Brief Report

First Insights into the Effect of Mycorrhizae on the Expression of Pathogen Effectors during the Infection of Grapevine with *Plasmopara viticola*

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Abstract: Grapevine (*Vitis vinifera* L.), widely used for berry and wine production, is highly susceptible to the pathogenic oomycete *Plasmopara viticola*, the etiological agent of grapevine downy mildew disease. The method commonly used to prevent and control *P. viticola* infection relies on multiple applications of chemical fungicides. However, with European Union goals to lower the usage of such chemicals in viticulture there is a need to develop new and more sustainable strategies. The use of beneficial microorganisms with biocontrol capabilities, such as the arbuscular mycorrhizal fungi (AMF), has been pointed out as a viable alternative. With this study, we intended to investigate the effect of AMF colonization on the expression of *P. viticola* effectors during infection of grapevine. Grapevine plants were inoculated with the AMF *Rhizophagus irregularis* and, after mycorrhizae development, plants were infected with *P. viticola*. The expression of *P. viticola* RxLR effectors was analyzed by real-time PCR (qPCR) during the first hours of interaction. Results show that pre-mycorrhizal inoculation of grapevine alters the expression of several *P. viticola* effectors; namely, *PvRxLR28*, which presented decreased expression in mycorrhizal plants at the two time points post-infection tested. These results suggest that the pre-inoculation of grapevine with AMF could interfere with the pathogen's ability to infect grapevine by modulation of pathogenicity effectors expression, supporting the hypothesis that AMF can be used to increase plant resistance to pathogens and promote more sustainable agriculture practices, particularly in viticulture.

Keywords: mycorrhizas; arbuscular mycorrhizal fungi; *Plasmopara viticola*; *Vitis vinifera*; RxLR effectors

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1. Introduction

During plant–pathogen interaction, several layers of defense are activated [1–3]. The first layer of plant defense consists of the recognition of Pathogen-Associated Molecular Patterns (PAMP) through Pattern Recognition Receptors (PRR) that trigger PAMP-Triggered Immunity (PTI). PTI is effective against non-adapted pathogens but not against adapted pathogens, because these are able to secrete effectors [2]. Effectors are low molecular weight proteins, secreted by pathogenic bacteria, fungi and oomycete into the plant cells, design to suppress or interfere with PTI response [4,5]. When the first layer of defense is overcome, plants activate a second layer, designed to recognize pathogen effectors. Resistance (R) proteins from the nucleotide-binding leucine-rich repeat (NB-LRR) family belong to this second layer, being able to recognize directly or indirectly pathogen effectors and activate Effector-Triggered Immunity (ETI) [2]. ETI activation may lead to hypersensitive response (HR), a programmed cell death (PCD) response caused by the plant in an attempt to control the spread of the infection. However, some of the effectors secreted by pathogens are capable of suppressing ETI also, resulting in an Effector-Triggered Susceptibility (ETS) [2].

Within plant–pathogen interactions, the grapevine downy mildew disease stands out due to the economic and social importance of grapevine. *Vitis vinifera* L., the cultivated *Vitis* specie widely used for berry and wine production, is highly susceptible to the pathogenic

oomycete *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni the causal agent of grapevine downy mildew disease [6,7]. This pathogen was introduced in Europe in mid-to late-19th century together with grapevine cuttings imported from America, the endemic region of this disease [6]. *P. viticola* spread rapidly across the European continent and by 1915 France reported a loss of 70% of its grape production to downy mildew disease [6]. *P. viticola* is an obligatory biotrophic oomycete that infects all green parts of the grapevine plant, such as leaves, twigs or young berries [8].

In the grapevine-*P. viticola* interaction, it is known that *P. viticola* secretes effectors, and several potential effector genes have been identified [9–15]. Oomycete effector proteins may act either in the apoplast (apoplastic effectors) or in the cytoplasm [5]. Within the cytoplasmic effectors, several studies highlight RxLR effectors due to their adaptative role to facilitate biotrophy [16] and to suppress PTI and/or ETI [17]. The *P. viticola* RxLR effectors described so far show different expression patterns throughout the infection of grapevine tissues [10,13,18], many of them targeting the plant nucleus [15,18,19] and acting to suppress plant immunity responses, such as the defense-related PCD, a hallmark of hypersensitive response-based immunity in plants [10,15,18–21]. Studies by Xiang et al. [18] suggest that *P. viticola* RxLR effectors suppress plant immunity by impairing H₂O₂ accumulation and reducing the transcriptional levels of defense-related genes, such as those involved in phytohormones essential for the immune response. Other studies on *P. viticola* effector function report an impairment of defense-related callose deposition and PCD at the site of infection, which were associated with effector targeting BKI1, an inhibitor of receptor-like kinases, resulting in the suppression of hormone signaling (brassinosteroid and ERECTA) involved in plant defense responses [20].

Currently, the main method to control downy mildew disease is through multiple applications of chemical fungicides, copper- and sulfur-based compounds [6]. However, the large quantities of fungicides used have led to environmental pollution, since these can accumulate in the soil and water. In 2003, Europe reported that 68 thousand tons of fungicides had been used to control grapevine diseases (Eurostat report, 2007). Therefore, the European Union has established goals to lower the usage of chemicals in viticulture (Directive 2009/128/EC) and so, there is a need to define innovative strategies to control this disease. Beneficial microorganisms with biocontrol capabilities, such as the arbuscular mycorrhizal fungi (AMF) constitute a promising way for reducing the application of fungicides [22,23].

AMF colonize about 80% of terrestrial plants, establishing a symbiotic relationship at the root level. Several studies have shown that mycorrhizal colonization leads to higher biomass and photosynthetic activity, along with alleviation of damage caused by pathogens through increased resistance of the host plant, mycorrhizal plants show a reduction in disease symptoms when compared to non-mycorrhizal plants [24–28]. The most important AMF-induced biocontrol mechanism seems to be the priming of the defense mechanisms of the host plant [22]. Priming is a phenomenon that sensitizes the plant leading to an alert state that allows a rapid and efficient defense activation when the plant is challenged by a pathogen [22]. Several studies have showed that when plants are pre-colonized by AMF, there is an induction of defense-related genes and proteins, increases in the level of defense phytohormones, antioxidant enzymes and defense compounds after pathogen attack, resulting in a better defense response when compared to non-mycorrhizal plants [24–27,29,30]. This mycorrhiza-induced priming effect is not restricted to the root being also systemically induced in the aerial parts of the plant when challenged with foliar pathogens [27,28].

While the above-cited studies have addressed the effect of mycorrhizas on the ability of plants to mount a defense response following pathogen attack, no studies have been performed on the effect of plant symbiosis with AMF on the pathogenic organism during infection. In this preliminary study we have evaluated the effect of AMF inoculation of grapevine plants on *P. viticola* pathogenicity by gene expression analysis targeting eight *P. viticola* RxLR effector genes. Here, we present for the first time, evidence that AMF

root colonization contributes for the modulation of *P. viticola* effector gene expression and hence AMF could be used to promote increased resistance to this pathogen, contributing to increase sustainability of agricultural practices, particularly in viticulture.

2. Materials and Methods

2.1. Plant Material and AMF Inoculation

Vitis vinifera cv Cabernet Sauvignon grapevine cuttings, grafted on Sélection Oppenheim 4 (SO4) rootstock, were obtained from the company VitiOeste (Leiria, Portugal). This grapevine cultivar (Vitis International Variety Catalogue number 1929) originated in France is highly susceptible to the pathogen *P. viticola*. SO4 is one of the most used rootstocks in viticulture due to its high number of roots, enabling high fruit quality and productivity. It is a hybrid between *Vitis berlandieri* and *Vitis riparia* that is able to adapt to different types of soil and environmental condition [31] being tolerant to phylloxera and nematodes [32]. A commercial inoculum of the *Rhizophagus irregularis* AMF was obtained from Symbiom (Lanškroun, Czech Republic). Grapevine cuttings were transferred to pots containing a sterile mix (3:1 v/v) of peat (pot plant substrate plus, Bord na Mona, pH 5.3–5.7) and vermiculite (grade 2). Inoculation with *R. irregularis* was performed by irrigation of the soil with a fungal spore suspension. Twenty-four cuttings were inoculated with 0.38 g of *R. irregularis* inoculum each, corresponding to approximately 1500 spores per cutting. The same number of cuttings was irrigated only with water to be used as controls. The cuttings were kept under natural conditions of light and temperature, from April to August 2019. Plants were irrigated when needed, and enough water was added to ensure that the soil was completely saturated.

2.2. Plant Growth and Mycorrhizal Colonization

A total of four months post AMF inoculation, the length and weight of the shoots of inoculated and non-inoculated plants was measured.

R. irregularis-inoculated and non-inoculated root samples were stained with trypan blue to evaluate the root colonization level of the inoculated plants. Briefly, roots from inoculated and non-inoculated plants were collected, washed, and cut into 1 cm segments. The segments were cleared in three different boiling 10% KOH solutions in sequence, during 5, 10 and 15 min, respectively, and then washed 4–5 times in distilled water and acidified in a 3.5% HCl solution for 20 min. Next, root segments were stained in a 0.05% Trypan blue solution (H₂O: lactic acid: glycerol; 1:1:1) for 7 min at an elevated temperature (without boiling). Stained root segments were washed in distilled water (4–5 times) and stored in distilled water at 4 °C. Six inoculated plants and five non-inoculated plants were used for mycorrhizal assessment. 80–100 segments were observed for each plant for the presence of AMF fungal structures such as hyphae, vesicles or arbuscules using microscopes SteREO Lumar V.12 (Zeiss, Oberkochen, Germany) and Olympus BX51 (Olympus, Tokyo, Japan) for higher resolution imaging. Mycorrhizal colonization was expressed as percentage of root segments containing mycorrhizal structures (internal hyphae, vesicle or arbuscule).

2.3. *Plasmopara viticola* Inoculation

A total of four months after AMF inoculation, grapevine plants were infected with *P. viticola* according to procedure described by Figueiredo et al. [33]. Briefly, *P. viticola* was collected from symptomatic leaves of field infected plants, after an overnight incubation in a moist chamber at room temperature. Fungal sporangia were carefully collected by brushing the abaxial surfaces of symptomatic leaves and stored at −25 °C. Prior to inoculation, sporangia viability was confirmed by microscopic observations as described in Kortekamp et al. [34]. A spore solution of 1.8×10^4 mL^{−1} was used to spray the abaxial leaf surface (3rd to 5th leaves from the shoot apex) of mycorrhizal and non-mycorrhizal grapevine plants. After inoculation, plants were kept in the dark for 8 h in a moist chamber (100% humidity) and then kept under greenhouse conditions during the infection time course. *P. viticola* inoculated leaves were collected at 6 and 24 h post inoculation (hpi),

snap frozen in liquid nitrogen and stored at -80°C . For each treatment (mycorrhized and non-mycorrhized) and time-point (6 hpi and 24 hpi) leaves from 10 different plants were collected. Disease development (infection control) was assessed using eight *P. viticola* inoculated plants by observation of downy mildew typical disease symptoms, such as “oil spots” and sporulation (Figure S1 in Supplementary Materials).

2.4. *Plasmopara viticola* Effector Gene Expression Analysis

2.4.1. RNA Extraction and cDNA Synthesis

From each treatment (mycorrhized and non-mycorrhized) and time-point (6 hpi and 24 hpi), five biological replicates were used for total RNA extraction using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's instructions. Total RNA was DNase treated using the On-Column DNase I digest set (Sigma-Aldrich, St. Louis, MO, USA). RNA concentration and purity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), while integrity was assessed by agarose gel electrophoresis. To detect contamination with genomic DNA (gDNA), treated RNA was used as a template for a real-time PCR (qPCR) reaction using specific primers for *V. vinifera* housekeeping gene *elongation factor 1-alpha* (*EF1α*). Primer sequences are available in Table S1.

First strand complementary DNA (cDNA) synthesis was performed as follows: 2 µg of total RNA were incubated with 2.5 µM oligo d(T) primer at 70°C for 10 min in a final volume of 20 µL. Next, 1X RT buffer, 1 mM of dNTP and 1 U/µL of Ribolock RNase Inhibitor were added, and the mixture was incubated 5 min at 37°C . Five U of RevertAid H Minus Reverse Transcriptase were added and the samples were incubated at 42°C during 90 min. The reaction was stopped by a 10 min incubation at 70°C . All reaction components were from Thermo Fisher Scientific (Waltham, MA, USA). cDNA was stored at -20°C .

2.4.2. qPCR Gene Expression Analysis

Specific primers for the amplification of selected *P. viticola* RxLR effectors and reference genes (*P. viticola* actin and β -tubulin) were design using the software's PrimerSelect (DNA Star Package, Madison, WI, USA) and PrimerBlast tool (NCBI) (Table S1). The effectors gene sequences were previously identified and studied by Mestre et al. [9] (*PvRxLR18*), Xiang et al. [18] (*PvRxLR16*, *PvRxLR22*, *PvRxLR27*, *PvRxLR28*, *PvRxLR61* and *PvRxLR67*) and Chen et al. [15] (*PvAVH52*). Accession numbers (Acc) of each sequence are shown in Table S1.

For each biological replicate two technical replicates were performed. Each qPCR reaction (with a 25 µL final volume) was composed of 2 µL of 1:5 diluted cDNA, 0.2 mM of primer reverse, 0.2 mM of primer forward, 1X of Maxima SYBR Green/Rox qPCR MasterMix (Thermo Fisher Scientific, Waltham, MA, USA) and H_2O . qPCR reactions were performed in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, Waltham, MA, USA). The conditions of the qPCR reaction started with an initial denaturation at 95°C during 10 min, followed by 45 cycles of a 15 s denaturation at 95°C and a 30 s annealing reaction at a specific temperature to each primer pair (Table S1). The melting curve of each reaction was analyzed to guaranty single product amplification (Figure S2). Gene expression values were calculated as fold changes between mycorrhizal and non-mycorrhizal plants, as described in Hellemans et al. [35]. The *P. viticola* housekeeping genes actin and β -tubulin were used to normalize gene expression.

2.5. Statistical Analyses

The statistical analyses were performed using IBM SPSS statistics software (version 26.0; SPSS Inc., Chicago, IL, USA). The *R. irregularis* colonization rate and the plant biomass data were analyzed for normality by the Kolmogorov–Smirnov test and for equal variances with Levene's test. Differences between mycorrhizal and non-mycorrhizal plants were analyzed by independent sample *t*-test at a significance of $p < 0.05$. The gene expression data was analyzed by independent samples Mann–Whitney U test at a significance of $p < 0.05$.

3. Results and Discussion

3.1. Mycorrhizal Colonization

Microscopic analysis after trypan blue staining confirmed grapevine mycorrhizal root colonization by the AMF *R. irregularis*. The typical AM fungal structures such as hyphae, vesicles and arbuscules were clearly observed in the inoculated roots (Figure 1). The colonization rate of inoculated plants (10%) was significantly higher when compared to the non-inoculated plants (2%) (Figure 2). Other studies that assess AMF colonization rate of SO4 rootstock refer to higher colonization rates [36–38]. Even with 10% colonization rate, grapevine plants still benefited from the AMF inoculation as plant biomass was significantly higher in *R. irregularis* inoculated plants. The 2% AMF colonization detected in the non-inoculated plants is probably related to the fact that some of the cuttings presented already some root development. The few developed roots may have been colonized by AMF fungi, a common phenomenon in nurseries and already reported in other studies [39].

Figure 1. Mycorrhizal structures observed in grapevine roots, 6 months after *R. irregularis* inoculation (Trypan blue staining). (A) Non-colonized root segment. (B) Colonized root with AMF hyphae. (C) Colonized root with AMF vesicle. (D) Colonized root with AMF arbuscules. (E) AMF root colonization on non-inoculated roots. Scale bar: 30 µm.

Figure 2. Colonization rate of mycorrhizal (Myc) and non-mycorrhizal (No Myc) grapevine plants 6 months after inoculation with *R. irregularis*. Statistical significance is represented by (*) (*t*-test $p < 0.05$).

3.2. Plant Biomass

To evaluate the effect of AMF inoculation on grapevine growth, the shoot length and weight of mycorrhizal and non-mycorrhizal plants were recorded, four months post-inoculation with *R. irregularis*. Results show that mycorrhizal inoculation significantly increased the biomass of the grapevine plants (Figure 3). Mycorrhizal plants showed a significantly higher shoot length (94.57 ± 23.40 cm) when compared to the non-mycorrhizal plants (74.26 ± 15.12 cm). Additionally, the shoot weight of the mycorrhizal plants (39.33 ± 7.94 g) was significantly higher, when compared to the non-mycorrhizal plants (24.19 ± 5.95 g). Mycorrhizal fungi are able to increase plant biomass by increasing the uptake of nutrients and water, as well as by reducing the effects of environmental stress in plants [40]. The small diameter of fungal hyphae allows AMF to reach pores in the soil that the plant's roots are not able to, covering large distances away from the root nutrient depletion zone [40]. An enhanced nutrient and water uptake lead to an increase in photosynthate production or hormonal balance resulting in an increased plant biomass. Several studies have reported an increase in grapevine plant growth upon AMF colonization [36,37,41–44]. Our results agree with the results from these studies and further confirm the benefits of AMF inoculation on the growth of grapevine plants.

Figure 3. Effect of mycorrhizal inoculation on grapevine shoot growth. (A) Shoot length of mycorrhizal (Myc) and non-mycorrhizal plants (No Myc). (B) Shoot weight of mycorrhizal (Myc) and non-mycorrhizal plants (No Myc). Statistical significance is represented by (*) (*t*-test $p < 0.05$).

3.3. *Plasmopara viticola* Effector Expression Is Altered in Mycorrhizal Grapevine Plants

This study aimed to investigate if grapevine mycorrhizal root colonization could interfere with the expression of *P. viticola* RxLR effector molecules. We analyzed the expression profile of eight *P. viticola* effector genes selected based on previous studies that showed high expression at early infection time-points and based on their capacity to interfere with plant defenses [9,10,15,18,45]. Expression was analyzed in both mycorrhizal and non-mycorrhizal (control) grapevine plants infected (6 and 24 hpi) with the oomycete *P. viticola*.

From the eight RxLR effectors analyzed, four presented no expression or very low transcript abundance in both mycorrhizal and non-mycorrhizal infected plants (*PvRxLR16*, *PvRxLR22*, *PvRxLR27* and *PvRxLR61*). In previous studies, these effectors reported to be expressed in the first 24 h upon infection [10,18]. Therefore, the lack of expression of these effectors in our study may be related to the detection limits within our experimental conditions being insufficient to detect low abundance genes. In a previous report by Gómez-Zeledón and Spring [45], these effectors were expressed at a lower level compared to other effectors in the study.

A total of four effector genes, *PvRxLR18*, *PvAVH52*, *PvRxLR28* and *PvRxLR67*, exhibited changes of expression in mycorrhizal plants when compared with non-mycorrhizal plants upon infection with *P. viticola* (Figure 4). These results show for the first time that pathogen effector gene expression may be altered by plant mycorrhization. Effector *PvRxLR18* showed a significant expression increase in mycorrhizal plants at 6 hpi (2.15 ± 0.11 -fold change), while at 24 hpi this effector was still up-regulated but the difference between mycorrhizal and non-mycorrhizal plants was not significant. This effector was studied in infected detached leaves of *V. vinifera* cv. Muscat Ottonel, where expression was detected early at 6 hpi, with peaks of expression reported at 24 hpi and 48 hpi [9]. A high *PvRxLR18* expression was also detected at 12 hpi and 48 hpi in inoculated detached *V. vinifera* cv. Cabernet Sauvignon leaves [10]. An early induction of *PvRxLR18* at 6 hpi was reported in two grapevine cultivars, one susceptible (Müller-Thurgau) and another tolerant (Regent) to *P. viticola* infection [45]. Like *PvRxLR18*, the expression of effector *PvAVH52* was also up-regulated by mycorrhizal inoculation on grapevine plants, with a significant increase at 24 hpi (2.54 ± 0.40 -fold change). This effector was reported to have a bimodal pattern of expression (increased expression at 6 hpi, that lowers at 12 hpi and is higher again at 24 hpi), being also expressed later 72 hpi [15].

Figure 4. Effect of mycorrhizal colonization on *P. viticola* RxLR effector gene expression on grapevine leaves, 6 hpi and 24 hpi. Statistical significance from comparing the expression of each effector on mycorrhizal and non-mycorrhizal plants, in each the time point, is represented by (*) ($p < 0.05$).

Studies have suggested that *PvRxLR18* and *PvAVH52* have a role in pathogen virulence. *PvAVH52* was shown to be located at the plant nucleoplasm being able to suppress PCD in *Nicotiana benthamiana* [15]. Although the function of effector *PvRxLR18* is not yet known, its early time of expression (6 hpi) may be an indicator of its importance for the infection establishment. The increased expression of these *P. viticola* effectors genes in the mycorrhizal grapevine plants suggests that mycorrhization was able to enhance the plant defense system, and that in response the pathogen could be trying to overcome plant defense mechanism by increasing effector gene expression. Gómez-Zeledón and Spring [45] reported that when *P. viticola* infects a tolerant grapevine cultivar, the expression of *PvRxLR18* was higher than that observed in a susceptible cultivar. Overall, the increased expression of these effectors in the mycorrhizal grapevine plants might indicate that the pathogen perceives the mycorrhizal grapevine as more tolerant, when in fact the plant used in our work is a susceptible cultivar.

In contrast, the expression of effector genes *PvRxLR67* and *PvRxLR28* was decreased in the mycorrhizal grapevine plants at both time points post-infection with *P. viticola* (Figure 4). Effector *PvRxLR67* was down-regulated in infected mycorrhizal grapevine leaves at both time-points post-infection, but the decrease in expression was not significant when compared to the non-mycorrhizal plants. This effector has been reported to be expressed at 24 h post infection [45], however Xiang et al. [18] reported a later expression, beginning at 72 hpi with a peak of expression at 96 hpi. Effector *PvRxLR28* was significantly down-regulated at 6 hpi (-1.64 ± 0.09 -fold change) in mycorrhizal grapevine plants when compared to non-mycorrhizal plants. This effector was also down-regulated at 24 hpi in mycorrhizal plants but the difference between mycorrhizal and non-mycorrhizal plants was not significant. Effector *PvRxLR28* was reported to be activated early during *P. viticola* infection process, showing a strong expression at 6 h post grapevine infection [18,45]. The inhibition of *P. viticola* effector gene expression detected in grapevine mycorrhizal plants in our study might suggest that AMF colonization results in the activation (priming) of defense mechanisms in the susceptible cultivar Cabernet Sauvignon, that could decrease pathogenicity in the first hours of infection, since this effector has been reported to interfere with plant's defense system [18]. For instance, effector *PvRxLR28* enhanced tobacco susceptibility to *Phytophthora parasitica* infection by repressing plant defense-related genes, such as the salicylic acid responsive pathogenesis-related genes *PR1* and *PR2*, the jasmonic acid responsive gene *LOX* and the ethylene responsive gene *EFR1* [18]. Expression of this effector was able to compromise plant defenses, such as the typical H_2O_2 accumulation in infected tissues by down-regulating plant genes encoding reactive oxygen species (ROS)-producing enzymes (*RbohA* and *RbohB*) [18]. Effector *PvRxLR28* was also shown to have the highest expression level, showing four times more expression when compared to other effectors studied during the course of grapevine infection [18]. The high and early expression of this effector may be essential for *P. viticola* infection progress or even to make the infection possible. The decreased expression of *PvRxLR28* effector detected in the mycorrhizal grapevines may be of great importance to the plant since less effector expression may result in less susceptibility to the pathogen. Overall, our results strongly suggest that the mycorrhizal-induced resistance can affect *P. viticola* pathogenicity by interfering with pathogenicity effectors expression.

Previous studies have reported variations in the expression of pathogen virulence genes when colonizing resistant and susceptible cultivars in several plant species. Pathogens presented lower expression of virulence genes (some of which were effector genes) in resistant plants, suggesting a natural capacity of resistant cultivars to recognize and interfere with the expression of pathogen effectors [46–48]. In cotton plants, a defense strategy that is able to interfere with pathogen gene expression was recently identified, reinforcing the idea that plants are able to interfere with pathogen effector expression [49]. In that study, a pair of microRNAs was identified that was able to silence specific genes essential to the fungal pathogen microsclerotium formation (critical for infection) and hyphal growth,

lowering its virulence [49]. These miRNAs are produced by the host plant after *Verticillium dahliae* infection and are exported to the fungal hyphae.

Regarding the effects of mycorrhizal colonization on pathogen gene expression and consequent mechanism underlying it, up to our knowledge, no studies were published so far. However, several mechanisms underlying the priming of the defense system observed in mycorrhizal plants that may be responsible for the alteration in the pathogen effector expression observed in our study, have been reported. One such mechanism is the activation of metabolic pathways that lead to the production of defense-related hormones, such as jasmonic-acid (JA) [25,50,51]. Studies also reported a higher expression of pathogenesis-related (PR) proteins when pre-mycorrhized plants are infected with pathogens [24,25,29,50]. An increased callose accumulation in mycorrhizal plants was also reported in leaves upon infection with pathogens [30,52]. In grapevine plants, pre-mycorrhizal inoculation with *R. irregularis* induced the expression of genes involved in stilbenoid biosynthesis, resulting in higher levels of stilbenoid compounds in leaves in response to downy mildew and grey mold infection [26]. Interestingly, susceptible grapevine cultivars were able to produce the more toxic stilbenoid compounds viniferin and pterostilbene, only when colonized by AMF, highlighting the effectiveness of AMF colonization for inducing defense compounds in such susceptible genotypes [26].

4. Conclusions

This preliminary study shows that pre-mycorrhizal colonization of grapevine plants with the AMF *R. irregularis* interferes with the expression of some pathogenic effectors during leaf infection with *P. viticola*. Mycorrhizal grapevine plants inoculated with *P. viticola* showed a decreased expression of the effector PvRxL28, which is described to be highly expressed at early stages of infection and is able to suppress plants defenses [18]. Therefore, inhibition of this effector in mycorrhizal grapevine plants may contribute to decrease *P. viticola* pathogenicity. These results suggest that pre-mycorrhization of grapevine plants may be of importance to decrease the pathogenicity of *P. viticola* and limit its ability to infect grapevine leaves. Further studies have to be conducted to test this hypothesis, namely through the evaluation of AMF priming effects in grapevine; however, these first results are promising and further support the use of mycorrhizal fungi as an alternative for a more sustainable control of the downy mildew disease in viticulture.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2071-1050/13/3/1226/s1>, Figure S1. Symptoms of *P. viticola* infection on grapevine leaves, Figure S2. Melting curves of target genes and housekeeping genes, Table S1. Genes, primers and amplification data for the qPCR analysis.

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