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Article

FAILURES COUNT TOO: EFFECT OF THE APPLICATION OF COMMERCIAL INOCULUM OF ARBUSCULAR FUNGI IN A VINEYARD DURING ITS PLANTATION

OS INSUCESSOS TAMBÉM CONTAM: EFEITO DA APLICAÇÃO DE UM INÓCULO COMERCIAL DE FUNGOS ARBUSCULARES NUMA VINHA DURANTE A SUA PLANTAÇÃO

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SUMMARY

Symbiosis with arbuscular mycorrhizal fungi (AMF) has long been recognized for its positive impact on plant health. Today, various companies market AMF-based commercial inoculants as biofertilizers or biostimulants for sustainable agriculture. However, their consistent efficacy in real-world field settings remains uncertain. This study investigated the influence of a commercial AMF inoculant on a newly planted vineyard featuring a local grape cultivar grafted onto a common rootstock (‘Ritcher 110’). Over two years, the physiological well-being, growth, and productivity of 20 inoculated vines compared to 20 control counterparts were monitored. The impact of inoculation on soil bacterial diversity and the infectivity of soil was assessed. Notably, AMF-inoculated plants exhibited consistently lower values in photosynthesis, growth, and grape production, although statistical significance was not always reached. Additionally, the total production remained unaffected, but there was a significant decrease in °Brix and pH values, suggesting delayed grape ripening in mycorrhizal plants, potentially promoting secondary metabolites accumulation. Regarding soil effects, the inoculation's impact was slight, with no substantial changes in soil mycorrhizal infectivity and only slight shifts in the microbial community's metabolic profile. Numerous studies highlight the context-dependent nature of AMF inoculation's effects, making it challenging to predict outcomes in field conditions. Failures found in trials like the present one provides valuable scientific information, contributing to determine the prerequisites for effective biofertilizer use in commercial viticulture. Ultimately, the effectiveness of AMF-based biofertilizers remains contingent on specific conditions, exposing the need for additional research to ensure their consistent and reliable application.

RESUMO

A simbiose com fungos micorrízicos arbusculares (FMA) é, há muito, reconhecida pelo seu impacto positivo na saúde das plantas. Atualmente, várias empresas comercializam inóculos comerciais à base de FMA como biofertilizantes ou bioestimulantes visando a sustentabilidade da agricultura. No entanto, a sua consistente eficácia em campo permanece incerta. Este estudo investigou a influência de um inóculo comercial de FMA numa vinha recentemente plantada, com uma variedade local, enxertada num porta-enxerto comum (‘Ritcher 110’). Ao longo de dois anos, foram monitorizados o bem-estar fisiológico, o crescimento e a produtividade de 20 videiras inoculadas em comparação com 20 videiras controlo. O impacto da inoculação na diversidade bacteriana do solo e na infecciosidade do solo foi avaliado. As plantas inoculadas com FMA apresentaram valores consistentemente mais baixos, na fotossíntese, no crescimento e na produção de uvas, embora estatisticamente nem sempre tenham sido detetadas diferenças. Além disso, a produção total de uvas não foi afetada, mas registou-se uma diminuição significativa nos valores do °Brix e do pH, sugerindo um atraso na maturação das uvas nas plantas micorrizadas, promovendo potencialmente a acumulação de metabolitos secundários. Relativamente aos efeitos no solo, o impacto da inoculação foi ligeiro, sem alterações substanciais na infecciosidade micorrízica do solo e apenas com ligeiras alterações no perfil metabólico da comunidade microbiana. Numerosos estudos realçam que os efeitos da inoculação do FMA depende do contexto, o que torna difícil prever os resultados em condições de campo. As falhas encontradas em ensaios como o presente facultam informações científicas relevantes, contribuindo para a determinação dos pré-requisitos para o uso eficaz de biofertilizantes na viticultura comercial. Por último, a eficácia dos biofertilizantes à base de FMA continua a depender de condições específicas, evidenciando a necessidade de investigação adicional para garantir a sua aplicação consistente e fiável.

Keywords: Rhizophagus irregularis, Funneliformis mosseae, vine plantation, ripening.
Palavras-chave: *Rhizophagus irregularis*, *Funneliformis mosseae*, plantação de vinha, maturação.

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INTRODUCTION

Mycorrhizae, particularly *arbuscular* mycorrhizae, represent a fungal-root symbiosis found in numerous plant species of agricultural and industrial importance (Smith and Read, 2008). This symbiotic relationship enhances the nutritional and physiological well-being of plants while simultaneously increasing their resilience against pathogens and environmental stresses (Aguilera *et al.*, 2022). The beneficial impact of arbuscular mycorrhizal fungi (AMF) has been well documented since the 1960s (Gerderman, 1968). Currently, commercial application of AMF has been shown to increase yield not only in horticultural plants (Baum *et al.*, 2015), but also in large-scale crop production (Ceballos *et al.*, 2013; Pellegrino *et al.*, 2015; Basiru *et al.*, 2021). AMF now holds a promising position in agricultural systems due to its capacity to enhance crop yield and maintain soil fertility in the long term. The global demand for AM fungi is increasing, leading to numerous businesses producing inoculants of mycorrhizal fungi for various applications (Sudheer *et al.*, 2023). Indeed, the biofertilizer market is projected to grow from $1,648.61 million in 2021 to $5,097.92 million in 2031, with a compound annual growth rate (CAGR) of 11.95% (The Business Research Company, 2023).

Similarly, the global organic wine market reached $10.5 billion in 2022 and is projected to reach $25.0 billion by 2030, with an expected CAGR of 11.5% during the forecast period of 2023-2030 (DataM Intelligence, 2023). This growth is driven by increasing consumer concern about the impact of chemical pesticides and fertilizers on both health and environment (Dhankhar and Kumar, 2023). In the search for sustainable viticultural practices that reduce dependence on chemical inputs, improve water management, and use green technologies, research has shown the numerous benefits of AMF on grapevines (Trouvelot *et al.*, 2015; Torres *et al.*, 2021; Aguilera *et al.*, 2022). Therefore, mycorrhizal inoculation is postulated as a good management strategy for sustainable viticulture (Trouvelot *et al.*, 2015; Popescu, 2016; Aguilera *et al.*, 2022).

Despite several studies highlighting the beneficial impact of AMF on grapevine development (Trouvelot *et al.*, 2015; Popescu, 2016; Aguilera *et al.*, 2022), it is crucial to recognize the multitude of external factors that must be considered to ensure the effectiveness and success of AMF in extended field applications (Baraza *et al.*, 2023). Different factors can influence the outcome of inoculation and the long-term presence of AMF in the soil. These factors include the characteristics and quality of the inoculum, the compatibility between the introduced AMF species and the grapevine cultivar/rootstock as well as the surrounding environment, the level of competition with other soil organisms, and subsequent agronomic practices (Verbruggen *et al.*, 2013; Berruti *et al.*, 2016; Chen *et al.*, 2018; Baraza *et al.*, 2023). Additionally, the quantity, timing, and method of application of the inoculum can significantly affect the extent of colonization (van Jaarsveld *et al.*, 2021). In fact, recent studies comparing various commercial inoculants in the same crop at the same time find diverse effects (Ganugi *et al.,* 2023).

This raises the question of both the potential and limitations associated with the use of these products in crop production, including grapevine cultivation (Baum *et al.*, 2015; Baraza *et al.*, 2023). Typically, positive outcomes are observed in response to plant performance under controlled greenhouse conditions (Baraza *et al.*, 2023). However, the benefits of AMF inoculants in field studies exhibit greater variability (Salomon *et al.*, 2022). The intricacies of real-world field conditions contribute to divergent research findings, with some studies reporting no discernible benefits to plant performance or even a suppressive effect on plant growth (Nogales *et al.*, 2019; Rosa *et al.*, 2020; Thomsen *et al.*, 2021).

Experts emphasize the importance of conducting field trials to determine whether a specific mycorrhizal inoculum can positively impact the growth of particular grapevine varieties when grafted onto specific rootstocks (Sinclair *et al.*, 2014; Baraza *et al.*, 2023). This study aims to assess the response of *Vitis vinifera* L. grafted onto a rootstock commonly used in Mediterranean regions to a commercial inoculum containing *Rhizoglomus irregularis* and *Funneliformis mosseae*, two of the most prevalent species found in commercial inoculants (Sudheer *et al.*, 2023). For this purpose, a farm with typical viticultural soil conditions was chosen, allowing to assess the effects of inoculation on this soil and its infectivity.

MATERIALS AND METHODS

Experimental site, plant material, and treatments

The experiment was carried out in a commercial vineyard in Sencelles (Mallorca; 93 m a.s.l.; 39°37'21.7"N 2°55'14.7"E) under organic management. The experimental plot was planted with 'Manto Negro', a local red cultivar, grafted on 'Ritcher 110' rootstock.

The plantation was established on April 5th 2019 with a training system of 2.80 m between rows and 1.20 m within plants in the same row. Seven days after planting, 40 plants were chosen and systematically distributed in alternative rows, with a line of plants no used in the experiment between the experimental ones (Figure 1).

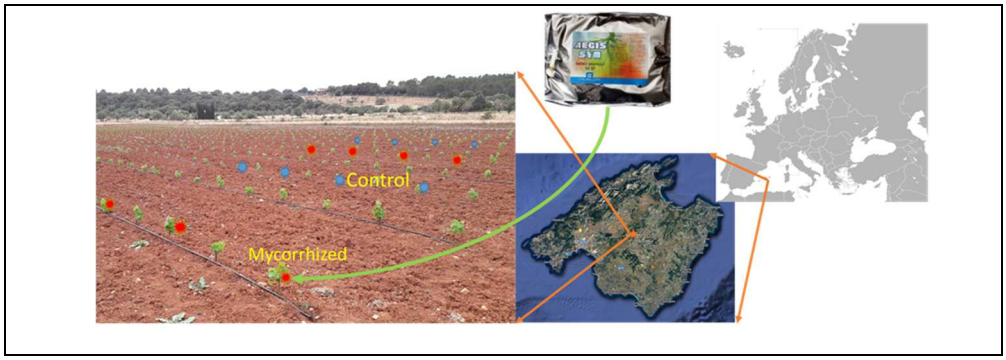

Figure 1. Geographical location and systematic distribution of measured plants in the vineyard. Red points correspond to inoculated plants, whereas blue points correspond to control plants.

Therefore, from the first row, plant 1 was inoculated, plant 2 was not considered in the experiment, and plant 3 was considered as the control. Plant 4 was not considered and plant 5 was inoculated again. This sequence was continued until 20 inoculated plants and 20 plants considered controls were completed. The inoculated plants were mycorrhized by adding 4 g of inoculum (AEGIS SYM © Microgranule, Agrotecnologias Naturales, SL Tarragona, Spain) in a 15 cm depth hole as close as possible to the roots of the vines. The commercial inoculum used contained 25 spores/g of *R. irregulare* BEG72 (synonym *R. irregularis*, basionym *Glomus irregulare*) and 25 spores/g of *F. mosseae* (basionym *G. mosseae*). Six soil samples composed of three subsamples, distributed throughout the experimental area, were taken one year after the moment of vine inoculation. The samples were air dried, homogenized and sieved with a mesh of 2 mm. of aperture size (IMPACT -UK).

Physicochemical analyses were performed by Eurofins Agroambiental, SA (Lleida, Spain), covering parameters such as pH, electrical conductivity, organic matter content, elemental nitrogen (N), and soil textural class determined through the USDA soil textural triangle (Soil Science Division Staff, 1993). Oxidable carbon content was determined using the Walkey-Black potentiometric titration method, and conductivity was measured in a soil extract with a water ratio of 1:5. The pH was determined in an aqueous soil suspension with a water ratio of 1:2.5. Total nitrogen in the soil was quantified using the Kjeldahl volumetric titration method. Phosphorus levels were determined through the Olsen method and UV-vis spectroscopy. Soil texture was characterized using the USDA Bouyoucos method (three fractions) and the internal gravity method. The analyses revealed that the vineyard soil exhibited a loamy-clay texture, with a low organic matter content, a moderate nitrogen concentration and a low, but highly variable, phosphorus concentration (Table I).

Growth parameters

Growth measurements were recorded in 40 plants (20 plants/treatment). Maximum plant height (cm), number of shoots, shoot length (cm) and diameter were measured at flowering, pea-sized berries and post-harvest (42 days, 72 days, and 170 days after inoculation, respectively). For the shoot growth parameters, for each plant, the sum of the diameters of all shoots and the sum of the lengths of all shoots were calculated. At harvest time (end of August 2020, which corresponded to day 513 after planting), the length of the shoot was also measured as a growth indication of the second period of study.

Gas Exchange

At the same moments of growth measurements, gas exchange was determined using one fully expanded leaf per plant using an infrared open gas exchange system (Li-6400, LI-COR Inc. Lincoln, Nebraska, USA), equipped with a 2 cm^2 leaf chamber fluorometer (Li-6400-40; LI-COR Inc. Lincoln, Nebraska, USA).

Table I

Soil properties

Parameter	mean ± SE
pH	8.38 ± 0.016
Conductivity (sS/m)	0.15 ± 0.002
OM (Walkey-Black %)	1.35 ± 0.072
N (Kjeldahl g/kg)	0.143 ± 0.007
P (Olsen mg/kg)	9.95± 3.48
Sand (%)	13.60 ± 0.72
Silt (%)	15.70 ± 0.51
Fine slime (%)	25.60 ± 0.44
Clay (%)	45.01 ± 1.18

Values are means ± standard errors of six random samples; OM: organic matter.

Net carbon dioxide (CO_2) assimilation (AN), stomatal conductance (g_S), transpiration (E) and internal CO_2 concentration (Ci) were determined in saturated light (1500 µmol/m^2/s) achieved with the red LED lamp of the system with an additional 10% blue light to maximize stomatal opening. The CO_2 concentration in the leaf chamber (Ca) was set at 400 µmol CO_2/mol air in the cuvette, and the relative humidity of the incoming air ranged between 40 and 60%. The block temperature was maintained at 30 ºC, while the water vapour pressure deficit (VPD) was not controlled. One year after planting, on 5 May 2020 (day 396 after planting), a new gas exchange measurement was recorded on the 40 plants flowing the same protocol.

Yield and quality parameters

At harvest time (end of August 2020, corresponding to day 513), the total yield per vine, the number of bunches, the mass of the bunch, and the mass of 100 berries were recorded in all plants with grapes. Fruits were processed into must for analysis on the same day. Grape musts °Brix were analysed using a portable refractometer Zuzi ATC 0-32 Brix ®. The pH was determined with a pH meter, and total acidity was evaluated by titration using a base (0.1 N sodium hydroxide) along with Panreac® Bromothymol Blue 0.4%, a pH indicator that changes of colour at pH 7. After inoculation, 72 days, soil samples were collected from the rhizosphere of five inoculated plants and five control plants for the assessment of bacterial functional diversity, employing Biolog EcoPlates ™. This technique has been used due to its effectiveness and speed in reflecting the functional and metabolic diversity of microbial communities (Baraza *et al.*, 2019). 5 g of each fresh sample was mixed with 45 mL of 0.9% sodium chloride solution. The mixture was homogenised by vortex and centrifuged at 2400 rpm for 10 min. The tubes were placed at 4 °C. After 20 min, the samples were diluted by adding 9.9 mL of 0.9 % sodium chloride solution to 100 µL of clear supernatant. 100 µL of each dilution was added to a Biolog EcoPlate™. Each 96-well plate consists of three replicate cells for the 31 sole carbon sources, three blank water wells, and a redox dye (tetrazolium violet) revealing oxidative catabolism (Baraza *et al.*, 2019). Finally, the plates were stored in the dark at 25 °C. Measurements were made using a photometer at 590 mm twice a day for the first three days and once a day for three more days (0 h, 24 h, 42 h, 48 h, 66 h, 72 h, 138 h, 144 h, and 168 h). Each absorbance value was corrected for the average value of the three control wells in each plate. The average value of the three wells of each substrate was used for the calculation of the Shannon index (H) and the Average Well Colour Development (AWCD). The index was determined considering only OD590 nm values higher than 0.15 as shown in Equations 1 and 2.

$$H = \sum [\, p_i \cdot \ln(p_i)] \qquad \textbf{Eq. 1}$$

where, p_i is the OD 590 nm value in well i divided by the sum of the OD 590 nm value for the 31 carbon sources on the Biolog EcoPlate™

$$AWCD = \sum (C_i \cdot R) / 31 \qquad \textbf{Eq. 2}$$

where, C_i is the OD 590 nm value for i in the 31 carbon wells and R is the OD 590 nm value of the water well on the Biolog EcoPlate™

Soil mycorrhizal infectivity

The infective capacity of the soil was measured in a test under controlled conditions with soil from the rhizosphere of the 10 inoculated and 10 non-inoculated plants taken 170 days after inoculation. 25 g of sieved soil (<2 cm) were mixed with 50 g of sterile sand in 100 cm^3 pots. In this case, lettuce (*Lactuca sativa*) was used as a trap plant and allowed

to grow for 2 months. After this period, roots were stained with trypan blue, following Phillips and Hayman (1970) protocol, and then visualised under a microscope to quantify the percentage of colonization according to the magnified intersection method (McGonigle *et al.*, 1990). Three replicates per plant were performed.

Statistical analysis

Differences between two independent treatments (control and mycorrhiza) were tested using generalized linear mixed models (GLMM) and linear mixed models (LMM) with the functions 'glmer' and 'lmer' from the 'lme4' R package (Bates *et al.*, 2015). After testing for normality of continuous variables, the Gamma distribution was found to be better-fitted for response variables such as 'sum of shoot diameters' and 'shoot length of all shoots'. LMM was used for the photosynthesis parameters. A Poisson distribution model was used to analysed the number of shoots. The model comprised the individual as a random factor, and the number of days since inoculation as an explanatory covariate, including the interaction with the treatment. In all cases, Least Squares Means comparison was also performed using the 'lsmeans' function of the 'lsmeans' package to compare treatments at each sampling moment. To identify differences in yield quantity and quality and plant growth between the two treatments, Generalized Linear Models (GLM) were used. A Gamma distribution was fitted for the models of total yield weight and berry weight, while a Poisson distribution was used for the response variables, including the number of clusters per individual and the number of shoots at harvest. Soil bacteria functional diversity was analysed in two ways. First, a GLMM fitted to the Gamma distribution was used to test for differences in Biolog EcoPlate™ between treatments, incubation time and their interaction for AWCD and H calculated from the OD at 590 nm for the 31 carbon substrates. Second, a principal component analysis (PCA) was used to identify patterns between treatments in the use of the 31 carbon substrates. PCA was completed using singular value decomposition via the 'prcomp' function of the 'stats' R-package. Data were centred and scaled using 'prcomp'. PERMANOVA with the adonis2 function of the vegan package was carried out in order to detect statistical effects of treatment on the rates of C-substrate utilization. A heat map was generated for better visualisation of similarities and dissimilarities in C-substrate utilization rates by soil microbial communities between the crop systems. Finally, to analyse the soil infectivity capacity results, a GLM was performed using a binomial distribution, comparing the percentage of mycorrhizal colonization between the two treatments. All analyses were performed in R v. 4.1.2 (R Core Team, 2023). The ANOVA function from the 'car' R-package was used to perform likelihood ratio tests to obtain the significance of the variables, and the 'emmeans' function of the R-package 'emmeans' was used for post hoc analyses (Lenth *et al.*, 2020).

RESULTS AND DISCUSSION

EFFECT OF MYCORRHIZA INOCULATION ON VEGETATIVE DEVELOPMENT AND GAS EXCHANGE OF GRAPEVINES

In general, inoculation had no influence on plant growth or physiological state during the first two months after inoculation (Table II). However, contrary to expectations, after summer (177 days), the AMF-inoculated plants reached a smaller size than the control (Figure 2A). This lower growth is corroborated by a lower rate of photosynthesis in AMF-inoculated plants compared to controls in autumn (Figure 2B). However, these differences disappeared over time. At the end of the second phenological year (513 days), no statistically significant effect of inoculation on the number of shoots was detected (Figure 2A). Even for trunk diameter, a significant interaction was found between time and treatment (Table II). Although control treatment increased by an average of 1.85 cm between D0 and D177, the AMF-inoculated plants only increased by 1.38 cm during the same period.

Despite numerous examples of positive effects of AMF inoculation on the growth and physiology of vine plants found in the literature (Aguilera *et al.*, 2022; Trouvelot *et al.*, 2015), it is also possible to find examples with neutral or even negative effects (Baraza *et al.*, 2023). In fact, Brunetto et al., (2023), studying the effect of pre-inoculation with several species of AMF, before planting in the field, found a negative effect on the growth of young vines after 316 and 500 days post-transplantation, similar to our findings. However, they observed a reduction in the accumulation of Cu in the plants, thereby reducing its toxicity. Several studies comparing different grapevine cultivars, rootstocks, or mycorrhizal species have yielded divergent results, showing positive effects in some cases and negative effects in others (Eftekhari *et al.*, 2010; Holland *et al.*, 2018; Rosa *et al.*, 2020). In this study, a positive effect was expected, since an increase in growth was detected in vine plants grafted onto "Richter 110" rootstocks under field conditions after inoculation of the same *R. irregulare* strain (BEG72; Camprubí *et al.*, 2008). Although other studies using the same AMF specie for inoculation, found that the response of the vine plant was positive or not depending on the soil characteristics, the used rootstock and the time after planting (Nogales *et al.*, 2009).

Numerous studies have shown that the beneficial effects of mycorrhizal associations on the growth and physiology of grapevines tend to manifest primarily

under stress conditions, such as drought (Valentine *et al.*, 2006) or nutrient deficiencies (Paul Schreiner, 2007).

However, in the current field study, although nutrient levels are low (Table I), soil and environmental conditions seemed adequate for grapevines cultivation, since no plant died and the growth and production of the first year were adequate.

Table II

Results of Generalized Linear Mixed Models for different plant growth and physiological variables over time after inoculation (mycorrhiza vs. control)

Response Variable	Predictive Variable	df	$\chi 2$	p-value
	Treatment	1	0.759	0.383
Number of Shoots	Days	3	23.563	**<0.0001**
	Treatment × Days	3	0.027	0.998
	Treatment	1	0.942	0.331
Trunk Diameter	Days	1	5.431	**0.019**
	Treatment × Days	1	3.932	**0.047**
	Treatment	1	0.428	0.513
Total Shoot Height	Days	3	772.259	**<0.0001**
	Treatment × Days	3	6.369	*0.094*
	Treatment	1	0.204	0.652
Photosynthesis (CO_2 m^{-2} s^{-1})	Days	3	94.045	**<0.0001**
	Treatment × Days	3	6.230	0.101

The identity of the individual was included as a random variable. The degrees of freedom (df), $\chi 2$, and p values are calculated based on likelihood ratio tests (LRT; $p<0.05$). Significant variables are shown in bold.

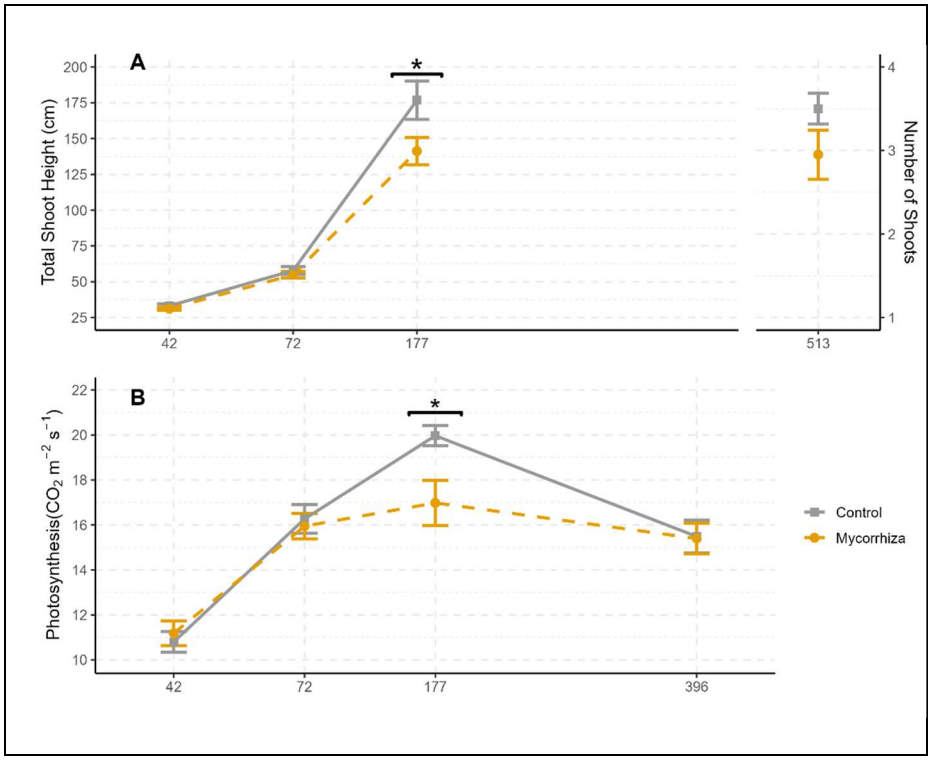

Figure 2. Mean and standard error (SE) of plant growth (as total shoot height and number of shoots; A) and physiological state (Photosynthesis; B) for each treatment (mycorrhiza vs. control) over time (days after inoculation). The asterisks show significant differences between mycorrhiza treatments (* $p<0.01$) for a post hoc test using Bonferroni pairwise comparisons.

This scenario raises the concern that the costs associated with maintaining the symbiosis might outweigh the benefits when the conditions for plants to grow are weak (Johnson *et al.*, 1997).

In addition, the impact of mycorrhization can vary over time. During their establishment, AMF consume carbon (C) for the development of new fungal structures and spores that are required for root colonization. Consequently, the costs incurred by grapevines during this period may exceed the nutritional benefits received from the symbiotic relationship (Smith *et al.*, 2010). For example, Mortimer *et al.* (2005) observed a negative effect of colonization by *G. etunicatum* on grapevines during the initial two months of their study. In contrast, in the present work, the negative effect on growth or photosynthesis was detected after six months, so it is less probable that this result from the carbon absorbed by the AMF during its development. Indeed, the effect of mycorrhization can change from negative to positive with time. For example, Linderman and Davis (2001) reported a substantial growth increase 112 days after inoculating seven rootstocks and two non-grafted cultivars with four different AMF.

Effect on the harvest of the first year after planting

As shown in Figure 3, grapes did not show differences in acidity (Figure 3A); however, both °Brix and pH were lower in AMF-inoculated plants (Figures 3B and C). Regarding overall production, there is a significant decrease in average weight per cluster for AMF-inoculated plants (Figure 3D). The higher average cluster weight of control plants may result from the number of berries per cluster, as no significant differences were observed in berry fresh weight (2.27 ± 0.17 g/berry in control plants and 2.13 ± 0.07 in inoculated plants). Additionally, no changes in berry water content were expected, as water availability was the same in both treatments, and no statistical differences in stomatal conductivity were found at any point during the study (data not shown). Despite this result, no differences in total harvest weight (Figure 3E) or in the number of clusters per vineyard (Figure 3F) between mycorrhiza and control individuals was registered. Studies analysing the impact of mycorrhizal inoculation on harvest parameters in vineyards under field conditions are relatively recent. In some of them, no significant differences were found between control and infected plants in performance metrics, such as the number of clusters and weight (Rosa *et al.*, 2020; Thomsen *et al.*, 2021). As reported by Karoglan *et al.* (2021), only one of the two years showed an increase in grape yield after AMF inoculation. However, both experimental years showed notable increases in total flavonoids, total anthocyanins, and total polyphenols in the skin of the berries. In wine grape production, prioritising fruit quality often outweighs considerations for yield. This trend is amplified by the ongoing impacts of climate change. Torres *et al.* (2016) highlighted the potential to improve the quality of grape berries through inoculation of grapevines with AMF. This enhancement was observed in several aspects of phenolic maturity, including increased anthocyanin content and increased antioxidant activity. Interestingly, they found different responses between different clones of the same grapevine variety, 'Tempranillo', when exposed to both high temperatures and inoculation with AMF. In particular, the typical decline in anthocyanins levels in berries, which occurs in non-mycorrhizal plants subjected to high temperatures and water deficit from veraison toripening, was conspicuously absent in plants that had received AMF inoculation (Torres *et al.*, 2018). According to Velásquez *et al.* (2020), AMF inoculation was found to improve the concentration of volatile organic compounds, which are closely related to improving grape quality. Symbiotic wines can also exhibit higher levels of bioactive compounds and improved oxidative stability, improving their nutritional and nutritional value (Gabriele *et al.*, 2016). Furthermore, research has shown that mycorrhizal inoculation can significantly improve grape quality, particularly when plants are exposed to environmental challenges such as water deficit (Torres *et al.*, 2021; Aguilera *et al.*, 2022; Goicoechea *et al.*, 2021, 2023;). In the context of climate change, as grape ripening occurs under warmer conditions, significant changes occur in grape composition. Grapes advanced their phenology, uncoupling technical and phenolic maturity, which results in berries with a high sugar content and less organic acids, resulting in higher pH (Rienth *et al.*, 2016). Results showed that the AMF inoculation induced significant modifications in quality attributes of the grape. Grapes from AMF-inoculated plants had lower values of total soluble sugars (ºBrix) (Figure 3B) and lower pH (Figure 3C) suggesting a delay in ripening and pointing to a beneficial effect under climate change conditions.

Soil infective capacity and soil microbial activity

72 days after inoculation, there is no significant effect of inoculum addiction on overall AWCD development (Treatment: df = 1, $\chi2 = 0.025$, p-value = 0.875; incubation time: df = 8, $\chi2 = 47.512$, p-value > 0.0001; Treatment: Time: df = 8, $\chi2 = 3.399$, p-value = 0.907; GLM Gamma). This result was consistent with no significant effect of inoculation on diversity (H index: Treatment: df = 1, $\chi2 = 2.445$, p-value = 0.118; Incubation time: df = 8, $\chi2 = 18.704$, p-value = 0.017; Treatment: Time: df = 8, $\chi2 = 7.205$, p-value = 0.515; GLM Gamma).

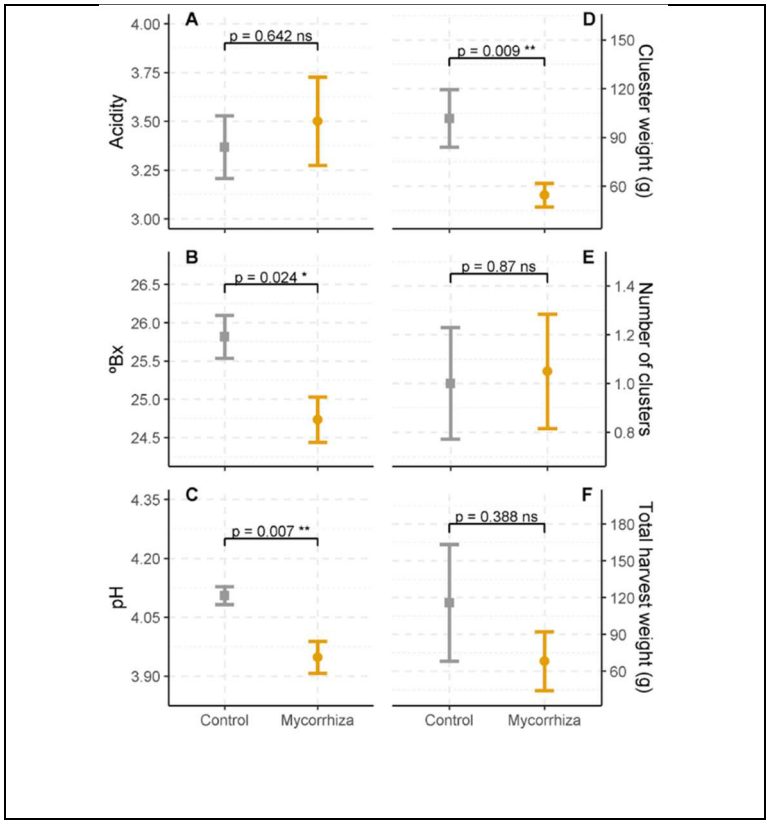

Figure 3. Mean and standard error (SE) of the plant harvest quality indicator (A: Acidity; B: °Brix; C: pH) and quantity (D: Cluster weight; E: Number of clusters per plant; F: Total harvest weight per plant) for each treatment (mycorrhiza vs. control); p-values for the GLMs are shown.

However, three plates for treatment showed low AWCD values and appeared grouped in PCA (C4; C7; C10; M2; M8; M11; Figure 4), while one plate per treatment showed different substrate oxidation (Figure 4). Control sample showed higher degradation of 4-hydroxybenzonic acid and D-malic acid. Meanwhile, the microbial communities in the soils of micorryzed plants showed higher activity on other substrates such as L-arginine and α-ketobutyric acid (Figure 4).

Nevertheless, the differences did not become significant, either considering the matrix of absorbances of all substrates (PERMANOVA adonis2 function of vegan package p=0.89) or each substrate separately (p>0.05 Wilcoxon test). The heat map of the average utilization of the C substrate types showed higher values for mycorrhiza treatment, but differences between treatments were not significant (p> 0.05 Wilcoxon test; Figure 5). The great influence of the microbiome on vine development, as well as the possible repercussions of inoculation on native microbial communities, should not be underestimated (Darriaut *et al.*, 2022). Although using a technique capable of detecting changes in bacterial function, only in the case of cultivable species, results indicate that the addition of commercial AMF inoculum could influence the indigenous soil microbiome. Cardinale *et al.* (2022) conducted an experiment in which they introduced a combination of AMF and plant growth-promoting rhizobacteria (PGPR) into vine plants. This intervention yielded markedly higher survival and significantly higher accumulations of 18 different elements. However, it also caused substantial changes in the bacterial communities present in the soil. Furthermore, the findings of Moukarzel *et al.* (2021) demonstrating that different AMF communities exerted varying effects on the development and uptake of nutrient from the rootstock. When these mycorrhizal species coexisted in equal proportions, competition ensued, ultimately resulting in reduced positive growth outcomes for vine plants (Moukarzel *et al.*, 2022)

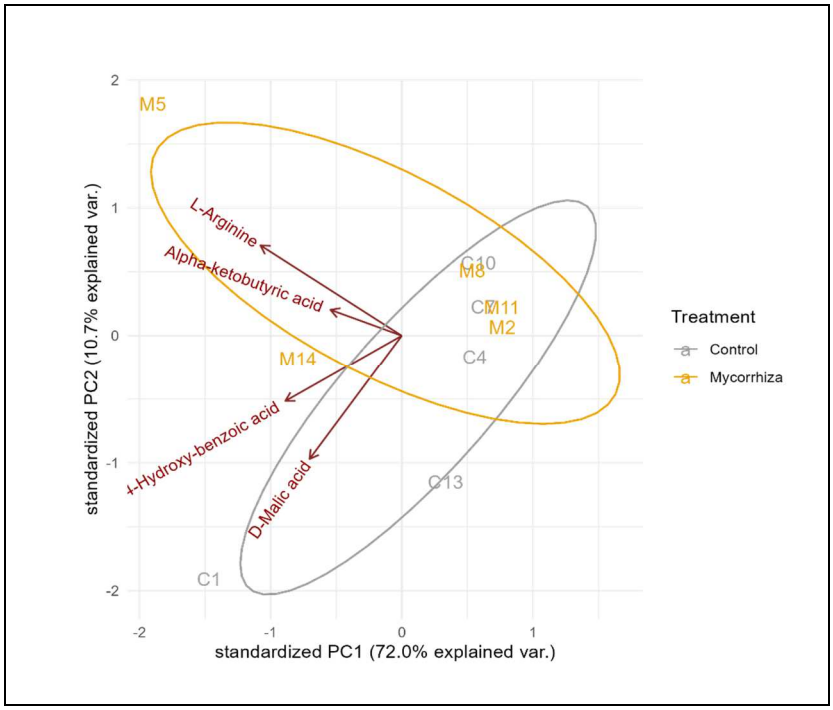

Figure 4. Biplot of the first two principal components (PC1 and PC2) of a PCA using OD at 590 nm for the 31 carbon substrates of Biolog EcoPlate™ after 168 h of culture. The proportion of variance explained is indicated for each component. Scores for the samples are plotted and the substrates with contributions >1% are shown.

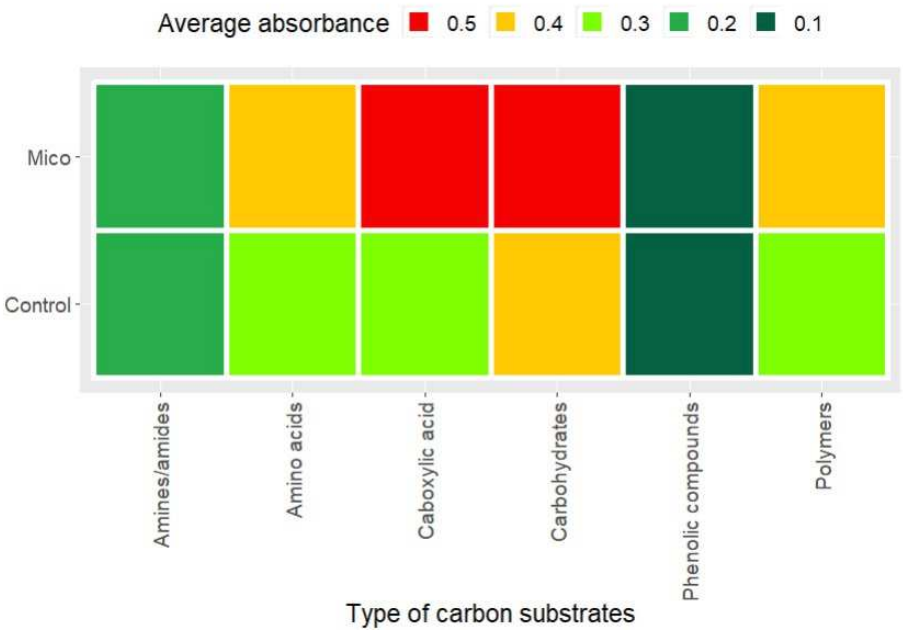

Figure 5. Heat maps generated to visualise the carbon utilization patterns of types of substrates on the Biolog EcoPlate. These patterns were based on the average sum of the 590 nm absorbance, after 168 hours of incubation, of the 31 substrates classified into 6 types.

The influence of the contribution of commercial mycorrhizal inoculum to the soil microbiome is due not only to the entry of new AMF species, but also to the fact that commercial inoculants are accompanied by a significant number of bacteria. In fact, the composition of the microbial community that accompanied the commercial inoculum used in this study has been studied and shows that it is home to a large and diverse community of bacteria (Agnolucci *et al.*, 2019).

These bacterial communities can include important functional PGP traits, possibly acting in synergy with AMF and providing additional services and benefits, but also affecting the natural bacterial community of soils. For example, Jacobs-Hoffman and Hills (2021) demonstrated notable variation in carbon substrates metabolized by bacteria in Biolog EcoPlates™ within the rhizosphere soil of inoculated *versus* control tomato plants after 56 days of using the commercial AMF inoculum Mycoroot™. In outhe present study, despite the trend towards greater average degradation of the different types of substrates shown in the inoculated treatment (Figure 5), the differences were not significant. This could be ascribed to either a genuine lack of effect of the inoculum used on bacterial functional diversity in this soil or to issues with the sampling system. It is possible that the spread of microorganisms through the soil was slow and that the sampling points were not consistently close to the inoculation site. Alternatively, the number of replicates used may have been insufficient to detect a significant effect given the expected effect size. Finally, the mycorrhizal colonization capacity of the rhizosphere soil of the vines was tested. No significant differences were observed between the mean percentage of colonization of lettuce roots growing in soil from inoculated vines or soil from the control (Control 51 ± 3.9% Inoculated 44 ± 4.4% df = 1, $\chi 2$ = 0.801, p-value = 0.813; GLM-binomial). This is not surprising since most viticultural soils contain populations of local mycorrhizae that can establish symbiosis and have diverse effects on vines (Schreiner *et al.*, 2007; Carbone *et al.*, 2021; Landi *et al.*, 2021).

Several works showed that inoculations with the AMF community of natural soil generate a stronger effect than commercial fungal inoculants of AMF (Berruti *et al.*, 2016; Frew, 2021). Indeed, research revealed that when comparing numerous commercial inocula, only a few produced a higher level of colonization by AMF, at its recommended application rates, compared to natural soil (Tarbell and Koske, 2007; Faye *et al.*, 2013; Salomon *et al.*, 2022). This could be related to the loss of viability of the propagules in the commercial product. Sometimes, the label does not include an expiration date or storage recommendations. In this study, the recommendations regarding the dosage and conservation of the product were followed.

The lack of differences may have resulted from the high infectivity of local soil (>50% root colonization), as it is a farm under biological cultivation with organic fertilization, no use of chemical products (Mäder *et al.*, 2000), and minimal tillage (Kabir *et al.*, 1997). This is consistent with the absence of observed effects of inoculation at both plant and harvest levels. Other studies analysing the impact of inoculation in the field have reported significant differences in the percentage of colonization of rice between AMF-inoculated plants and the control. For example, Nicolás *et al.* (2015) found values exceeding 40%, even one year after inoculation, and up to 89% with re-inoculation, compared to 10% in control plants. Consequently, they also observed significant improvements in the growth and nutrition of inoculated plants. However, Nogales *et al.* (2021), despite confirming positive mycorrhizal infection in inoculated grapevine plants, observed positive effects on photosynthesis and harvest only under stress conditions.

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CONCLUSIONS

Despite several references in the literature of positive results associated with inoculation of grapevines onto different rootstocks with *R. irregulare* BEG72 (synonym *R. irregularis*, basionym *G. irregulare*) and *F. mosseae*, (basionym *G. mosseae*), the final outcome of each individual trial depends on various factors. The assay described showed that the effects on growth vary over time and can even be negative at certain moments with a neutral final result. In the case of the vineyard, the improvement of grape characteristics seems to be a more significant effect of mycorrhizal inoculation than the effects on plant growth or harvest. In the present study, a year and a half after inoculation, an effect on the quality of the grapes was observed, with a tendency to improve the ripening conditions for wine production. The use of the inoculant in this study appears to be safe and has a low impact on the local microbiome. However, the results presented show that the application of AMF in commercial vine plantations should be carried out with caution, and small-scale case studies are recommended before large-scale application. The use of AMF can relieve stress or delay grape ripening without improving plant growth or increasing harvest. The divergent impacts on various plant parameters underscore the importance of employing AMF with a precise purpose. This approach enables the careful selection of the inoculum, timing, and application method to achieve specific goals.

The findings of this research provide valuable insight into the performance of the commercial inoculum studied in field conditions, thus contributing to understanding its potential applications in similar contexts. It is essential to enhance knowledge of how commercial inoculations work in the field in order to identify the factors that influence results and improve their effectiveness for future use.

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