissue used for the acetone and ethanol extract.

### Statistical analysis

A log-transformation analysis of the data was conducted to check for normal distribution, and each experiment was run at least three times. Tukey's HSD post-hoc test was used after a two-way ANOVA for statistical analysis. Significant results were defined as a p-value of less than 0.05. For statistical analysis, R software (version 4.3.3) was used.

## Results and discussion

The induction of multiple shoots was significantly affected, as observed from the fifth week of *in vitro* culture (Figure 2). There were noticeable variations in carbohydrate concentrations. Specifically, the 30 g $L^{-1}$ concentration of fructose produced the most shoots when compared to the other carbon sources evaluated (Figure 2A). Moreover, fructose and maltose significantly increased the number of leaves produced, according to the effects of various carbon sources and concentrations (Figure 2B). Additionally, we found a positive impact of maltose on the number of roots that developed at both concentrations (Figure 2C).

Significant differences in shoot length were detected among the carbohydrate sources and concentrations (Figure 3A). Concentrations of 15 g $L^{-1}$ and 30 g $L^{-1}$ of maltose, as well as 30 g $L^{-1}$ of glucose and fructose, had a positive effect, in contrast to the limited effectiveness of sucrose, which is typically used as a carbon source (Figure 3A). However, sucrose at both concentrations, along with glucose and maltose at higher concentrations, had a beneficial effect on leaf length (Figure 2B). Interestingly, lower concentrations of 15 g $L^{-1}$ glucose and galactose were the most effective in promoting greater root length (Figure 3C).

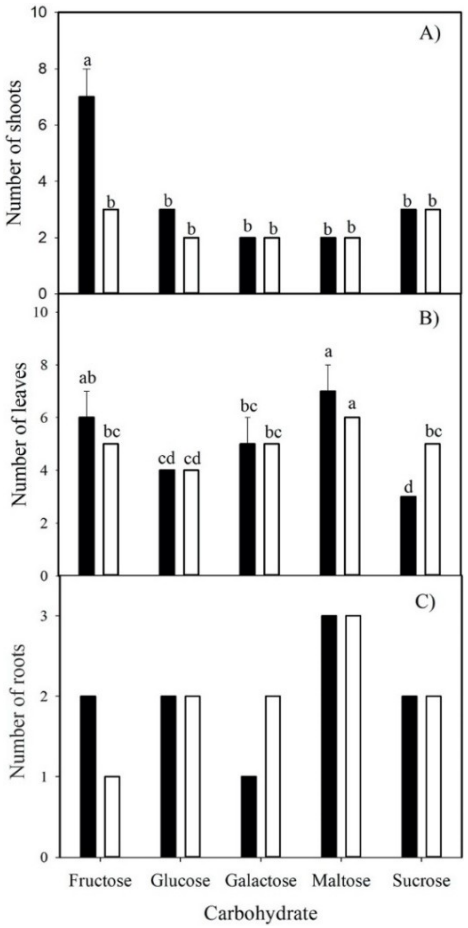


**Figure 2.** Number of shoots, leaves, and roots of *Vanilla planifolia in vitro* plantlets after five weeks of *in vitro* culture at different concentrations (15 and 30 g $L^{-1}$) and sources of carbohydrates (fructose, glucose, galactose, maltose, and sucrose). White bars correspond to the 15 g $L^{-1}$ concentration and black bars to the 30 g $L^{-1}$ concentration. Different letters above the bars indicate significant differences ($p < 0.05$) by Tukey's test. The standard error (SE) for n = 180 is represented by the error bars in the graphs.

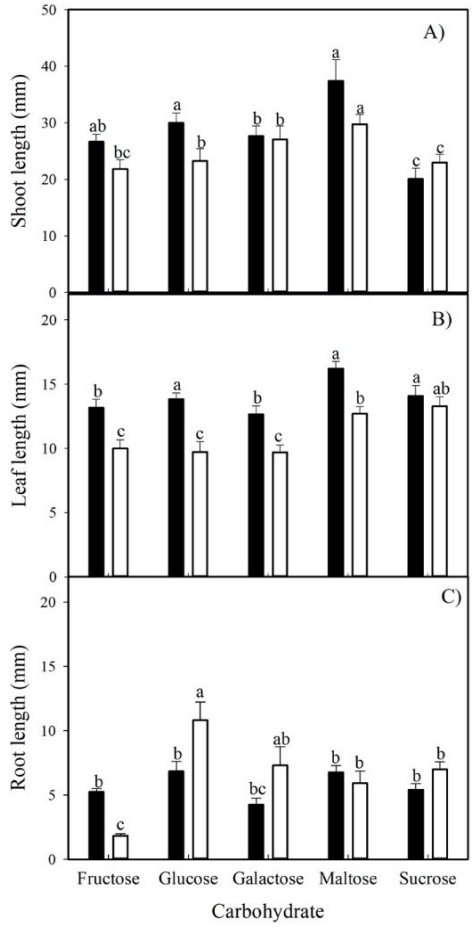


**Figure 3.** Shoot, leaf, and root length of *Vanilla planifolia* after five weeks, subjected to different concentrations (15 and 30 g $L^{-1}$) and sources of carbohydrates (fructose, glucose, galactose, maltose, and sucrose). White bars correspond to the 15 g $L^{-1}$ concentration and black bars to the 30 g $L^{-1}$ concentration. Different letters above the bars indicate significant differences ($p < 0.05$) by Tukey's test. The standard error (SE) for n = 180 is represented by the error bars in the graphs.

The effect of the two concentrations (15 and 30 g $L^{-1}$) and the five carbohydrate sources (maltose, fructose, glucose, sucrose, and galactose) on the morphological characteristics of *V. planifolia* $RH_{350}$ *in vitro* plants can be observed in Figure 4.

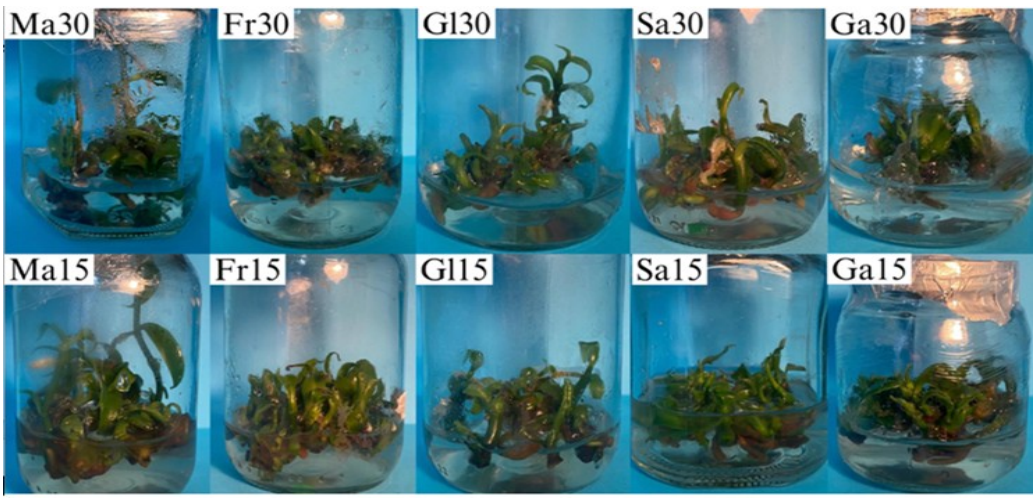


**Figure 4.** Effect of two concentrations (15 and 30 g $L^{-1}$) and five carbohydrate sources (maltose, Ma; fructose, Fr; glucose, Gl; sucrose, Sa; galactose, Ga) on the characteristics of *in vitro* plants of *Vanilla planifolia* $RH_{350}$.

When analyzing the impact on photosynthetic pigment content, the important effects of concentrations and sources of carbon were also observed in the in vitro plants of *V. planifolia* $RH_{350}$ evaluated (Figures 5A and 5B). The results showed that *in vitro* plants cultivated in the presence of maltose, glucose, and sucrose had the highest content of chlorophyll A (Figure 5A). Meanwhile, the highest content of chlorophyll B was achieved with a maltose concentration of 15 g $L^{-1}$ (Figure 5B). Similar findings were found for carotenoid content (Figure 5C). Notably, both maltose concentrations significantly impacted the total chlorophyll A and B content (Figure 5D).

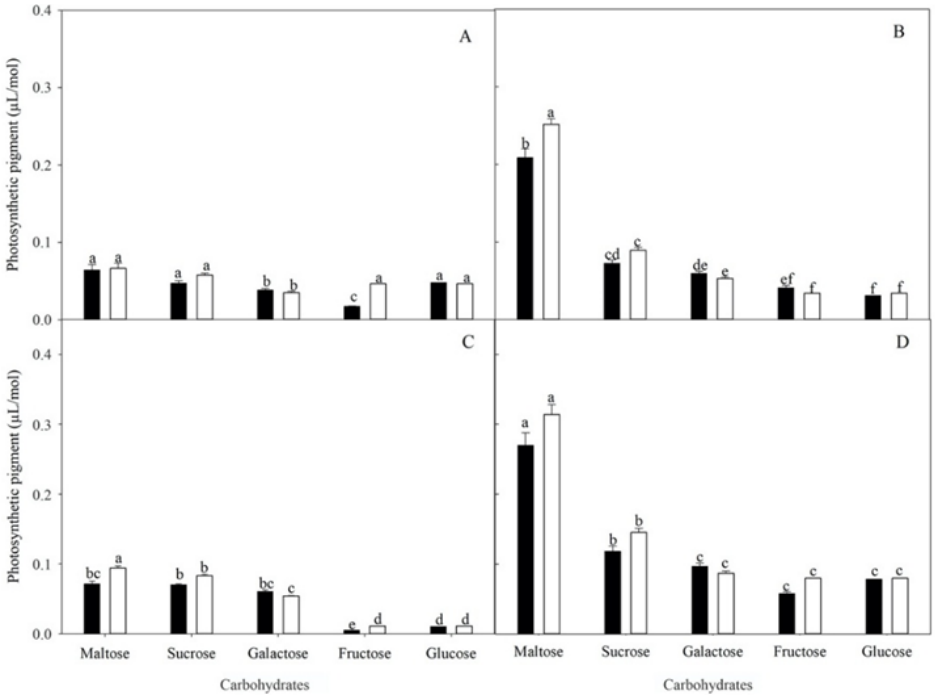


**Figure 5**. Effect of different concentrations (15 and 30 g $L^{-1}$) and carbohydrate sources (maltose, sucrose, galactose, fructose, and glucose) on the photosynthetic pigments content after five weeks of in vitro culture. White bars indicate a concentration of 15 g $L^{-1}$; black bars represent a concentration of 30 g $L^{-1}$. A) chlorophyll A; B) chlorophyll B; C) carotenoids; D) chlorophyll A+B. Different letters above the bars indicate significant differences ($p < 0.05$) by Tukey's test.

Various authors (Buah et al., 2000; Silos-Espino et al., 2023) have pointed out that carbohydrates in the culture medium act as an energy source that partially replaces photosynthesis. While this facilitates shoot growth, it can also inhibit the production of photosynthetic pigments. Figure 5D shows the highest chlorophyll A+B pigment content with the lowest maltose concentration tested.

The results of this study confirm the significant role that carbohydrate content and source play in the morphological and physiological characteristics of micropropagated plants of the *V. planifolia* genotype $RH_{350}$. Contrary to expectations, however, adding 30 g $L^{-1}$ sucrose to the culture medium was not the most effective source of carbon for promoting shoot induction or enhancing other morphological and physiological characteristics of the shoots. This finding is noteworthy, as sucrose is commonly used in micropropagation protocols, particularly for *V. planifolia*. Previous studies have reported that the initial sucrose concentration can affect growth and biomass accumulation in certain orchid species (Zahara et al., 2017).

High concentrations of certain carbohydrates can be toxic when serving as a carbon source and inhibit *in vitro* plant growth and development (Teixeira da Silva, 2004). According to recent studies by Nowak et al. (2004), sucrose can lead to cell disruption (plasmolysis) due to osmotic pressure. This disruption can hinder nutrient uptake and impair cell division in aerial parts (Garcia et al., 2002). It can also negatively affect chlorophyll content and stomatal structure, resulting in stunted plant growth. In the present study, it was found that the addition of 30 g $L^{-1}$ sucrose produced *in vitro* plants that were smaller and had fewer leaves (Figures 2 and 3).

Some authors have reported the beneficial effects of using various sugars as carbon sources for the *in vitro* cultivation of different plant species (Ondo-Ovono et al., 2009; Gabryszewska, 2010). In this context, fructose and glucose have been identified as two monosaccharides that decompose more easily than sucrose. However, aside from findings by Teixeira da Silva (2004) indicating that glucose enhances shoot and root growth, there

is limited information on their effects in micropropagation studies. These findings align with those of the present study.

In this study, we observed the beneficial effects of using maltose as a carbon source, like findings in other species of *Cymbopogon schoenanthus* L. (Abdelsalam et al., 2018). Maltose is a disaccharide composed of two glucose molecules that plays a role in sucrose synthesis and transport throughout the plant (Bernal et al., 2022). However, maltose hydrolyzes at a rate that is twenty times slower than that of sucrose (Blanc et al., 2002). The slower absorption and metabolism of maltose compared to sucrose may lead to reduced secretion of phenolic compounds into the culture medium. This phenomenon is often associated with detrimental effects in the *in vitro* culture of this species.

The type and concentration of carbohydrate chosen are crucial to optimizing both *in vitro* growth and subsequent plant survival during the acclimatization process. Based on our results, *in vitro* plantlets grown in 15 g $L^{-1}$ maltose solution are expected to have greater survival rates upon acclimatization.

Maltose has demonstrated remarkable potential in promoting somatic embryogenesis and facilitating plant regeneration in various species. Studies have highlighted its significant effects on *Triticum aestivum* L. (Ren et al., 2010); *Oryza sativa* L. (Joyia & Khan, 2013); *Pinus* spp. (Pullman & Bucalo, 2014); *Gossypium* spp. (Juturu et al., 2015; Kumar et al., 2015); *Saccharum officinarum* L. (Kaur & Kapoor, 2016); *Cymbopogon schoenanthus* L. (Abdelsalam et al., 2018); and *Urochloa brizantha* (A. Rich.) R. D. Webster (Carrión-Pereira et al., 2019).

The findings of this study emphasize the crucial role of maltose in the micropropagation of important species, such as *V. planifolia*. As we know, the use of maltose affects key enzymatic activities in cultured plant tissues, particularly those involved in carbohydrate metabolism. The presence of maltose in the medium can influence the activity and expression of maltase/α-glucosidase, responsible for degrading maltose into glucose (Silos-Espino et al., 2023). This could modify the plant's metabolic pathway to prioritize maltose use. When maltose is supplied externally, it can affect the plant's internal starch metabolism.

Furthermore, evidence suggests that adding maltose to the culture medium affects the balance between starch degradation and synthesis. This balance is crucial for energy management and plant development. This natural metabolic connection may explain why maltose often leads to better regeneration and more robust development in micropropagation, preparing seedlings more effectively for acclimatization. Nevertheless, maltose performed poorly in chestnut tissue culture (Akyüz, 2025). These findings confirm the importance of sugar concentration levels in plants because they enable effective responses to environmental changes. Plants utilize various signaling pathways to modify gene expression and protein activity, which can affect overall chromatin structure through histone modification (Zhu et al., 2020). In this regard, sugar levels regulate metabolism, hormonal responses, and overall plant development (Sami et al., 2016). Sugar levels also contribute to growth under stressful conditions (Hernández-Bernal, 2022).

These results will improve the quality of *in vitro V. planifolia* $RH_{350}$ plants, which are in high demand by producers due to their resistance to *Fusarium oxysporum* f. sp. *vanillae* (*Fov*), which causes significant damage to vanilla plantations. The results will also confirm the impact of maltose on the *in vitro* culture of this valuable genotype. Therefore, maltose is recommended for use in *V. planifolia* micropropagation due to its slow hydrolysis rate, which provides a stable, sustained energy source. This can help prevent osmotic stress and metabolic imbalances that could result from the rapid decomposition of sucrose.

Future studies will elucidate the role of enzymatic activities related to starch metabolism. These studies may explain how carbon sources achieve greater effectiveness in micropropagation valuable genotypes, such as *V. planifolia* $RH_{350}$.

## Conclusion

This study emphasizes the importance of carbon sources and concentrations in improving the *in vitro* propagation of *V. planifolia* $RH_{350}$, a genotype highly valued by vanilla producers due to its resistance to *Fov*. The study found that, compared to sucrose, which is typically used in micropropagation protocols for this species, certain carbohydrates, such as maltose, positively influenced shoot growth and the content of photosynthetic pigments. Based on these findings, we recommend proceeding with large-scale micropropagation of plants derived from *V. planifolia* $RH_{350}$ for future field evaluations by crop producers. Additionally, ongoing molecular studies will clarify the effects of varying doses and carbon sources on these processes, as well as the genes involved. This knowledge will enable the optimization of mass propagation for valuable genotypes, such as $RH_{350}$, and facilitate their rapid exploitation in the field.

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