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From Plant Nursery to Field: Persistence of Mycorrhizal Symbiosis Balancing Effects on Growth-Defence Tradeoffs Mediated by Rootstock

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Abstract: The plant domestication process led to crops with strongly modified growth-defense tradeoff features, and crops that were much more pampered in terms of nutrition, irrigation and defense measures, showing less ability to trigger adaptation strategies with respect to their wild relatives. It is worth noting that plants are not alone, they share their environment with a myriad of microbes supporting them with many relevant functions. We have already demonstrated that an arbuscular mycorrhizal fungal (AMF) inoculum (formed by two AMF species, i.e., *Rhizophagus irregularis* and *Funneliformis mosseae*) is able to balance growth and defense responses in two grapevine rootstocks with opposite tradeoff features. In the present study, we evaluated the persistence of AMF-mediated balancing effects under field conditions, confirming the positive impact of the symbiosis in vineyards. In detail, some genes related to nitrogen (N) uptake and metabolism were specifically modulated by the presence of the symbionts, while others were not. Additionally, photosynthetic performances and stilbenes accumulation were influenced by the AMF presence. Overall, our results open new questions about the timing of AMF inoculation in grapevine to obtain a stable and functional symbiosis, suggesting that an early inoculation can facilitate the interaction between grapevine roots and these beneficial microorganisms.

Keywords: growth-defense tradeoffs; AM fungi; leaf gas exchange; grapevine; stilbenes

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1. Introduction

Plants are continuously subjected to biotic and abiotic environmental stimuli that activate a sophisticated plethora of responses, involving diverse molecular and biochemical pathways to protect themselves [1]. In this regard, many cultivated crops are the results of a long co-evolution history which led to the selection of useful traits for human requirements. This so-called plant domestication process led to plants with strongly modified growth-defense tradeoff features, and plants that were much more pampered in terms of nutrition, irrigation and defense measures, showing less ability to trigger adaptation strategies with respect to their wild relatives [2]. Grapevine is one of the world's most economically important crops and, in turn, one of the agricultural systems with higher agrochemical application and a consequent negative environmental impact [3]. It is worth noting that plants are not alone in soil. They share their environment with a myriad of microbes that support them to improve plant physiological functions and to cope with environmental stresses [4–7]. Thanks to Next Generation Sequencing (NGS) technologies, several studies analyzed the microbiome composition of several grape tissues in diverse environments (defining the so-called microbial *terroir*) as well as, at least for some of them, their functional roles *in planta* [8–10]. Among belowground-living microbes, particular

emphasis should be given to arbuscular mycorrhizal fungi (AMF) that are obligate symbionts able to colonize more than 80% of land plants, including grapevine [11,12]. It is well known that AM symbiosis plays a key role in providing nutrients (e.g., phosphorus, —P— and nitrogen, —N—), water and other elements to their hosts as well as other important ecological services [13–15]. The association between AMF communities and grape, a typical fruit crop in the Mediterranean basin [16,17], has already been reported suggesting that they might represent a sustainable alternative in viticulture to reduce chemical and water inputs in the future. Recently, positive effects on both root growth and branching, coupled with enhanced expression of two grape phosphate transporters (PTs), have been observed in acclimatized grape plantlets colonized by *Funneliformis mosseae* [18]. It has also been demonstrated that grapevines colonized by AMF displayed more efficient photosynthetic performances and water absorption and transport, compared to non-colonized ones, making them more resilient to heat and water stresses [17,19]. Apart from the well-known AM-mediated priming against abiotic stresses, the triggering of the so-called mycorrhiza-induced resistance (MIR) [20,21] against biotic enemies is still poorly explored in grapevine. In this regard, Bruisson and colleagues [22] suggested that inoculation of *R. irregularis* at the cutting stage of several potted grape varieties grafted onto 41B led to improved molecular and biochemical (e.g., stilbenoids) defense pathways 48 h after *Plasmopara viticola* and *Botrytis cinerea* inoculation. This suggests that AM symbiosis can potentially lead grape plants in a priming status against necrotrophic and biotrophic fungal pathogens. Recently, Cruz-Silva et al. [23] demonstrated an altered expression of several *P. viticola* effectors in Cabernet Sauvignon plants inoculated at the cutting stage with *R. irregularis* (in pot inoculation), further confirming the AM-mediated improved plant defense to control downy mildew. Similarly, a recent study, using a non-targeted metabolomic approach combined with targeted transcriptomics in *Vitis vinifera cv*. Gewurztraminer colonized with *R. irregularis* (at the woody cutting stage), revealed a strong reprogramming in root primary metabolism and defense responses in leaves, attesting the priming status mediated by the AM symbiosis [24]. Additionally, some reports showed that AM symbiosis improved tolerance to root fungal pathogens such as *Armillaria mellea* (AMF inoculated in grapevine plantlets *in vitro*) [25], *Cylindrocarpon macrodidynum* (AMF inoculated at the cutting stage in pot) [26] and the soil nematodes such as *Meloydogine incognita* (AMF inoculated at the grapevine seedling stage in pot) [27] and *Xiphinema index* (AMF inoculated at the cutting stage in pot) [28], although the mechanisms behind these interactions need to be elucidated. Most of the studies on plant interaction with AMF have been in fact conducted in controlled conditions using *in vitro* techniques or sterilized substrates that simplify the variables occurring in an open field system [17]. This approach is useful for basic research purposes, but it is far from the natural environment conditions where, at different levels, complex interactions, which can compromise the resident microbial community and/or beneficial microbial associations with their hosts, take place [29,30].

To favor grapevine adaptation in diverse soil-scape and climate features, vines are commonly grafted with rootstocks that strongly influence scion responses to biotic and abiotic factors [31–33]. The modulation of physiological performances is reflected on quality features in the berry [33] and are also able to shape root bacterial microbiome and networking [34]. Additionally, our recent work demonstrated that an AMF inoculum (formed by *R. irregularis* and *F. mosseae*), applied in two rootstocks with opposite tradeoff features (i.e., 1103 Paulsen and SO4), is able to balance growth and defense responses [12]. In addition, an 'Inducer' (I, D-glucose at low dose; INOQ GmbH, patent EP2982241A1), previously reported to stimulate native AMF associations [35], was used in a non-sterilized vineyard soil (used as plant substrate). Growth traits were improved in all treatments (AMF inoculum, Inducer and their combinations) when looking at the rootstock genotype characterized by a low vigor (SO4), while defense priming responses were potentiated in the high vigor genotype (1103 Paulsen).

In the present study, the aim was to evaluate the persistence of the observed growth-defense tradeoff effects [13] under field conditions using plants already assessed for the

priming responses (from Nerva et al. [12]) and then transplanted in a vineyard and followed for two consecutive seasons. To achieve this goal, in addition to the expression analyses on root tissues considering N uptake and metabolism-related genes before the transplanting, the main growth (indole acetic acid—IAA) and defense (stilbenoids and abscisic acid—ABA) features were studied using a combined ecophysiological and targeted metabolomics approach at véraison (BBCH 81). The role of IAA in plants is well known since it represents the predominant and most indispensable auxin in plants [36]. Its role is not only restricted to the plant, but it also plays crucial functions during plant-microbe and microbe-microbe interactions [37]. In parallel, the ABA hormone is also pivotal in plants due to its extensive involvement in physiological processes and stress adaptation [38]. In more detail, ABA is the central regulator of abiotic stress resistance in plants and coordinates physiological responses such as stomatal closure, cuticular wax accumulation, leaf senescence, bud dormancy, seed germination, osmotic regulation, and growth inhibition [39]. Finally, the role of stilbenes in protecting grapevine from fungal diseases is well documented in the literature [40]. Among stilbenes-derived compounds, resveratrol and viniferin exert the major antifungal effects [41] and, for this reason, were analyzed in our study. The véraison stage was chosen as the sampling timepoint since downy mildew pressure is commonly high in the Mediterranean climate [3] and the N uptake reaches the maximum rate influencing growth performances (e.g., photosynthesis) [5,42].

2. Materials and Methods

2.1. Plant Materials and Experimental Design

In this study two hundred *Vitis vinifera* cv 'Glera' plants grafted onto 1103 Paulsen (1103P, 100 plants) and SO4 (100 plants) were subjected to diverse treatments (see below) as previously described [12]. In detail, prior to field planting, the viability and functionality of the AMF symbiosis were checked by means of root microscopy observations and molecular analyses, confirming that mycorrhizal vines were successfully colonized as previously reported [12]. These plants were transferred in an experimental field, courtesy offered by Villa Sandi S.p.A., and located at Nervesa della Battaglia, TV, Italy (GPS coordinates: 45.84377N, 12.18452E). The soil composition was the same used for the previous pot experiment (sandy loam soil, pH 7.8; available P 10.4 mg kg^{-1}; organic matter 1.80%; cation exchange capacity 20.11 mew 100 g^{-1}; Nerva et al. [12]). Measurements were taken in 2- and 3-year-old vines planted at a spacing of 2.80 × 1.30 m. Plants were trained to a vertical trellis system with spur pruning; conventional agronomic management was applied in the vineyard and water was supplied 3 times per week for 2 h, delivering 6 L h^{-1} per plant over the vegetative season by means of a subsoil irrigation system. As previously reported, for both rootstock genotypes, three treatments were compared to the uninoculated controls (CTRL, 25 plants for each rootstock): (i) plants inoculated with the AMF-mixed inoculum (Advantage Grade II—INOQ GmbH) (25 for each rootstock, M); (ii) AMF-inoculated plants + Inducer (D-glucose low dose, INOQ GmbH; patent EP2982241A1) (25 plants for each rootstock, M+I); (iii) 25 plants for each rootstock amended with the Inducer only (I).

Plants were transplanted in rows and each treatment was located in adjacent blocks positioning in distal parts of the vineyard AMF-inoculated and uninoculated plants. At least 5 randomly selected plants were monitored for ecophysiological performances and targeted metabolites production (see below) for two consecutive seasons at véraison (July 2020 and 2021; BBCH 81 stage). During the two seasons, weather conditions were monitored using the CREA-VE weather station coupled to a Watch Dog 1400 datalogger instrumentation (Spectrum Technologies, Bridgend, UK) (Supplementary Figure S1). Moreover, to further elucidate the N fate in AMF-colonized roots, prior to transplantation, root tissues were sampled to analyze the transcript modulation of some N-related target genes (e.g., nitrate transporters) previously found to be regulated by AMF symbiosis in grapevine roots [43].

2.2. RNA Isolation and RT-qPCR Analysis

Root tissues from at least 3 cuttings for each treatment were sampled prior to planting them in the field. Collected samples were immediately frozen, freeze-dried and stored at −80 °C until use. Total RNA was isolated from the lyophilized samples and cDNA synthesis was performed as previously reported [44]. The absence of genomic DNA contamination was checked before cDNA synthesis by qPCR using specific grapevine ubiquitin (*VvUBI*) primers. RT-qPCR reactions were carried out in a final volume of 10 µL containing 5 µL of SYBR® Green Master Mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 1 µL of 5 µM specific primers and 1 µL of 1:5 diluted cDNA. Reactions were run in the CFX 96 apparatus (Bio-Rad Laboratories, Inc.) using the following program: 10 min preincubation at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 30 s at 60 °C. Each amplification was followed by melting curve analysis (60–94 °C) with a heating rate of 0.5 °C every 15 s. All reactions were performed with at least two technical replicates. The comparative threshold cycle method was used to calculate relative expression levels using plant (ubiquitin and cytochrome oxidase, *VvUBI* and *VvCOX*) reference genes. Oligonucleotide sequences are listed in Supplementary Table S1. Gene expression data were calculated as expression ratio (Relative Quantity, RQ) to Control 1103P or SO4 plants (C 1103P or C SO4, respectively).

2.3. Leaf Gas Exchange Measurements

For both seasons (July 2020 and 2021), gas exchange analyses were performed on warm and sunny days (see above). In general, a clear sky with moderate-to-high solar radiation and vapor pressure deficit (VPD) and some rainfall events were recorded over the véraison stage, as commonly observed in the Veneto region [45,46] (Figure S1). Measurements of net photosynthesis (Pn), transpiration rate (E), intercellular CO_2 concentration (Ci), water use efficiency (WUE, obtained as the ratio between Pn and E) and apparent carboxylation efficiency (ACE, obtained as the ratio between Pn and Ci; Flexas et al. [47]) were carried out on 6 randomly selected vines from each treatment. For each plant, two fully developed non-senescent leaves at the same physiological age (fourth to fifth leaf from the shoot apex) were measured using a portable infrared gas analyzer (ADC-LCi T system; Analytical Development Company, BioScientific Ltd., Hoddesdon, UK) in the hottest hours of the day as previously reported [45]. During measurements, ambient parameters were maintained: light intensity ranged from 1.700 to 1.900 µmol photons m^{-2} s^{-1}, the temperature ranged from 27 to 29 °C and the concentration of the CO_2 in the air ranged from 420 to 440 ppm.

2.4. HPLC-DAD Targeted Metabolites Analysis

Concentration levels of indole-acetic acid (IAA), abscisic acid (ABA) and stilbenoids (resveratrol and viniferin) were measured in leaves using a high-performance liquid chromatographer (HPLC) as previously described [3,12]. Briefly, for both seasons, at least 5 independent biological replicates (one leaf per plant) from each treatment and rootstock genotype were collected and lyophilized. Extraction was performed using 100 mg in 1 mL of methanol:water (8:2 v/v) acidified with 0.1% (v/v) of acetic acid in an ultrasonic bath for 1 h. Once centrifuged, the supernatant was analyzed by an HPLC apparatus (Agilent 1220 Infinity LC system; Agilent R, Waldbronn, Germany) model G4290B equipped with a gradient pump and auto-sampler. A 170 Diode Array Detector (Gilson, Middleton, WI, USA) was used. Original standards of *t*-resveratrol, viniferin, ABA and IAA (Merck KGaA, Darmstadt, Germany) were used for the identification by comparing the UV spectra with samples. A C18 analytical column (250 mm Å–4.6 mm i.d., 5 µm, Macherey Nagel) was used and the concentration was determined by the external calibration method.

2.5. Statistics

For each treatment and rootstock genotype, the mean values and ± standard deviations (SD) were calculated. For treatment comparisons, data were subjected to one-way ANOVA and Tukey's HSD as a post-hoc test ($p \leq 0.05$) using the SPSS statistical software package (version 23; SPSS Inc., Cary, NC, USA).

3. Results

3.1. Expression Analysis of Nitrogen-Related Genes in Cuttings

To deepen the molecular facets of N uptake and transport mediated by AM symbiosis in the two rootstock genotypes, the relative expression of three key genes involved in these processes was evaluated in roots (Figures 1 and 2, Table S1). Transcripts analyses showed diverse regulations depending on the gene and treatment applied. Looking at *VvNRT1.3* and *VvNRT2.4*, the first one was mainly affected by the M (i.e., the AMF inoculation) and Inducer treatments, although in an opposite way, with M stimulating the upregulation and the Inducer treatment a downregulation. Indeed, higher values were observed in AMF-inoculated plants with respect to all the other treatments (Figures 1a and 2a), suggesting a role in nitrate uptake during symbiosis, particularly in 1103P genotype (Figure 1a). Conversely, Inducer and M treatments significantly affected the *VvNRT2.4* expression showing a rootstock genotype-dependent regulation, with Inducer1103P plants showing significantly higher values with respect to the other treatments, while Inducer SO4 did not differ with respect to C (i.e., control untreated plants) SO4 (Figures 1b and 2b). It is also worth noting that a *VvNRT2.4* higher expression level was observed in M+I SO4 plants (Figure 2b) with respect to the other treatments. The high-affinity nitrate transporter *VvHNT1* showed a peculiar expression pattern with a significantly higher expression only in SO4 genotype after treatment with the Inducer (Figure 2c), while in both genotypes and treatments, transcripts were generally down-regulated with respect to their controls (Figures 1c and 2c).

3.2. Leaf Gas Exchange Performances in Field

To evaluate the persistence of growth-related features mediated by the treatment application, photosynthesis and water exchange with the surrounding atmosphere were monitored. In detail, gas exchanges were measured for two consecutive seasons at véraison (BBCH 81), a specific stage in which grapevine metabolism is very active to support growth and defense features, at least in our experimental context.

Pn slightly increased in all the treatments for both 1103P and SO4 rootstocks, although with a significant difference only in Inducer1103P and M+I SO4 with respect to their C (Figures 3a and 4a). Conversely, E rates significantly decreased in M and M+I treated plants with respect to C and Inducer plants in both 1103P and SO4 suggesting a key role in E modulation mediated by AMF (Figures 3b and 4b). These observed trends led WUE values significantly higher in M and M+I treatments for both genotypes with greater values recorded for M, though significant only for M 1103P (Figures 3c and 4c). Ci rates slightly decreased for both 1103P and SO4 plants in all treatments with respect to their C, although significant differences were noticed only in M+I 1103P and SO4 (Figures 3d and 4d). Conversely, ACE values did not differ significantly among treatments for both 1103P and SO4 genotypes (Figures 3e and 4e).

3.3. Growth—And Defence—Related Metabolites Content in Leaves

Persistence of growth and defense tradeoffs mediated by AM symbiosis was further investigated through a targeted metabolomics approach, quantifying key growth (IAA) and defense (ABA and stilbenoids) metabolites. Regardless of the treatment applied, ABA concentration was not detectable in all samples analyzed (data not shown). Conversely, IAA concentration increased in all the treatments with respect to C in both 1103 and SO4 plants (Figure 5). In detail, M+I 1103P showed the higher values, significantly different with respect to C and Inducer plants, whilst M SO4 and M+I SO4 ones were significantly higher with respect to C SO4. These data suggest a role of AM symbiosis in maintaining growth-related metabolite production and accumulation also in SO4 genotype, commonly considered a rootstock inducing low vigor in the scion (Figure 5).

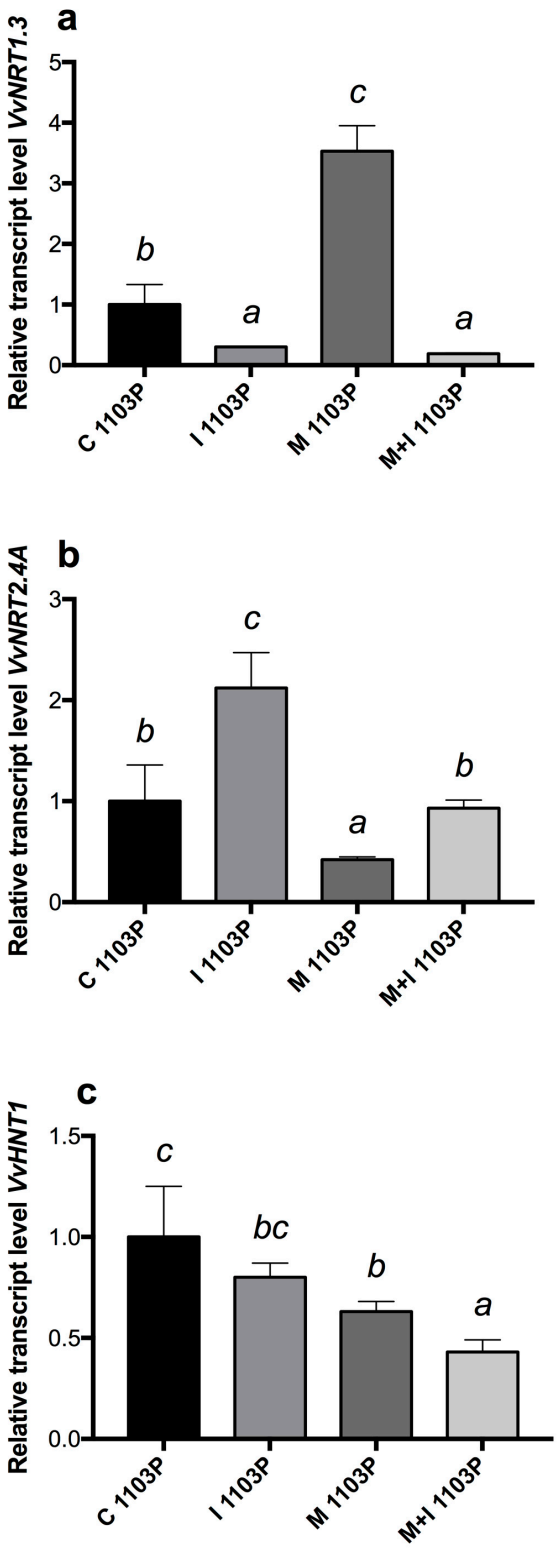

Figure 1. Expression changes of nitrogen-related genes in 1103P root. All data are expressed as mean ± SD (n = 3). Different lowercase letters above the bars indicate significant differences according to Tukey's HSD test ($p < 0.05$). C, control plants; I, Inducer-treated plants; M, AMF mixed inoculum-treated plants; M + I AMF mixed inoculum + Inducer-treated plants. (**a**) is the relative expression level of *VvNRT1.3* (**b**), is the relative expression level of *VvNRT2.4A* (**c**), is the relative expression level of *VvHNT1*.

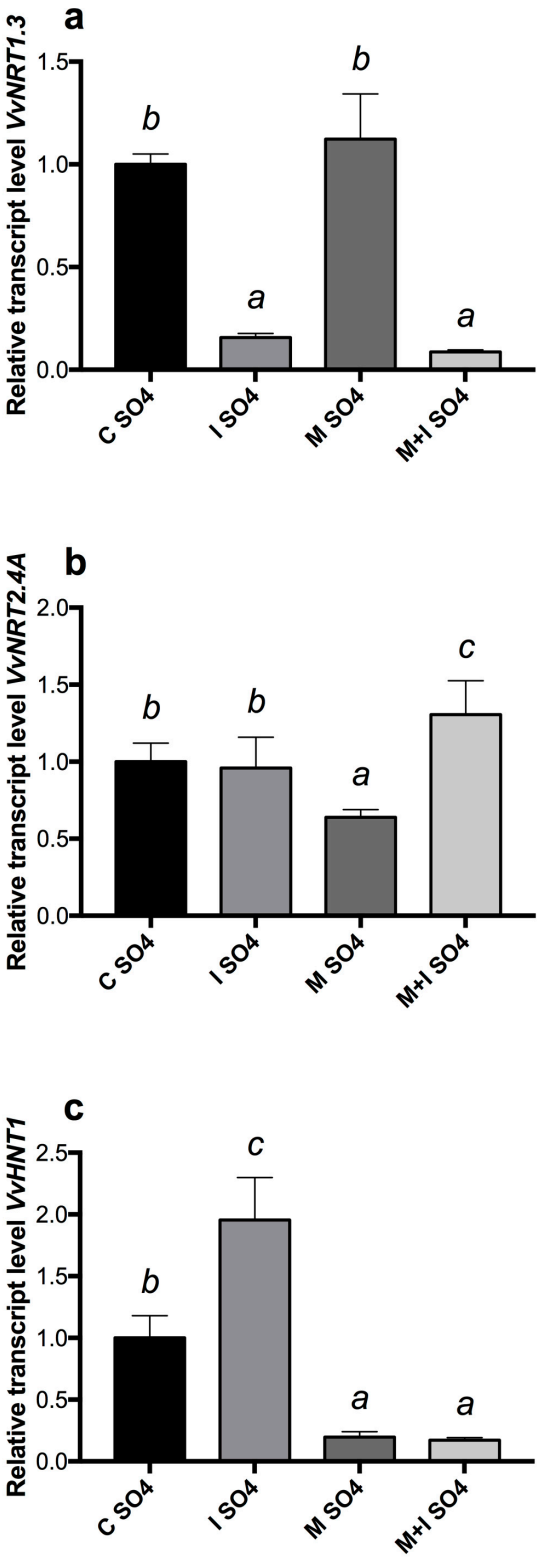

Figure 2. Expression changes of nitrogen-related genes in SO4 root. All data are expressed as mean ± SD ($n = 3$). Different lowercase letters above the bars indicate significant differences according to Tukey's HSD test ($p < 0.05$). C, control plants; I, Inducer-treated plants; M, AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants. (**a**) is the relative expression level of *VvNRT1.3*, (**b**) is the relative expression level of *VvNRT2.4A*, (**c**) is the relative expression level of *VvHNT1*.

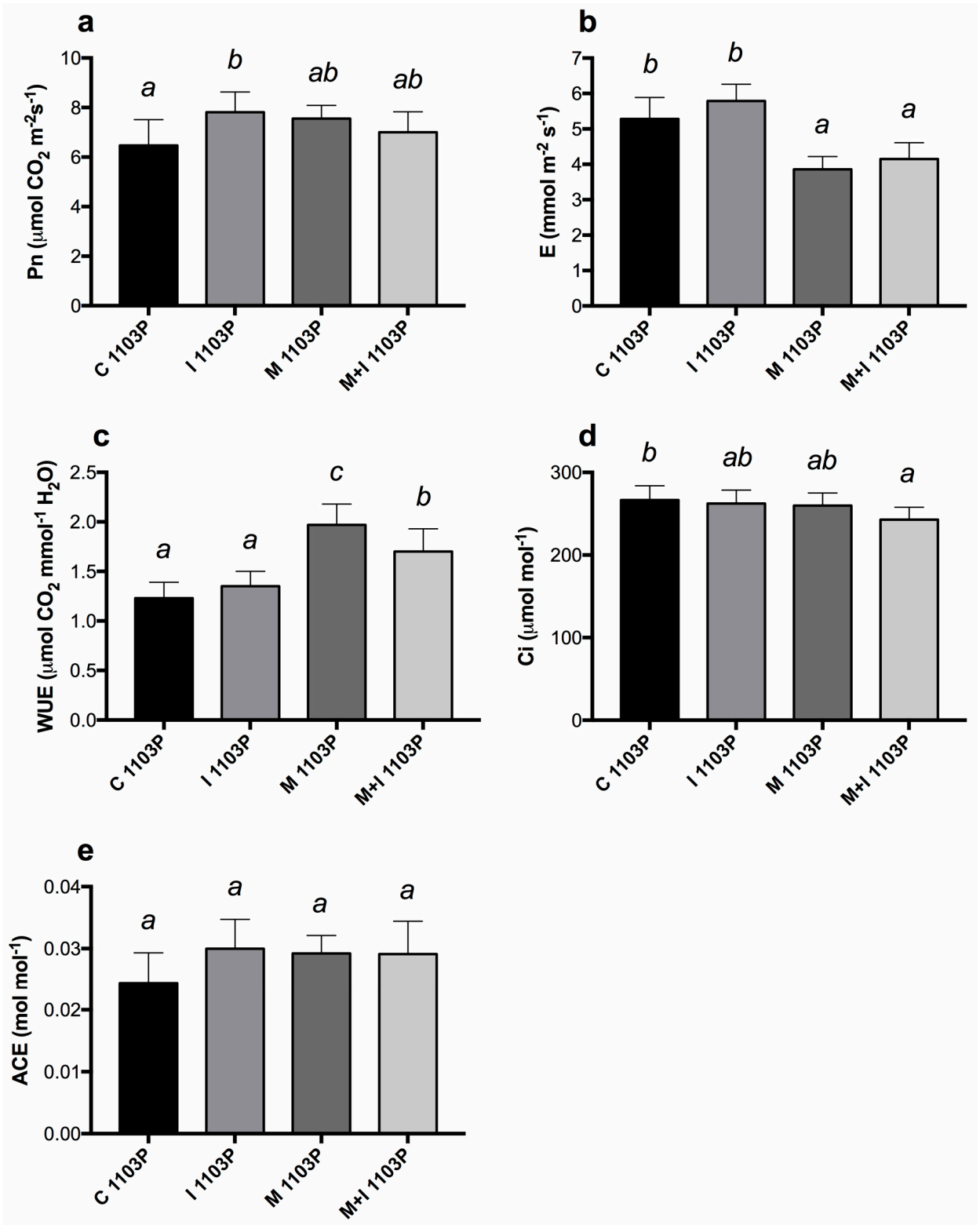

Figure 3. Leaf gas exchange in 'Glera' plants grafted onto 1103P rootstock at véraison. Performances of net photosynthesis (Pn) (**a**), transpirations (**b**), water use efficiency (WUE) (**c**), intercellular CO_2 concentration (Ci) (**d**), apparent carboxylation efficiency (ACE) (**e**). Data are expressed as mean ± SD (n = 10) of randomly selected plants over 2020 and 2021 seasons. Different lowercase letters above the bars indicate significant differences according to 'Tukey's HSD test ($p < 0.05$). C, control plants; I, Inducer-treated plants; M, AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants.

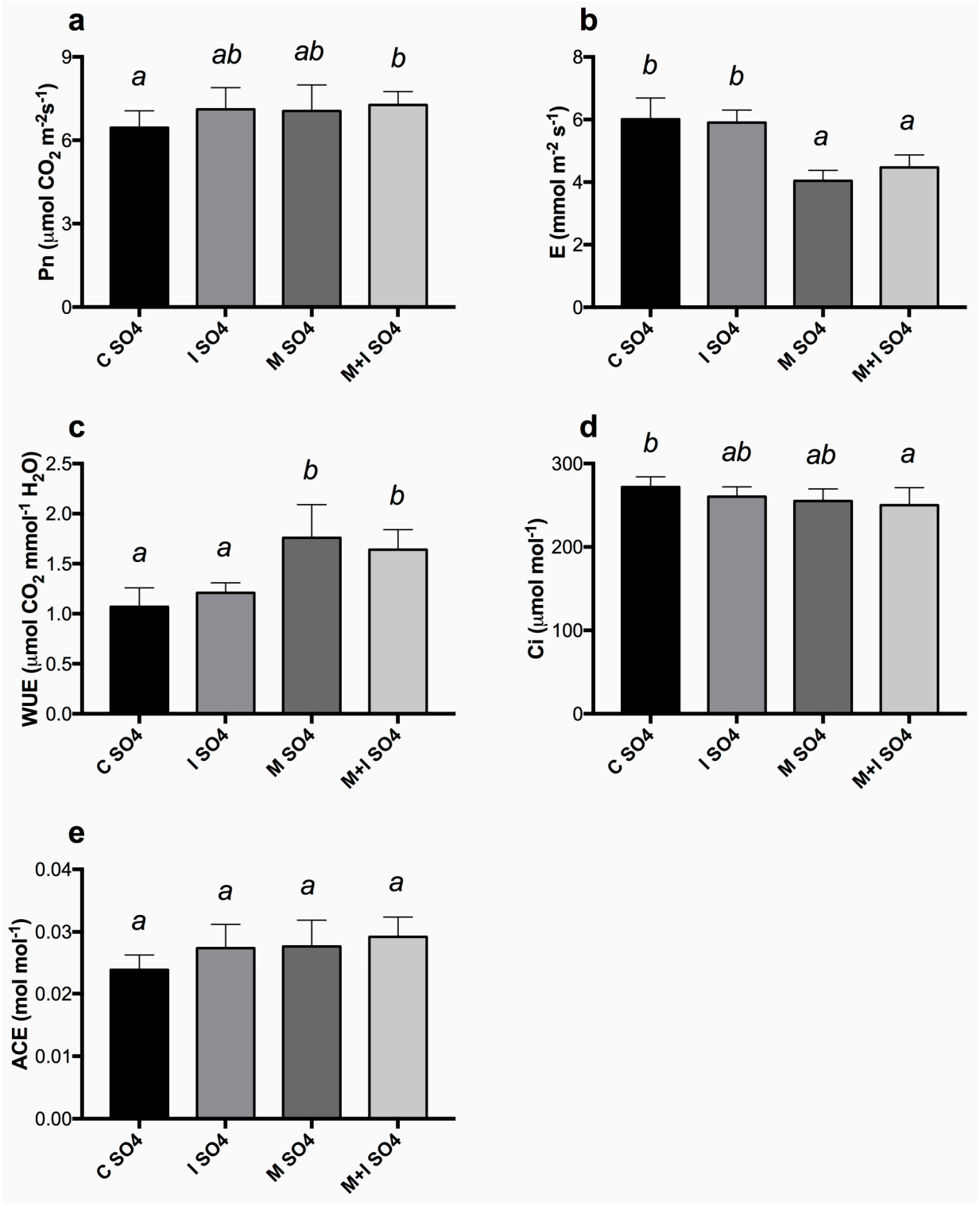

Figure 4. Leaf gas exchange in 'Glera' plants grafted onto SO4 rootstock at véraison. Performances of net photosynthesis (Pn) (**a**), transpirations E (**b**), water use efficiency (WUE) (**c**), intercellular CO_2 concentration (Ci) (**d**), apparent carboxylation efficiency (ACE) (**e**). Data are expressed as mean ± SD (n = 10) of randomly selected plants over 2020 and 2021 seasons. Different lowercase letters above the bars indicate significant differences according to Tukey's HSD test ($p < 0.05$). C, control plants; I, Inducer-treated plants; M, AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants.

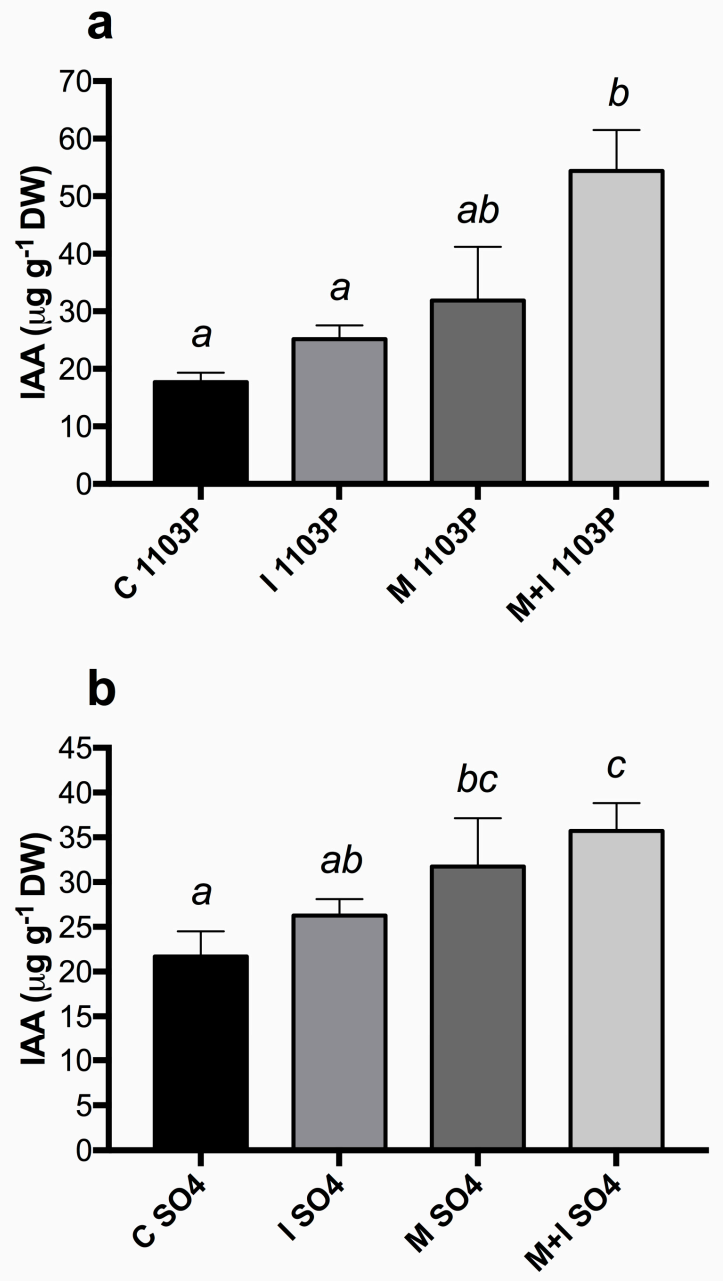

Figure 5. Analysis of the endogenous leaf IAA concentration. (**a**) IAA concentration in leaves of 'Glera' plants grafted onto 1103P and SO4 (**b**) rootstock genotypes. Data are expressed as mean ± SD (n = 10) of randomly selected plants over 2020 and 2021 seasons. Different lowercase letters above the bars indicate significant differences according to Tukey's HSD test ($p < 0.05$). C, control plants; I, Inducer-treated plants; M, AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants.

Looking at the concentration of the defense-related stilbenoids (Figures 6 and 7), *t*-resveratrol did not differ significantly among treatments in both 1103P and SO4 grafted plants (Figures 6a and 7a). Contrariwise, viniferin was significantly more accumulated in M and M+I 1103P leaves with respect to C (Figure 6b), while this metabolite did not differ among treatments in SO4 samples (Figure 7b) further confirming the AMF-mediated balancing effect in 1103P plants.

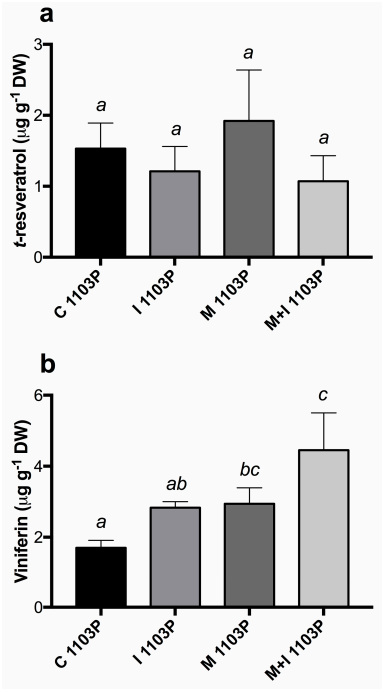

Figure 6. Stilbenes concentration in leaf tissues of 'Glera' plants grafted onto 1103P rootstock. (**a**) *t*-resveratrol quantification and (**b**) Viniferin quantification. Data are expressed as mean ± SD (n = 10) of randomly selected plants over 2020 and 2021 seasons. Different lowercase letters above the bars indicate significant differences according to Tukey's HSD test ($p < 0.05$). C, control plants; I, Inducer-treated plants; M, AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants.

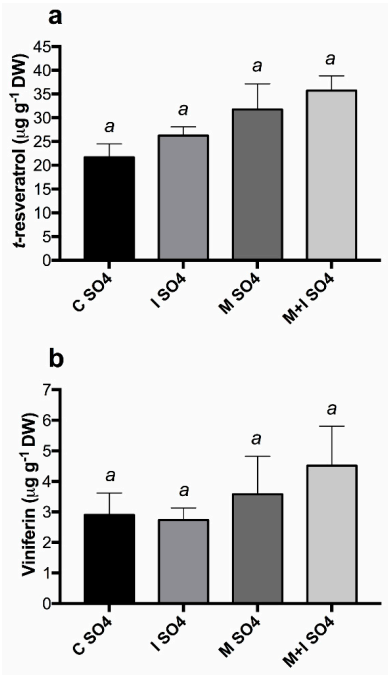

Figure 7. Stilbenes concentration in leaf tissues of 'Glera' plants grafted onto SO4 rootstock. (**a**) *t*-resveratrol quantification and (**b**) Viniferin quantification. Data are expressed as mean ± SD (n = 10) of randomly selected plants over 2020 and 2021 seasons. Different lowercase letters above the bars indicate significant differences according to Tukey's HSD test ($p < 0.05$). C, control plants; I, Inducer-treated plants; M, AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants.

4. Discussion

The growth-defense tradeoff concept became increasingly important during the last decade, when global change negatively impacted the agricultural sector. The tradeoff principle is one of the most important 'plant economics' ways to adjust growth and defense pathways [48]. This is valid for the natural conditions where wild plants are 'programmed' to survive and reproduce in diverse ecological contexts. Thus, deciphering the mechanisms underlying growth-defense tradeoff features becomes essential for future breeding strategies, particularly those activated by beneficial root-associated microorganisms [49]. Beneficial traits delivered by plant growth-promoting bacteria (PGPBs) and AMF are largely documented for several crops, including grapevine, where they can positively affect both growth and defense paths [49–51]. As cited before, grapevine roots can establish functional symbiosis with AMF that, in turn, may influence the surrounding microbiome structure forming the so-called mycorrhizosphere [52].

In a recent study, AM-mediated growth-defense tradeoff balancing effects were noticed in both the considered rootstocks (i.e., 1103P, SO4) by the activation of diverse pathways and involving the whole root-associated microbiota [12]. Here, the plants previously considered in Nerva et al. [13] were transplanted in the field with the aim to evaluate the balancing persistence effects mediated by AMF symbiosis, analyzing functional targets directly involved in grapevine growth (e.g., photosynthesis, IAA) and defense (stilbenoids). Additionally, since several nitrate transporters were previously found to be regulated by an AMF inoculum [43], the attention was focused on the expression of some of these N-related genes in cutting roots, prior to the transplanting, to deepen the still-debated AMF-mediated effects in N transport/uptake and their potential correlation with photosynthesis performances recorded in the field. Nitrogen (N) is in fact an essential element for all grapevine processes and N transporters were found among the genes upregulated by both a single AMF inoculum and a mixed bacterial-fungal one through transcriptomics in grapevine roots [43]. However, AMF symbiosis has been reported to have positive, negative or neutral effects on N nutrition in grapevine [53]. In the previous experiment in pots and greenhouse conditions [13], lower values of nitrate uptake with respect to uninoculated controls were observed among all treatments, independently from the considered genotypes. Furthermore, no-significant effect on N accumulation in leaves was observed, suggesting that a positive correlation between N content and growth is not relevant in the considered system or likely due to a biomass dilution effect, particularly in SO4-treated plants [45]. AMF have been reported to show $NH4^+$ preference to be assimilated in extraradical mycelium and translocated to plant roots after completion of the GS-GOGAT cycle [5]. In this respect, the lower net nitrate uptake (NNU) observed in M plants suggests a role of AMF in regulating root N uptake strategies helping plants in its acquisition [12]. Here, two nitrate transporters (*VvNRT1.3* and *VvNRT2.4*) showed a different treatment-mediated modulation: *VvNRT1.3* was conversely affected by mycorrhizal symbiosis (M) and the Inducer (I), with the higher values in AMF-inoculated plants in comparison with all the other treatments, while *VvNRT2.4* was mainly influenced by the treatment with the Inducer. Thus, the considered N-related genes were differently modulated between 1103P and SO4 treated with I- and M+I, to more extent than in the M ones, suggesting that they could be specifically involved in N uptake mainly in AMF-colonized cells, as previously demonstrated for other N-related genes [54]. Conversely, a high-affinity nitrate transporter (*VvHNT1*) showed a peculiar expression pattern with a higher expression, particularly in I-SO4 plants. This could be related to the significant diversity in I-SO4 root-associated bacteria, in agreement with a previous investigation showing the induction of *VvHNT1* in grapevine roots treated with a mixed inoculum containing plant growth-promoting bacteria [12,43].

The positive effects on plant growth and tolerance mediated by beneficial soil microorganisms have been largely demonstrated in several crops. These effects can be due to microbial capability to influence host metabolism, growth and water relations mediated by leaf gas exchanges [55,56]. In this respect, gas exchanges reflect the photosynthetic status and sensitivity to biotic and abiotic factors becoming a good indicator of the physiological

processes occurring in plants in response to various environmental settings [57]. It has been already reported that AMF symbiosis can change gas exchanges in colonized plants (e.g., Pn, E), improving or modulating differently their rates on the base of pedo-climatic environments and stresses that may arise [55]. In our study, for both rootstocks, Pn and ACE only slightly increased (although not significantly) in all the treatments, according to the shift of the root-associated microbiome previously observed [12]. The Pn raising observed in I-treated plants (particularly in 1103P) might be in fact likely due to the enrichment of microbial genera with plant growth-promoting features as noticed in the metagenomic analysis previously performed on grapevine roots from the same plants used here [13]. However, no modulation in E rates was found and, consequently, a lower WUE with respect to AMF-inoculated plants have been recorded, thus negatively impacting water relations and resilience to drought events [58,59]. Additionally, the absence of significant differences of Pn rates between C- and M-treated vines (with the only exception of M+I SO4) suggests that M-plants invested in new leaves formation and photosynthetic area by means of photosynthetic energy use efficiency as observed in the woody evergreen *C. flexuosa* plants inoculated with a mixed AMF inoculum [59]. These findings are in line with what has been reported in the literature for AMF-colonized plants maintained in a healthy status (often used as controls in stress responsive studies), while promotive effects of Pn rates have been mostly observed when mycorrhizal plants were measured during the imposition of abiotic stresses [55]. WUE can reflect the photosynthetic energy conversion in plants and is related to g_s or E rates. Many studies noticed WUE increased in several crops, including grapevines, when challenged against stresses as a strategy for plants' adaptation to the surrounding environment (e.g., [45,58,60]). It is worth noting that, in our study, AM-inoculated plants showed significantly higher WUE values (due to lower E rates) with respect to C and the Inducer treatment, according to what was previously observed in tomato and maize submitted to water stress [58,61]. This finding may be of particular interest to growers, considering that plants were grown under field conditions and irrigated regularly. This, in fact, suggests that the water management schedule can be reduced, saving water resources and maintaining unaltered (or improved) photosynthetic performances. This was consistent with previous studies where AMF-inoculated plants significantly increased this index, thus improving the water status by E limitation, efficiently controlling water loss [62,63].

Beneficial microorganisms also influence the host's hormonal status either directly or indirectly [56]. Among phytohormones, IAA has been well recognized as a growth-related marker since its involvement in growth promotion and developmental events in plants [48,64]. AMF are not reported as IAA producers, although several plant species react to increasing IAA content following AMF inoculation. Mechanisms behind this phenomenon are still under debate; some studies reported an enhancement in host IAA biosynthesis with ABA that seems to play a pivotal role in triggering IAA signaling pathways. This results in the formation of more lateral roots that represent the preferred penetration sites for AMF hyphae [65]. According to these findings, IAA concentration in AMF-plants (M and M+I) confirmed the persistence of balance in the field for the less vigorous SO4 rootstock genotype with respect to its control. Additionally, IAA concentrations were not significantly different in C and Inducer samples of both rootstock genotypes in field conditions. Similarly, the previously observed growth levels in pots (by means of BBCH scale) were analogous in both Inducer and M plants, likely due to the abundance of PGPBs associated with the Inducer roots, which can be involved in stimulating growth processes and plant nutrition [12]. In this line, many root-associated microorganisms have been classified as IAA-producing species able to improve the root IAA pool, leading to increased growth and stress resilience to counteract diverse environmental stresses and providing relevant benefits to their hosts [56]. Overall, IAA evaluation in the interactions between beneficial microbes and plants can represent a good marker to define the growth and defense priming (mainly against abiotic stresses) status of treated plants in several experimental contexts.

Although a direct correlation among N transporter genes, growth targets (e.g., IAA in this study or NNU and BBCH scale observed in Nerva et al. [12]) and Pn rates cannot be clearly stated, our findings further highlight the complex interactions involving root-associated microbes, AMF and rootstock genotypes. These findings provided a proof of concept for future studies to unveil the direct and indirect role(s) of AMF symbiosis in N uptake/transport and growth features in grapevines.

Many grapevine varieties are susceptible to fungal pathogens that can cause severe damages and production loss if not massively treated with fungicides (e.g., powdery and downy mildews, grey mold). Conventional fungicide treatments contribute to an increase in environmental pollution and serious risks for human health, thus necessitating the discovery of new sustainable alternatives such as inducers and/or beneficial microorganisms [3,49]. Among them, AMF has proved to efficiently confer tolerance to biotic and abiotic stresses in diverse horticultural and woody crops (including grapevine), leading plants in a priming status by stimulating the immune system and defense responses [12,22]. These priming effects mediated by AMF symbiosis can be attributed to the potentiation of plant defense-related metabolites, such as phenolic compounds, antioxidant enzymes or pathogenesis-related (PR) proteins [58,66,67]. Looking at the defense-related compounds, grapevine stilbenoids (i.e., *t*-resveratrol and viniferin) represent the most important target compounds because of their proven antioxidants and antimicrobial activities [33]. In this study, the balancing effects were still observed in AMF-inoculated 1103P plants, particularly for the viniferin.

These results are consistent with a previous study where three AMF-colonized grapevine cultivars (Chasselas, Pinot noir and the interspecific hybrid Divico, all grafted onto 41B rootstock) showed higher amounts of active forms of stilbenoids (i.e., resveratrol, viniferin and pterostilbene) in comparison with non-mycorrhizal vines [22]. These findings provide further functional evidence of the so-called mycorrhiza-induced resistance (MIR) in grapevine, a phenomenon where mycorrhizal plants better tolerate potential biotic (and abiotic) stresses [68].

5. Concluding Remarks

Grapevine, like many other plants, lives in close association with microbes that strongly influence its physiology and fitness. Here, the persistence of AMF-mediated balancing effects has been observed in field conditions, at least for the two years of monitoring for both rootstock genotypes, confirming the positive impact of AMF symbiosis in vineyards. Conversely, the I-treated plants confirmed what was previously observed in potted experiments, leading to a diverse response with respect to those reported for functional AMF symbiosis. Although further studies are needed, these findings open new questions about the timing of AMF inoculation in grapevine to obtain a stable symbiosis. In this study, the analyzed plants were inoculated at the young cutting stage, when the association with the soil microbiota is still yet to be fully established and plant niches can be easily colonized by microbes. This less competing condition probably allows a better AMF accommodation in root cells, forming a stable and functional symbiosis (together with the associated mycorrhizosphere) since microbial community assembly is historically contingent on priority effects, as previously demonstrated [69]. This was further confirmed in our study, where AMF-colonized vines showed improved WUE in both rootstock genotypes and the balancing effects of defense-related compounds (i.e., stilbenes) were maintained in the field with respect to control plants.

Additionally, a recent study conducted in Pinot noir vines grafted onto 3309C rootstock, evaluated the priority effects of different AMF inoculation timing (using a commercial AMF inoculant): (i) at the cuttings stage in a pot containing a sterile substrate for 4 weeks before field transplanting; (ii) in the hole at the time of cuttings planting in the field, and (iii) in the soil after one year of field planting. Unexpectedly, the introduced strain did not successfully establish in any treatment, likely due to the ineffective AMF inoculant used with the selected rootstock genotype and the over-fertilized soil (phosphorus in particular) [70].

These findings highlight that, to exploit AMF potential benefits in vineyards, prior to the inoculation a careful site evaluation (e.g., soil fertility and management) and genotype selection are required.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13010229/s1, Table S1: Oligonucleotides used in this study; Figure S1: Climatic data recorded during the experimental trial (July 2020 and 2021). Average daily rainfall (blue dashed lines), average daily air temperature (mT, red lines) and average Vapor Pressure Deficit (mVPD, black lines) retrieved from the weather station during July 2020 and 2021.

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