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Research Article

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Posted Date: October 28th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11144478/v1

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Additional Declarations: No competing interests reported.

Arbuscular mycorrhizal fungi provide better agronomic performance in the production of 'Hass' avocado seedlings: a multivariate approach

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ABSTRACT

Among the fruit trees that stand out in the current scenario is the avocado tree, which is widely cultivated worldwide due to the nutraceutical properties of its fruit, which are beneficial to human health. In this context, the adoption of sustainable practices at different stages of the avocado production chain is essential, aiming at reducing inputs and minimizing environmental impacts. This research aims to validate the use of mycorrhizal fungi in the I) production of rootstocks and II) production of 'Hass' avocado seedlings. For this purpose, the following treatments were proposed: 0 $g.L^{-1}$ (T1), 2 $g.L^{-1}$ (T2), 4 $g.L^{-1}$ (T3), and 6 $g.L^{-1}$ (T4) of the commercial product EcoRhiza® (*Glomus* spp. 1 x 10^3 CFU g^{-1}), composed of four strains of arbuscular mycorrhizal fungi from the genus *Glomus* (*Glomus aggregatum*, *G. mosseae*, *G. intraradices*, and *G. etunicatum*) in a completely randomized design. The results showed significant responses when using the dose of 4 $g.L^{-1}$ for variables associated with gas exchange in rootstocks. For the seedlings, the same dose provided not only improvements in gas exchange variables but also increases in variables associated with chlorophyll indices, leaf area, and dry mass of the root system. These results indicate that

the dose of 4 g.L^{-1} of EcoRhiza® is the most effective for optimizing the development of 'Hass' avocado rootstocks and seedlings, contributing to a more sustainable avocado nursery production.

Keywords: *Persea americana* Mill., *Glomus* spp., Rootstocks, Seedlings, Chlorophyll.

1 Introduction

A cultivation of the avocado tree has been expanded and intensified at the worldwide and national levels (FAOSTAT, 2024) due to the high added value of the fruit (Bhore et al., 2021), versatility of use in the food industry (Charles et al., 2022; Nyakang'i et al., 2023) and cosmetics (Charles et al., 2022; Kupnik et al., 2023; Garcia-Vallejo et al., 2023), and for containing nutraceutical components and fatty acids, which offer scientifically proven health benefits (Sánchez-Quezada et al., 2023; Cárdenas-Castro et al., 2023). Among the genetic materials used, 'Hass' cultivation stands out for presenting commercially relevant characteristics that boost exports in all producing countries (AAB, 2024; Aguirre-López et al., 2024).

For the cultivation expansion to occur, it is extremely important to invest in the production of quality seedlings (Dorizzotto and Santoro, 2022). Nursery owners seek alternatives to meet the growing demand (Kasana et al., 2024), including reducing the time for seedling formation, which averages 8 to 10 months (Bender et al., 2012; Dorizzotto and Santoro, 2022).

In this sense, a good strategy lies in the use of biofertilizers containing arbuscular mycorrhizal fungi (AMF), which has increased in recent decades for being a low environmental impact management, with positive results in plant development (Diagne et al., 2020; Bennett and Groten, 2022; Mahbou et al., 2022). Furthermore, it is a sustainable alternative aligned with the Sustainable Development Goals (SDGs) proposed by the UN (Cf, 2015), aiming to meet the growing global demand for reducing chemical inputs (Messa and Savioli, 2021; Schaefer et al., 2021).

Mycorrhiza inoculation has also been widely studied and documented in the seedling production process of various crops (Schenck et al., 1984; Tawaraya, 2003; Feng et al., 2024). Benefits of applying *Glomus aggregatum*, *G. mosseae*, *G. intraradices*, and *G. etunicatum* were observed in the development of avocado rootstocks, through phosphate solubilization and greater allocation of carbohydrates and nutrients to the plants, resulting in a significant increase in leaf area compared to plants not treated with these microorganisms (Silveira et al., 2002; Bañuelos et al., 2017; Sotomayor et al., 2019). In addition, mycorrhizas have provided vegetative growth and formation of new roots in avocado seedlings also affected

by *P. cinnamomi*, the main pathogen of the crop (Silveira et al., 2002; Solórzano-Acosta et al., 2024; Shu et al., 2017).

Other crops have also shown improvements in agronomic characteristics through the use of the *Glomus* genus. In apple trees, mycorrhizas were effective in mitigating salt stress (Yang et al., 2014); while in rootstocks, they aided in suppressing soil-borne diseases in replant areas (Himaya et al., 2021), nutrient availability (Khan et al., 2022), and gas exchanges (Chandrasekaran et al., 2019; Khan et al., 2022). In citrus seedlings, the species *Glomus etunicatum* increased root weight by up to 250.6% compared to the control. For acerola seedlings, inoculation with *Gigaspora margarita*, *Glomus clarum*, and *Claroideoglomus etunicatum* favored an increase of up to 40% in aerial dry mass (Pinheiro et al., 2019), while in grapevines, beneficial effects were observed in the root system (Anzanello et al., 2011).

In this context, it is paramount to conduct a thorough analysis of the implementation of these new sustainable practices, aiming to highlight their real impacts on agriculture and promote them as essential inputs for nursery owners (Nelson and Rawlings, 1983; Krupnik et al., 2019). For this, various areas of statistics have advanced significantly, especially in studies employing multivariate approaches, seeking to comprehensively understand the results of experiments (Soulé et al., 2023).

Multivariate models can holistically capture the effects of the proposed treatments and show their respective interrelationships (Ajaykumar et al., 2023). By employing multivariate techniques, such as principal component analysis, factor analysis, and cluster analysis, it is possible to explore the complex interactions between multiple variables, providing a more complete and detailed view of the experimental data (Johnson and Wichern, 2007; Abdi et al., 2017; Backhaus et al., 2021).

These statistical approaches allow not only to assess the impact of a single variable on the outcome but also the identify hidden patterns, detect potential correlations between variables, and even predict future trends (Burdejova et al., 2023). For this reason, this study aimed to validate the adoption of AMF on the agronomic performance of 'Hass' avocado rootstocks and seedlings through a multivariate approach.

2 Materials and Methods

2.1 Influence of arbuscular mycorrhizal fungi on the development of avocado rootstocks

2.1.1 Acquisition of material and experimental conditions

The seeds were obtained from fruits of non-grafted (seedling) avocado mother plants from the Cordeirópolis-SP region and stored for two months in cold chamber conditions (5ºC and relative humidity of 85-90%) for better conservation until sowing.

The experimental period spanned from November 2022 to February 2023. During this period, the rootstocks were kept in a greenhouse located at the Department of Plant Production (LPV) – ESALQ/USP, under the climatic conditions described below (Fig. 1).

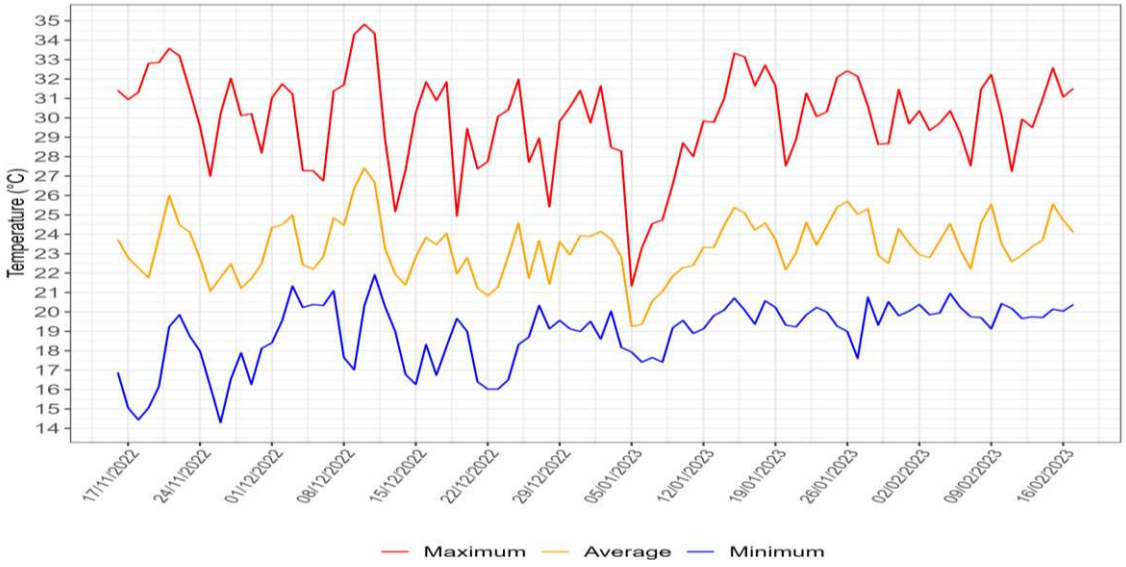


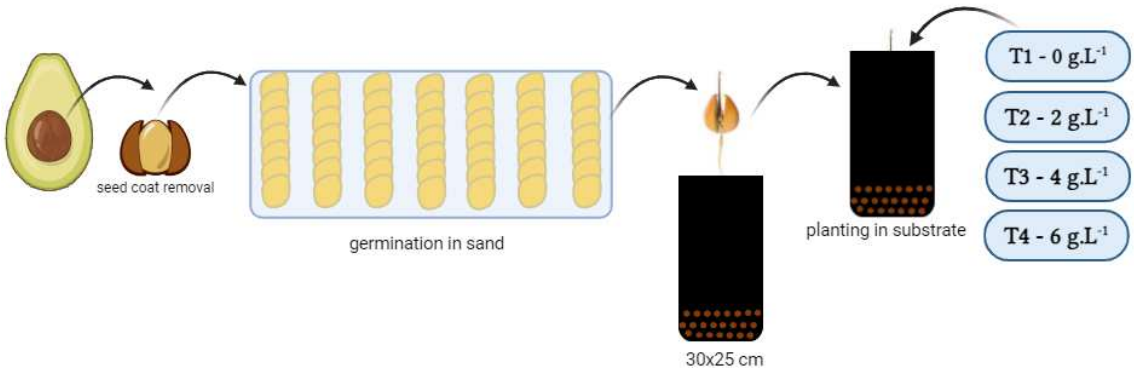
Fig. 1. The minimum, average, and maximum temperature during the experimental period. Experimental station ESALQ/USP. Piracicaba, Sao Paulo. Brazil.

For seed preparation, the tegument (Figure 2) covering the seeds was removed, and then the lower part was scarified using a grater, techniques that facilitate root emission and water absorption (Bergh, 1988). Next, 120 seeds were placed in Styrofoam boxes filled with a sand-based substrate, with openings at the bottom to facilitate drainage.

Subsequently, in November 2022, the seedlings up to 5 centimeters in height were transplanted into 30x25 cm plastic bags containing commercial substrate based on *Pinus* spp. bark, with the seedlings placed in the center and selected for uniform size, forming Lot 1. Plants >5cm and <10cm formed Lot 2. After transplanting, the seedlings were labeled and fertilized with 5g of Basacote Plus 9M NPK 16-08-12(+2), a slow-release fertilizer.

111 *2.1.2 Composition of treatments*

112 One week after transplanting, on 12/29/2022, the following treatments were applied: 0 $g.L^{-1}$ (T1),
113 2 $g.L^{-1}$ (T2), 4 $g.L^{-1}$ (T3) e 6 $g.L^{-1}$ (T4) of the commercial product EcoRhiza® (*Glomus* spp. 1 x 10^{3} CFU
114 g^{-1}), composed of four strains of arbuscular mycorrhizal fungi of the genus *Glomus* (*Glomus agregatum*,
115 *G. mosseae*, *G. intradices* e *G. Etunicatum*). The dose of 4 $g.L^{-1}$ (T3) represents the manufacturer's
116 recommendation, along with doses 50% higher (T4) and lower (T2) than the recommended dose. The
117 product was weighed on an analytical balance and diluted in 250 mL (per plant) of water to prepare the
118 doses (Fig. 2).
119



120

121 **Fig. 2.** Methodology used in seed preparation and germination, transplanting, and treatment application.
122 Piracicaba, Sao Paulo. Brazil.
123

124 *2.1.3 Vegetative development*

125 Weekly after the application of the treatments, all rootstocks were measured to monitor development in
126 terms of plant height (PH, cm), diameter at the collar height (CD, mm), and diameter 15 cm above the collar
127 (D15, mm), a height commonly used for grafting (Oliveira et al., 2008). For height and diameter
128 evaluations, a millimeter ruler and a digital caliper (Zass Precision) were used.
129

130 *2.1.4 Gas exchange and chlorophyll index*

131 At the end of the experiment, before destructive analyses, a gas exchange evaluation was conducted on two
132 fully expanded leaves, located in the East-West direction of 10 plants per treatment, totaling 80 leaves. For
133 this, an infrared gas analyzer (IRGA), model LI-6400XT (LI-COR Inc.) (ADC-BioScientific Ltd.), was
134 used in an open system with a CO_2 concentration of 385 µmol mol^{-1}. The variables observed were: CO_2
135 assimilation (A) [µmol (CO_2) m^{-2} s^{-1}]; transpiration (E), [mmol (H_2O) m^{-2} s^{-1}]; stomatal conductance (gs)

[mol (H_2O) m^{-2} s^{-1}] and intercellular CO_2 concentration (Ci) [µmol (CO_2) mol^{-1}]. From the data obtained, the carboxylation efficiency (A/Ci), [µmol m^{-2} s^{-1} Pa^{-1}], and water use efficiency (A/E) [µmol $mmol^{-1}$] were calculated. In the same leaves, chlorophyll content was also evaluated using a digital chlorophyll meter, model CFL 1030 Falker, expressed in a dimensionless unit by the Falker Chlorophyll Index (FCI).

2.1.5 Destructive analyses

For the destructive analyses, dry mass values were obtained using a precision balance, expressed in grams (Ohaus® Explorer); leaf area (LA, cm^2) was obtained using a LICOR leaf area integrator, model LI-3100; drying to obtain the aerial and root system dry mass (DMA and DMR, in grams) was performed in a forced air circulation oven (Tecnal, Model TE-394/3) at 65°C for 72 hours. Data on surface area (RSA, cm^2), total length (TRL, cm), total volume, and average root diameter (TRV, cm^3, and ARD, mm) were obtained by image scanning using an Epson® (XL 10000) scanner and WinRHIZO® software (Regent Instruments INC.).

2.2 Influence of arbuscular mycorrhizal fungi on the development of grafted 'Hass' avocado seedlings

2.2.1 Acquisition of material and experimental conditions

This experiment was conducted in a commercial nursery for avocado seedling production in Cordeirópolis, Sao Paulo, Brazil (latitude 22º28'13.3''S, 47º25'07.8''W). The experimental period spanned from January 2023 to July 2023, totaling 6 months in the nursery under the climatic conditions described in Figure 3.

Two months after grafting, 'Hass' avocado seedlings received 250 mL of a solution containing the commercial product EcoRhiza® (*Glomus agregatum*, *G. mosseae*, *G. intradices* e *G. Etunicatum*), following the same treatments used in the rootstock experiment: control (T1), 2 $g.L^{-1}$ (T2), 4 $g.L^{-1}$ (T3) e 6 $g.L^{-1}$ (T4), totaling 15 plants per treatment. The same variables proposed in the previous experiment were also evaluated, with the variable D5 being replaced by the measurement of the diameter 5 cm (D5) above the grafting region (cm) using a digital caliper.

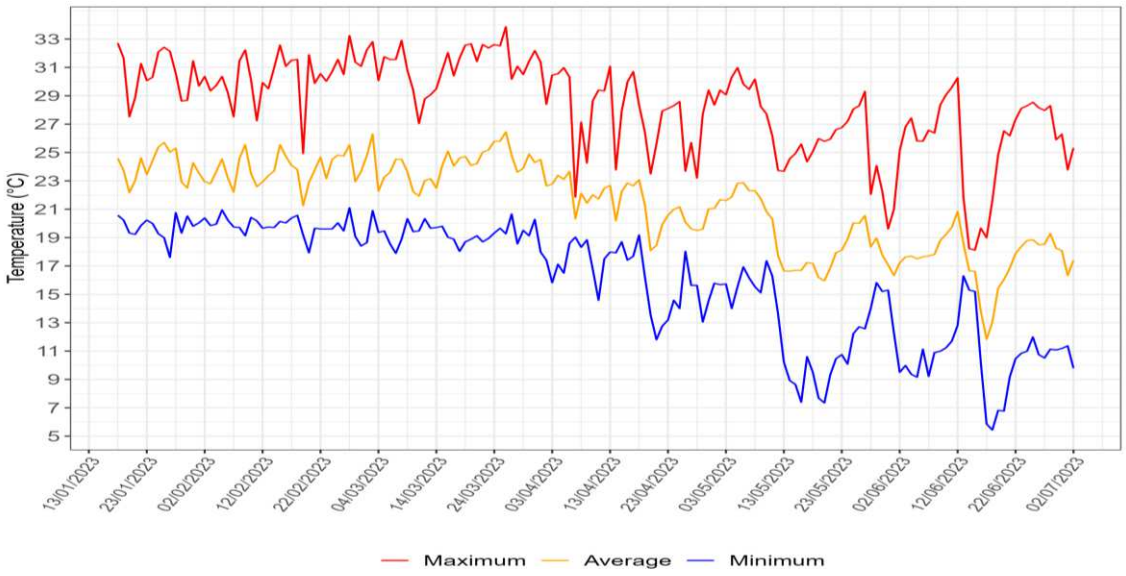


Fig. 3. Minimum, average, and maximum temperature during the experimental period. Experimental station Cordeirópolis, Sao Paulo. Brazil.

2.3 Experimental design and data analysis

The experiments followed a completely randomized design (CRD) with four treatment doses, each applied to 15 plants, totaling 60 plants per experiment. Data were initially analyzed using ANOVA to evaluate treatment effects. Assumptions for ANOVA were verified, testing residual normality with the Shapiro-Wilk test (Shapiro and Wilk, 1965) and variance homogeneity with Bartlett's test (Bartlett, 1960). Upon satisfying these assumptions, the CRD structure was defined as follows:

$$Y_{ij} = \mu + d_i + \epsilon_{ij}$$

where Y_{ij} is the observed response for the $j-th$ plant in the $i-th$ treatment, μ is the overall mean, d_i is the fixed effect of the iii-th treatment (dose), and ϵ_{ij} is the residual error term.

In parallel with the rootstock production experiment, a complementary study was conducted to account for variability due to seedling size. Plants were grouped into two categories based on size (Batch): those above 5 cm and those below 10 cm. This classification was adopted to capture the variability arising from seed germination, as the seeds used, although derived from the same maternal source, exhibited inherent differences (Olano et al., 2007). When the batch effect was found to be non-significant ($p>0.05$), the data from both batches were combined into a single dataset for subsequent analyses. This approach ensured that the observed treatment effects were not confounded by variability across batches.

Regression analysis was performed to investigate the dose-response relationship. Polynomial models up to the third degree were tested to identify the best fit. The polynomial regression model was defined as:

$$Y_i = \beta_0 + \beta_1 d + \beta_2 d^2 + \beta_3 d^3$$

where Y_i is the observed response variable, d is the dose level, $\beta_0, \beta_1, \beta_2, \beta_3$ are the coefficients. Regression deviations were tested to confirm the adequacy of the model, and the coefficient of determination (R^2) was calculated to measure the goodness of fit. Additionally, the derivative of the regression function was calculated and solved for $\frac{dy}{dx} = 0$ to identify the dose level (d) corresponding to the maximum rate of change in the response (Y). This analysis provided insights into the most responsive dose range, helping to determine the optimal application level. To complement the regression analysis, a joint analysis of the variables was performed using principal component analysis (PCA). Before applying PCA, the Kaiser-Meyer-Olkin (KMO) measure was calculated to assess sampling adequacy, and only variables with KMO values greater than 0.5 were retained. Eigenvalues greater than 0.5 were used as the retention criterion for principal components. PCA reduced the dataset's dimensionality while retaining the most relevant information. A biplot was generated following the methodology described by Gabriel (1971), allowing the visualization of relationships between treatments and variables.

Finally, once the established assumptions were met, the data were subjected to Pearson correlation, which allowed an understanding of the linear relationships between them (Benesty et al., 2008; Schober, 2018). A heatmap was constructed using the Mahalanobis distance, as it accounts for correlations among variables, providing a robust grouping of treatments (Maesschalck et al., 2000). All statistical analyses were performed using the open-source software R (R Core Team, 2023).

3 Results

3.1 Influence of arbuscular mycorrhizal fungi on the development of avocado rootstocks

3.1.1 Vegetative development

No statistically significant differences were observed for the variables plant height (PH), collar diameter (CD), and diameter at grafting height (D15) between treatments ($p>0.05$) for both batches of avocado rootstocks (Table S1 and Fig. 4).



Fig. 4. (A) Avocado seeds from Batch 1 (right) and Batch 2 (left) and (B) Seedlings from Batch 1 at 40 days after transplanting. Piracicaba, Sao Paulo, Brazil.

3.1.2 Gas exchange and chlorophyll index

Fig. 5 shows that, regardless of the batch, the effects of the treatments were similar, thus generating a mixed batch with all observations, as the trends of increase or variation in physiological variables remained consistent across the batches.

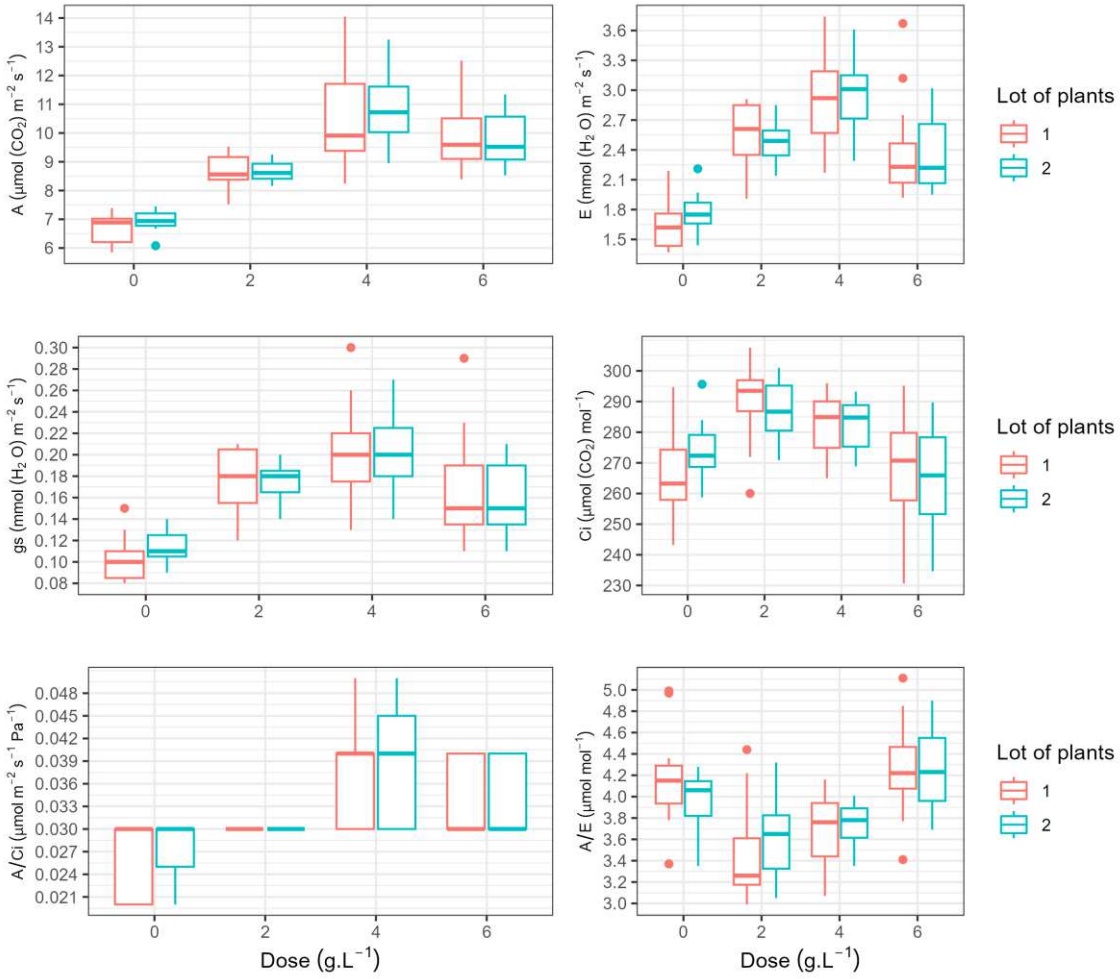


Fig. 5. Boxplot for exploring the effects of batches on CO_2 assimilation (A), transpiration (E), stomatal conductance (gs), intercellular CO_2 concentration (Ci), carboxylation efficiency (A/Ci), and water use efficiency (A/E) in avocado rootstocks 90 days after transplanting. Piracicaba, Sao Paulo, Brazil.

Significant differences ($p < 0.01$) were observed in the means of CO_2 assimilation (A), transpiration (E), stomatal conductance (gs), and intercellular CO_2 concentration (Ci) (Table S2). It was observed that the 2 $g.L^{-1}$ dose resulted in a 28.1% higher CO_2 assimilation compared to the 0 $g.L^{-1}$ dose. This trend was even more pronounced at the 4 $g.L^{-1}$ dose, which showed a 57% increase compared to the 0 $g.L^{-1}$ dose. The 6 $g.L^{-1}$ dose, in turn, was 45.4% higher than the control dose (0 $g.L^{-1}$).

The 2 $g.L^{-1}$ dose resulted in a 50.6% increase in transpiration compared to the 0 $g.L^{-1}$ dose. The 4 $g.L^{-1}$ and 6 $g.L^{-1}$ doses showed a significant increase of 73.2% and 41.7%, respectively, compared to the control dose.

For the stomatal conductance variable, the 2 $g.L^{-1}$ dose was 63.6% higher than the 0 $g.L^{-1}$ dose, while the 4 $g.L^{-1}$ dose resulted in an 81.8% increase compared to the control dose, and the 6 $g.L^{-1}$ dose was 54.5% higher.

The intercellular CO_2 concentration (Ci) increased slightly, by about 7.7% and 5.1% when the 2 $g.L^{-1}$ and 4 $g.L^{-1}$ doses were applied, respectively, compared to the 0 $g.L^{-1}$ dose. However, the 6 $g.L^{-1}$ dose resulted in a slight reduction of 0.6% compared to the control dose.

Despite the significant statistical differences observed between treatments, carboxylation efficiency showed a low variation of 1.66% between doses (0.03 to 0.04 $\mu mol\ m^{-2}\ s^{-1}\ Pa^{-1}$), indicating a marginal increase. For water use efficiency, the 0 $g.L^{-1}$ dose resulted in an average of 4.09 $\mu mol\ mmol^{-1}$, which, when compared to the average of the other doses (2, 4, and 6 $g.L^{-1}$) of 3.82 $\mu mol\ mmol^{-1}$, was reduced by approximately 6.6%.

The analysis of chlorophyll indices (A, B, and Total) for the different doses did not result in any statistical difference between treatments (Table S3). It was observed that the overall mean for Chlorophyll A was 39.68, for Chlorophyll B was 14, and for Total Chlorophyll was 53.75. The coefficients of variation for these variables indicate low data variability.

Based on the R^2 values presented in Figure 6, a quadratic fit to the observed data was confirmed. The variation in R^2 from 0.8683 to 0.9984 suggests that most of the variation in physiological responses can be explained by the treatment dose. The stomatal conductance (gs) graph has an R^2 of 0.998375, indicating that over 99% of the variation in gs is explained by the fitted model.

In addition to stomatal conductance, other variables also showed strong fits. The CO_2 assimilation rate (A) has an R^2 of 0.96, transpiration (E) has an R^2 of 0.99, and the internal CO_2 concentration (Ci) has

an R^2 of 0.95. The CO_2 use efficiency (A/Ci) and water use efficiency (A/E) are also well explained by the fitted models, with R^2 values of 0.87 and 0.98, respectively.

The analysis of the equations through derivation equal to zero provided the indication of the dose with the highest response. For the CO_2 assimilation rate, the slope is zero when the dose is approximately 4.675 $g.L^{-1}$. For transpiration, this point occurs at a dose of 3.71 $g.L^{-1}$. Stomatal conductance reaches a zero slope at approximately 3.81 $g.L^{-1}$, while the internal CO_2 concentration reaches this point at a dose of 2.86 $g.L^{-1}$.

Furthermore, the CO_2 use efficiency (A/Ci) and water use efficiency (A/E) also have significant zero slope points. For A/Ci, the slope is zero at a dose of approximately 4.4 $g.L^{-1}$, while for A/E, this point occurs at 2.76 $g.L^{-1}$.

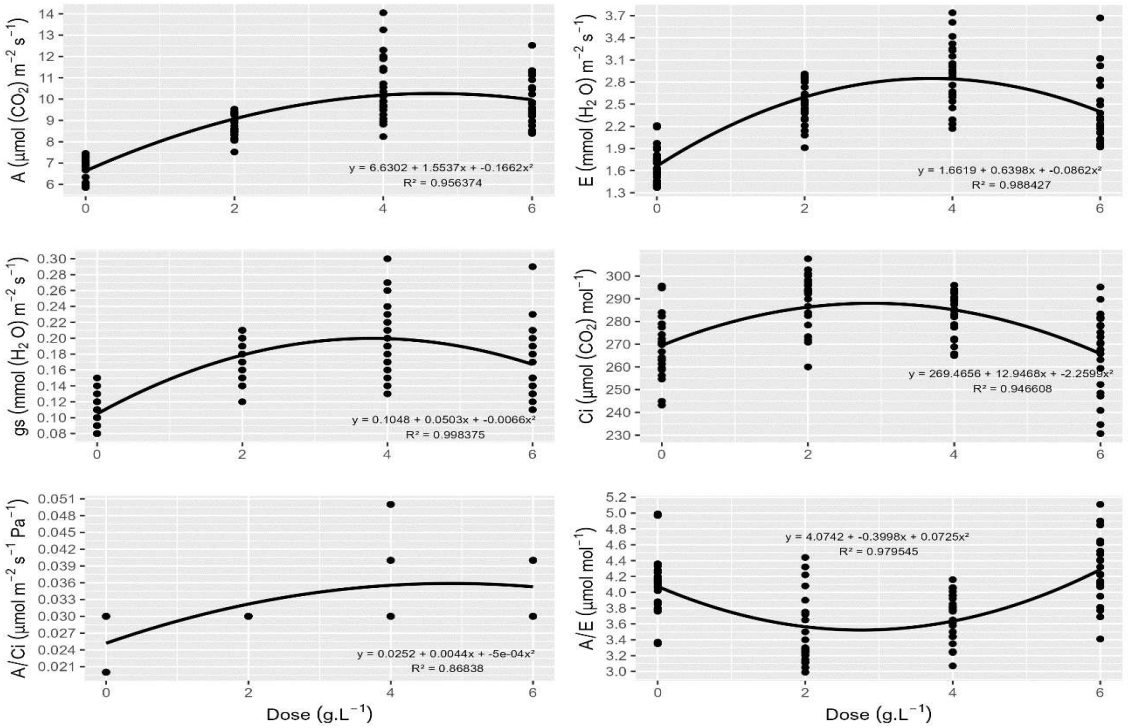


Fig. 6. Regression analysis for exploring the effects of treatment on CO_2 assimilation (A), transpiration (E), stomatal conductance (gs), intercellular CO_2 concentration (Ci), carboxylation efficiency (A/Ci), and water use efficiency (A/E) in avocado rootstocks 90 days after transplanting. Piracicaba, Sao Paulo, Brazil.

In the correlation shown in Fig. 7, only correlations above 0.5, whether positive or negative, were plotted. In this regard, the variable CO_2 assimilation (A) demonstrated a significant positive correlation of 0.91 with transpiration (E), 0.95 with stomatal conductance (gs), and 0.99 with intercellular CO_2 concentration (Ci). Additionally, a correlation of 0.9 with Chlorophyll A and a negative correlation of -0.93

with Chlorophyll B were observed. The same was noted for the variable transpiration (E) and Chlorophyll A (0.99) and B (0.98) contents, respectively. It was also evidenced that the variable CO_2 assimilation (A) had a correlation of 0.99 with stomatal conductance (gs) and 0.91 with CO_2 assimilation (A), as well as a significant correlation of 1 with the diameter measured at 15 cm height (D5).

The carboxylation efficiency (A/Ci) variable demonstrated correlations of 0.91 with stomatal conductance (gs), 0.98 with CO_2 assimilation (A), 0.94 with water use efficiency (A/E), 0.91 with transpiration (E), and 0.96 with intercellular CO_2 concentration (Ci). Finally, the water use efficiency (A/E) variable showed correlations of 0.99 with CO_2 assimilation (A) and 0.91 with transpiration (E).

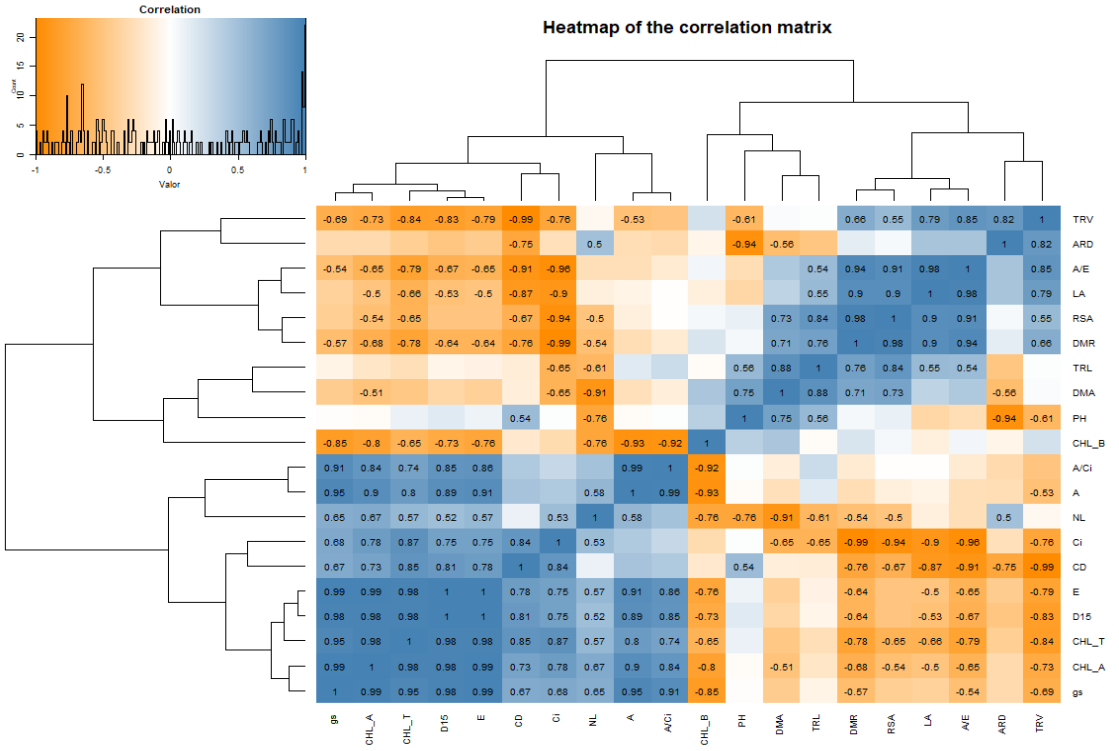


Fig. 7. Heatmap of the correlation matrix and clustering of variables evaluated in avocado rootstocks 90 days after transplanting. Piracicaba, Sao Paulo, Brazil. Legend: gs (stomatal conductance), CHL_A (chlorophyll A), CHL_Tl (total chlorophyll), D15 (diameter at 15 cm), E (transpiration), CD (collar diameter), Ci (internal CO_2 concentration), NL (number of leaves), A (CO_2 assimilation), A/Ci (carboxylation efficiency), CHL_B (chlorophyll B), PH (plant height), DMA (aerial dry mass), TRL (total root length), DMR (root dry mass), RSA (root surface area), LA (leaf area), A/E (water use efficiency), ARD (average root diameter), and TRV (total root volume).

Principal Component Analysis (PCA) and subsequent dimension decomposition (Figure 8) were performed only for the variables that showed statistically significant differences and correlations greater than 0.89, following the methodology proposed by Francisco (1995).

The use of two components (x and y) explains a significant portion of the data variation: the x-axis (Dimension 1) explains 31.2% of the variance, while the y-axis (Dimension 2) explains 26.7% of the variance, and with 4 dimensions, more than 80% of the total variation can be explained using PCA. Based on the orientation and proximity of the arrows representing the variables in the PCA plot, the 4 $g.L^{-1}$ dose is related to the significant variables (E, gs, A, Ci, and A/Ci) and correlated variables (Chlorophyll A and Total Chlorophyll). In the opposite direction, the variable A/E showed a direct relationship in the absence of mycorrhizae.

The first dimension (Dim.1) is strongly influenced by variables such as transpiration (E 17.51), stomatal conductance (gs 17.24), and CO_2 assimilation (A 11.90). In the second dimension (Dim.2), the dominant variables are CD (17.73), DMR (15.69), and D15 (15.92), while the third dimension (Dim.3) shows a strong contribution from chlorophyll-related variables, mainly Chlorophyll A (14.60) and Total Chlorophyll (19.19). Finally, the fourth dimension (Dim.4) is clearly dominated by Total Chlorophyll (25.35), Chlorophyll B (13.74), and internal CO_2 concentration (Ci 13.78).

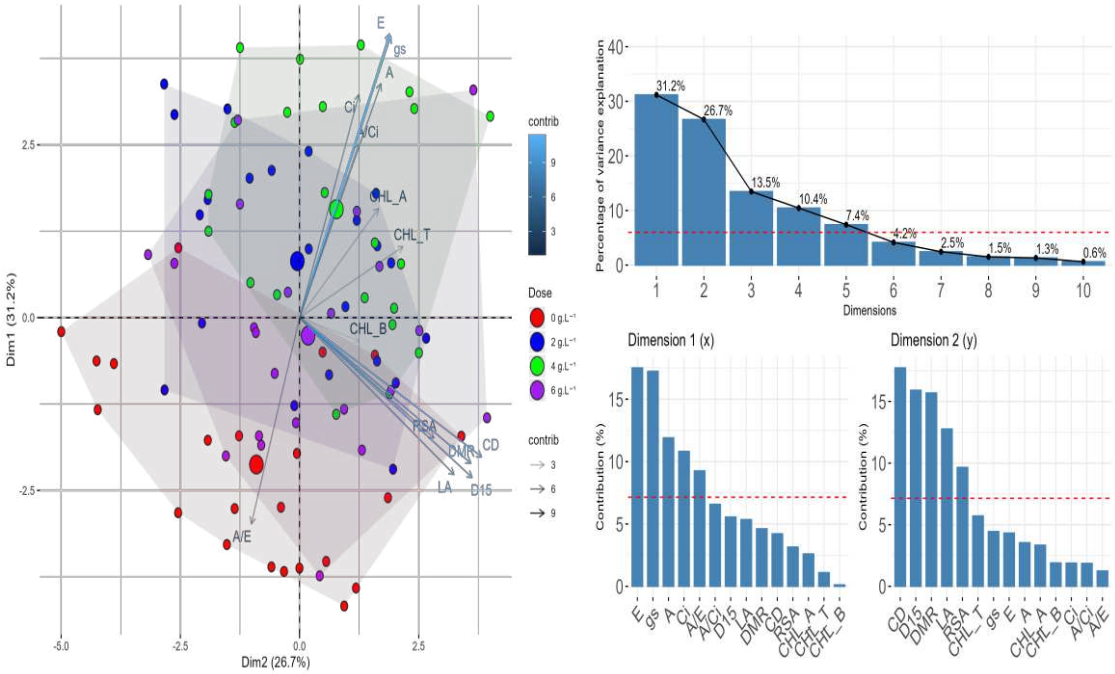


Fig. 8. (A) PCA-Biplot for the variables of interest listed in the legend and (B) variance breakdown for Dimensions 1 and 2 in avocado rootstocks 90 days after transplanting. Piracicaba, Sao Paulo, Brazil.

Legend: gs (stomatal conductance), CHL_A (chlorophyll A), CHL_Tl (total chlorophyll), D15 (diameter at 15 cm), E (transpiration), CD (collar diameter), Ci (internal CO_2 concentration), NL (number of leaves), A (CO_2 assimilation), A/Ci (carboxylation efficiency), CHL_B (chlorophyll B), PH (plant height), DMA (aerial dry mass), DMR (root dry mass), RSA (root surface area), LA (leaf area) and A/E (water use efficiency).

3.1.3 Destructive analyses

Despite the significant influence of the batch, no significant differences were observed between treatments for the number of leaves (NL), leaf area (LA), aerial dry mass (DMA), and root dry mass (DMR) (Table S4) obtained using the WinRHIZO software (Fig. 9).

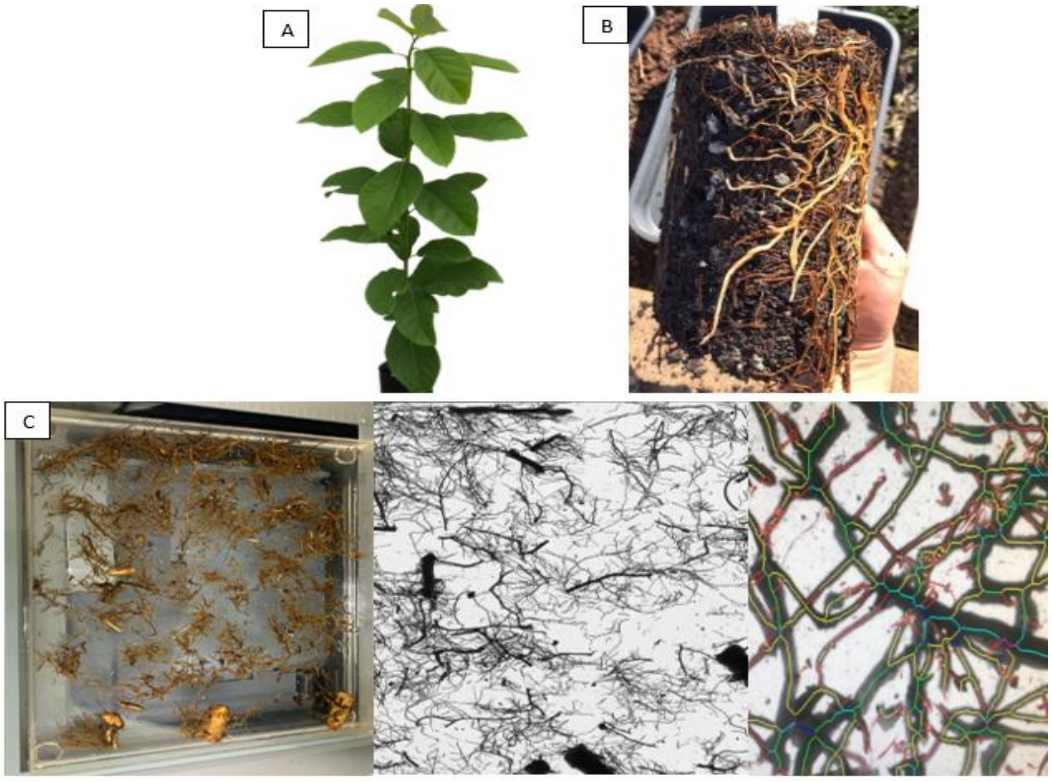


Fig. 9. (A) Images of the aerial and (B) root system of avocado rootstocks. (C) Analysis of the root system of avocado rootstocks using the WinRHIZO software. Piracicaba, Sao Paulo, Brazil.

The different doses of treatments applied to the avocado rootstocks also did not show a statistically significant effect on the variables root surface area (RSA), total root length (TRL), total root volume (TRV), and average root diameter (ARD) for both batches (Table S5).

3.2 Influence of arbuscular mycorrhizal fungi on the development of grafted 'Hass' avocado seedlings

3.2.1 Vegetative development

The mean plant height values ranged from 55.6 to 57.5 cm, collar diameter from 13.1 to 13.2 mm, and grafting height diameter from 11.7 to 12.1 mm (Table S6), without showing statistically significant differences between treatments.

3.2.2 Gas exchange and chlorophyll index

Significant differences between treatments were observed in the gas exchange variables (Table S7) and chlorophyll index (Table S8). The 2 $g.L^{-1}$ and 4 $g.L^{-1}$ doses resulted in 20% higher CO_2 assimilation compared to the 0 $g.L^{-1}$ dose. Conversely, the 6 $g.L^{-1}$ dose resulted in a lower response, reducing CO_2 assimilation by 24.7% compared to the 0 $g.L^{-1}$ dose.

The 2 $g.L^{-1}$ dose reduced transpiration (E) by 50% compared to the 0 $g.L^{-1}$ dose, while the 6 $g.L^{-1}$ dose was 25% higher than the 0 $g.L^{-1}$ dose. The proposed doses did not affect the stomatal conductance (gs) variable.

The intercellular CO_2 concentration (Ci) increased by 34% when compared to the average of the 4 $g.L^{-1}$ and 6 $g.L^{-1}$ doses and the 0 $g.L^{-1}$ dose. As for the carboxylation efficiency (A/Ci) variable, the doses that provided the best response were 2 $g.L^{-1}$ and 4 $g.L^{-1}$, with an average 79% higher than the control dose. Carboxylation efficiency showed low variation between treatments, from 0.02 for the 6 $g.L^{-1}$ dose to 0.05 for the 2 $g.L^{-1}$ and 4 $g.L^{-1}$ doses, respectively, showing a minimal increase.

Statistically significant differences were also observed between treatments for chlorophyll levels (Table S8), with the 4 $g.L^{-1}$ dose reaching 46.46, slightly higher than the 2 $g.L^{-1}$ dose, which was 46.16, showing an increase of 12.37% and 11.65%, respectively, compared to the 0 $g.L^{-1}$ dose, which was 41.35. The 2 $g.L^{-1}$ dose resulted in a significant increase in Chlorophyll B levels, with a value of 20.46, compared to the 0 $g.L^{-1}$ dose of 14.64, corresponding to a 39.76% increase. On the other hand, the 6 $g.L^{-1}$ dose also showed an increase in Chlorophyll B levels (19.13), representing a 30.73% increase compared to the 0 $g.L^{-1}$ dose.

The Total Chlorophyll levels in the 2 $g.L^{-1}$ and 4 $g.L^{-1}$ doses (Table S8) correspond to increases of 18.97% and 23.52%, respectively, compared to the 0 $g.L^{-1}$ dose. At the highest dose, 6 $g.L^{-1}$, there was a reduction in Total Chlorophyll levels to 62.47, which represents an increase of 11.57% compared to the 0 $g.L^{-1}$ dose.

The regression curves presented in the graphs of Figure 10 provide a quadratic fit for each variable, indicating the seedlings' response to the different doses applied. These fits have high coefficients of determination (R^2) ranging from 0.7 to 0.9, suggesting that the quadratic models significantly explain the observed variation in the data. By deriving these equations and setting them to zero, it was identified that the points of maximum slope for each variable varied approximately from 1.66 $g.L^{-1}$ for internal CO_2 concentration (Ci) to 2.83 $g.L^{-1}$ for Chlorophyll B. The specific doses for each variable were: 2.26 $g.L^{-1}$ for CO_2 assimilation rate (A), 2.31 $g.L^{-1}$ for transpiration (E), 2.25 $g.L^{-1}$ for CO_2 use efficiency (A/Ci), 2.26 $g.L^{-1}$ for water use efficiency (A/E), 2.66 $g.L^{-1}$ for Chlorophyll A, and 2.5 $g.L^{-1}$ for Total Chlorophyll (Fig. 10).

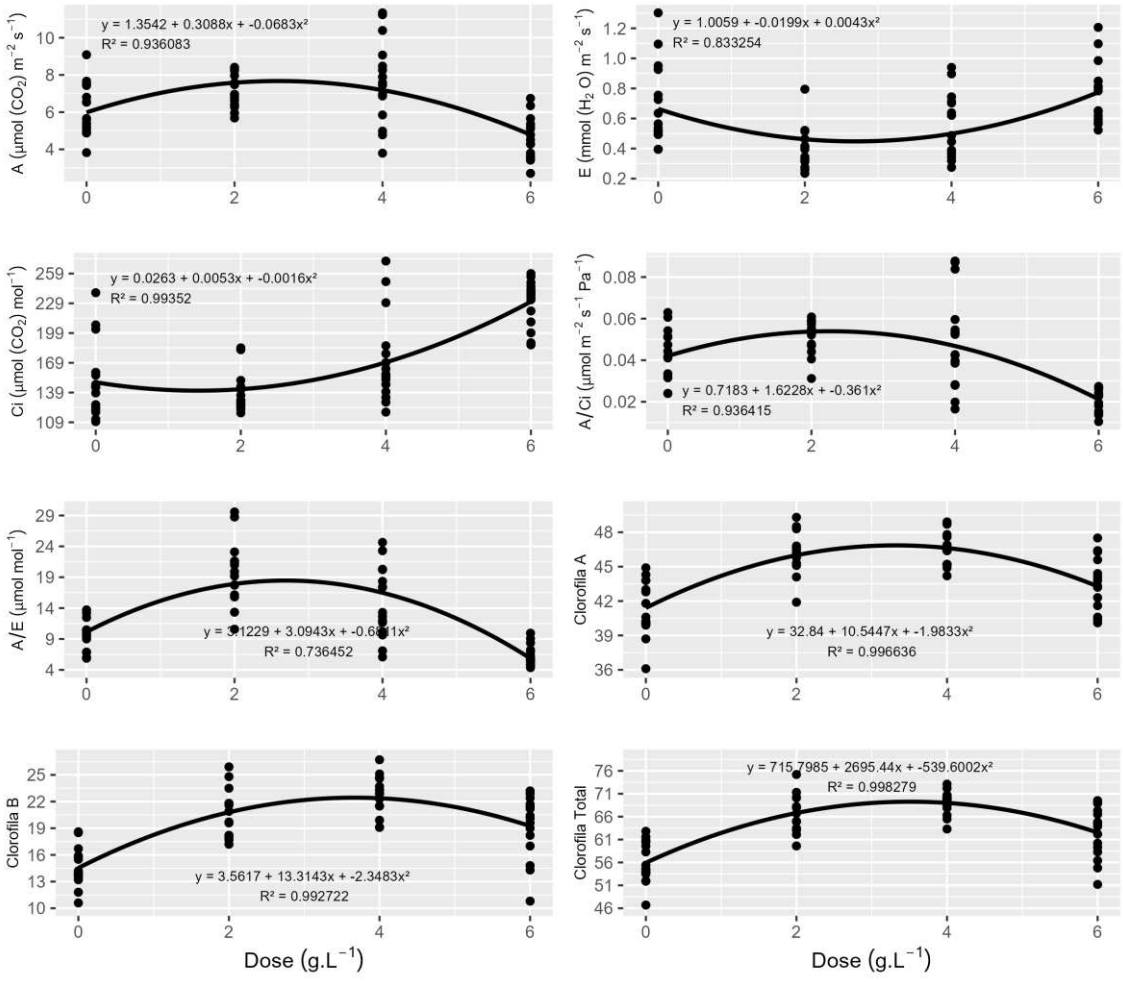


Fig. 10. Regression analysis for exploring the effect of treatments on CO_2 assimilation (A), transpiration (E), stomatal conductance (gs), intercellular CO_2 concentration (Ci), carboxylation efficiency (A/Ci), water use efficiency (A/E), and Chlorophyll A, B, and Total in 'Hass' avocado seedlings at the end of the experiment. Cordeirópolis, Sao Paulo, Brazil.

3.2.3 Destructive analyses

The application of treatments did not influence the variables number of leaves (NL), root surface area (RSA), total root length (TRL), total root volume (TRV), and average root diameter (ARD), but resulted in statistically significant differences for leaf area and aerial dry mass in 'Hass' avocado seedlings (Table S9).

The 2 g.L^{-1} and 4 g.L^{-1} doses increased leaf area, with values of 909.3 cm² and 1123.9 cm², respectively, compared to the 0 g.L^{-1} dose, which had a value of 693.3 cm², representing increases of 31.2% and 62.1%, respectively. Conversely, increasing the dose to 6 g.L^{-1} resulted in a smaller increase in leaf area, with a value of 1133.2 cm², representing a 63.4% increase compared to the 0 g.L^{-1} dose.

The 2 g.L^{-1} dose also resulted in a significant increase in aerial dry mass, with a value of 31.2 g compared to the 24.8 g observed at the 0 g.L^{-1} dose, an increase of 25.8%. On the other hand, the 6 g.L^{-1} dose also provided an increase in aerial dry mass (35.8 g), representing a 44.4% increase compared to the 0 g.L^{-1} dose, while the 4 g.L^{-1} dose showed the greatest increase, with a value of 39.7 g, representing a 60.1% increase compared to the control. The application of doses (2, 4, and 6 g.L^{-1}) resulted in an average root dry mass (DMR) of 11.7 g, representing an increase of 2.2 g or approximately 23.2% compared to the control (9.5 g).

The derivation of a linear equation for leaf area results in a constant slope of 3184.23 (Fig. 11). In contrast, the quadratic equation for aerial dry mass has a derivative resulting in a dose of 3.25 g.L^{-1}.

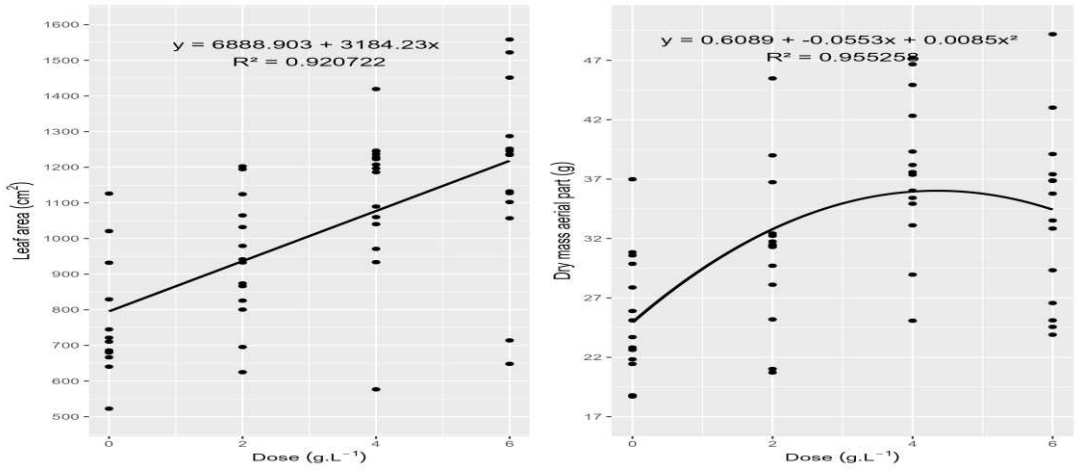


Fig. 11. Regression analysis for exploring the effects of treatments on leaf area (LA) and aerial dry mass (DMS) in 'Hass' avocado seedlings at the end of the experiment. Cordeirópolis, Sao Paulo, Brazil.

The correlations presented in the heatmap revealed correlations between intercellular CO_2 concentration (Ci) and stomatal conductance (gs) (0.94). Similarly, the photosynthesis rate (A) is positively correlated with transpiration (E) (0.88) and leaf area (LA) (0.88) (Fig. 12).

Leaf area (LA) showed a negative correlation with intercellular CO_2 concentration (Ci) (-0.74). Additionally, chlorophyll levels (A, B, and total) showed positive correlations with the photosynthesis rate (A). Chlorophyll A indicated a correlation of 0.82 with A, suggesting that higher levels of Chlorophyll A are associated with a higher photosynthesis rate. Aerial dry mass (DMS) showed positive correlations with Chlorophyll A (0.95) and Chlorophyll B (0.96), suggesting that higher levels of these variables are associated with greater dry mass (Fig. 12).

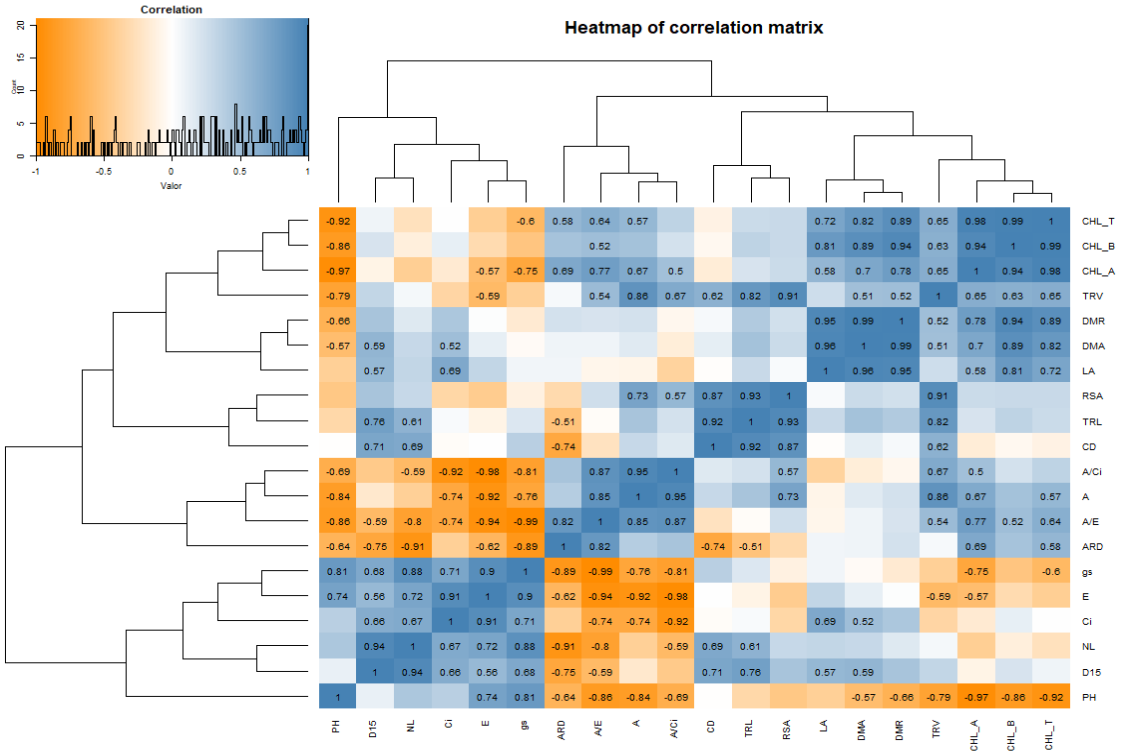


Fig. 12. Heatmap of the correlation matrix and clustering of variables evaluated in 'Hass' avocado seedlings at the end of the experiment. Cordeirópolis, Sao Paulo, Brazil. Legend: PH (plant height), D15 (diameter at 15 cm), NL (number of leaves), Ci (internal CO_2 concentration), E (transpiration), gs (stomatal conductance), ARD (average root diameter), A/E (water use efficiency), A (CO_2 assimilation), A/Ci (carboxylation efficiency), CD (collar diameter), TRL (total root length), RSA (root surface area), LA (leaf area), DMA (aerial dry mass), DMR (root dry mass), TRV (total root volume), CHL_A (chlorophyll A), CHL_B (chlorophyll B) and CHL_T (total chlorophyll).

The PCA analysis (Fig. 13) indicated that the first two principal components explain a significant portion of the data variation, with Dimension 1 explaining 22.5% and Dimension 2 explaining 20.2%. In total, the first four dimensions together explain more than 75% of the variation. The contributions of the variables to these dimensions were analyzed, highlighting that NL, TRL, RSA, and TRV are the most influential variables in Dimension 1, while A, A/E, A/Ci, Chlorophyll A, Chlorophyll B, and Total Chlorophyll dominate Dimension 2. It is also possible to observe the formation of two groups, with the 0 and 6 g.L^{-1} doses being similar, while the 2 and 4 g.L^{-1} doses follow the same trend (Fig. 13).

The 2 and 4 g.L^{-1} doses are also, according to PCA analysis, strongly associated with the significant variables of TRL, RSA, TRV, DMR, DMS, as well as showing a positive correlation with Chlorophyll A (CLH_A) and Total Chlorophyll (CLH_T), A, A/Ci, and A/E. Furthermore, the contribution of the variables in the different dimensions highlights their importance in the observed variation. In Dimension 1, NL (10.17), TRL (15.06), RSA (15.02), and TRV (12.12) are the most influential variables, while in Dimension 2, A (10.18), A/E (16.37), A/Ci (11.8), Chlorophyll A (14.06), Chlorophyll B (11.01), and Total Chlorophyll Total (13.59) predominate. The third dimension highlights the variables related to chlorophyll, mainly with leaf area (18.99), Ci (16.67), and Chlorophyll B (10.53).

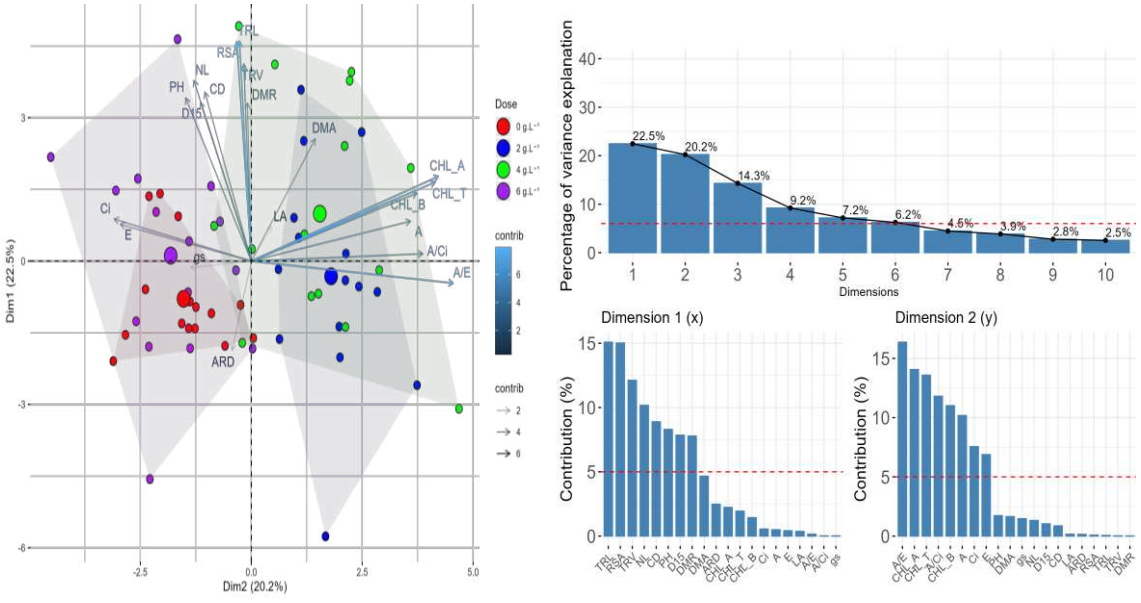


Fig. 13. (A) PCA-Biplot for the variables of interest listed in the legend and (B) variance breakdown for Dimensions 1 and 2 in 'Hass' avocado seedlings at the end of the experiment. Cordeirópolis, Sao Paulo, Brazil. Legend: PH (plant height), D15 (diameter at 15 cm), NL (number of leaves), Ci (internal CO_2

concentration), E (transpiration), gs (stomatal conductance), ARD (average root diameter), A/E (water use efficiency), A (CO_2 assimilation), A/Ci (carboxylation efficiency), CD (collar diameter), TRL (total root length), RSA (root surface area), LA (leaf area), DMA (aerial dry mass), DMR (root dry mass), TRV (total root volume), CHL_A (chlorophyll A), CHL_B (chlorophyll B) and CHL_T (total chlorophyll).

4 Discussion

Vegetative characteristics, such as the size and diameter at the grafting height of rootstocks, are crucial in the production of fruit tree seedlings, including avocados, as they indicate the maturity necessary for grafting (Darikova et al., 2011). Although these variables did not show significant differences between the proposed treatments, positive correlations (>0.5) were observed between these variables and those significant ($p<0.05$) variables associated with gas exchange (Fig. 7). Additionally, the principal component analysis (PCA) demonstrated an association with the application of 4 and 6 $g.L^{-1}$ doses in the rootstock experiment (Fig. 8). Similar results were observed for destructive variables, such as leaf area, dry mass, and root surface area (Fig. 8 and 9).

In this context, supplementation with arbuscular mycorrhizal fungi (AMF) proved effective, corroborating the results obtained by Lemus-Soriano et al. (2020), who observed a 39.78% increase in height in recently grafted avocado seedlings treated with Glumix® compared to the control, while plants treated with Mycoroot® showed a 23.36% increase compared to the control. The authors used 5 g of the commercial product, which has the same concentration of mycorrhizae ($1x10^3$ CFU g^{-1}) as in this study, at 21-day intervals, totaling nine applications.

The contribution of high doses (4 and 6 $g.L^{-1}$) was significant for all gas exchange variables (Fig. 13), which is consistent with research on AMF of the genus *Glomus*. Alemán et al. (2001) observed more than a 150% increase in CO_2 assimilation and a 50% increase in stomatal conductance in avocado rootstocks treated with a product containing mycorrhizae compared to the control. This is because the presence of AMF increases the absorption and utilization capacity of phosphorus, essential for producing energy compounds such as ATP (Solórzano-Acosta et al., 2024). Additionally, AMF can improve the passive flow of photosynthesis products in cortical cells (Gupta et al., 2021).

The non-significance observed in vegetative and destructive variables may be related to the fact that the plants remained in their containers for 90 days, an insufficient period for the mutualistic association, which according to Gomide et al. (2009), may take 90 to 180 days. In this regard, countries like Spain and

the USA use substrate inoculation during preparation for commercialization, as this technique can reduce the colonization time of AMF, making the inoculants used more effective and benefiting plant growth and nutrient uptake (Menge et al., 1978, 1980; Vidal et al., 1992; Riviera Espinosa et al., 2015; Solórzano-Acosta et al., 2024).

Another important point to consider is that during the symbiotic process, the host plant root releases strigolactones, phytohormones that stimulate hyphal branching (Akiyama et al., 2005). In response, AMF release molecular signals such as lipochito-oligosaccharides, which induce the activation of "symbiosis genes" in the plant, enabling the formation of the symbiotic association (Maillet et al., 2011). However, the excessive use of inputs, such as herbicides (Novais et al., 2019), insecticides (Hage-Ahmed et al., 2019), and fertilizers (Treseder and Allen, 2002), can hinder the colonization of fungi in the root system. Furthermore, plant domestication has resulted in an indirect selection of plants with low affinity or resistance to the mutualistic association (Hornstein, 2022).

In this regard, Bárcenas et al. (2007), Raya et al. (2019), and Montaño et al. (2019) identified that in more than 20 avocado production areas, the genus *Glomus* naturally prevails. Additionally, Yildiz (2010) emphasizes that inoculation with AMF from cultivated areas, i.e., native, is more efficient than commercial AMF sources. This is because selected AMF have a monospecific behavior, and the species may not be adapted to the edaphoclimatic conditions of the cultivation area (Viera et al., 2017; Ibrahim, 2018).

Furthermore, AMF form a network of hyphae and arbuscules in root cells, allowing the plant's root system to explore the soil/substrate further (Hamel, 2004). This enhances the roots' ability to intercept essential nutrients in the crop, improving plant development and reducing the need for fertilizers (Smith and Smith, 2012; Gou et al., 2023). In this regard, the experimental period for the seedlings was 6 months after grafting, showing that the AMF proposed in the treatments resulted in significance for gas exchange variables (Fig. 10), as already observed in the rootstocks, as well as variables associated with the root system (dry mass and surface area) and leaf area (Fig. 11).

Plants with a larger leaf area (Fig. 11) can benefit from greater light interception (Liu et al., 2021). Furthermore, when these plants are inoculated with AMF, there is an increase in stomatal density, thus improving parameters associated with this structure, such as gas exchange (Haghighi et al., 2023), which was also confirmed in this research. Due to this increase in leaf area, AMF can help the plant form a better root system architecture (Hetrick, 1991). This optimizes the activity of enzymes linked to the defense system and increases the nutritional content of the leaves (Yang et al., 2023). In addition to the benefits

mentioned, the presence of mycorrhizal hyphae in the substrate can improve aeration, increase water retention, and suppress phytopathogens such as *Phytophthora cinnamomi*, which is one of the main diseases in avocado orchards (Farooq et al., 2024).

The use of bio-inputs yields positive results and enables more sustainable crops, as the effective application of AMF can help reduce inputs in orchards. However, it is necessary to make adjustments in plant management and acquire knowledge about the biology and development of structures formed in the soil to achieve this.

5 Conclusion

The application of 4 $g.L^{-1}$ of a mixture of arbuscular mycorrhizal fungi (AMF) of the genus *Glomus* resulted in significant improvements in parameters related to gas exchange during the formation of avocado rootstocks.

For the Hass cultivar seedlings, this same dosage not only favored gas exchange but also increased the levels of Chlorophyll A, B, and total, promoted greater leaf area, and increased root system dry mass.

Data availability All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This research was funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Process No. 147435/2021-8) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/PROEX, Process No. 88887.635436/2021-00).

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