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Does Inoculation with Arbuscular Mycorrhizal Fungi Reduce Trunk Disease in Grapevine Rootstocks?

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Abstract: *Ilyonectria* is a weak pathogen known for causing black foot disease in young vines, infecting roots and vascular tissues at the basal end of the rootstock and restricting the movement of water and nutrients. This negatively impacts vine establishment during transplant into the vineyard. Arbuscular mycorrhizal (AM) fungi are symbiotic fungi that associate with most plants and have been shown to mitigate the infection and effect of pathogens. This greenhouse study was designed to determine if the mycorrhizal fungi could mitigate *Ilyonectria* infection and whether this was dependent on inoculation timing. 'Riparia gloire' grapevine rootstocks (*Vitis riparia*) were infected with *Ilyonectria* either after AM fungi, at the same time as AM fungi, or to roots that were not inoculated by AM fungi. We measured the abundance using specific markers for both the pathogen and AM fungi. Colonization by AM fungi did not suppress *Ilyonectria*, but instead increased the abundance of *Ilyonectria*. Further, mycorrhizal rootstocks did not have enhanced growth effects on physiological parameters when compared to non-mycorrhizal rootstocks. These findings stand in contrast to the general perception that AM fungi provide protection against root pathogens.

Keywords: digital PCR; ddPCR; root mycobiome; arbuscular mycorrhizal fungi; *Ilyonectria*; grapevine; trunk disease; pest management; microbial-interaction

1. Introduction

Black foot disease (BFD) is a grapevine trunk disease contributing to poor growth or death of young grapevines. Black foot is caused by several soil-borne fungal species belonging to the genera *Campylocarpon* (Halleen, Schroes and Crous), *Cylindrocarpon* (Wollenw), *Cylindrocladiella* (Boesew), and *Ilyonectria* (Chaverri & Salgado) [1,2]. Black foot impacts young grapevines during field establishment by infecting root vascular tissues at the basal end of the rootstock, or scion in self-rooted vines, restricting the movement of water and nutrients [1–3]. Young vine transplants are highly likely to encounter these pathogens in the vineyard as the BFD causal agents exist as facultative saprobes or are dormant within agricultural soils. Alternatively, materials coming from a nursery can be already infected by BFD agents, though asymptomatic. The severity and spread of BFD is enhanced by environmental stress, and while management practices can improve the performance of diseased grapevines, there are no known practices that can fully eliminate BFD [4].

Arbuscular mycorrhizal (AM) fungi (phylum *Glomeromycota* (Walker and Schüssler)) are obligate root symbionts found in the majority of plant species, including grapevine rootstocks [5,6]. These fungi enhance plant nutrient uptake, water use efficiency and overall growth [6,7]. They are also known to provide protection against many grapevine fungal pathogens in the genera, *Armillaria* (Fr. Vahl) [8],

Fusarium (Link), *Phytophthora* (de Bary), *Pythium* (Pringsh), *Rhizoctonia* (DC), *Sclerotium* (Tode), and *Verticillium* (Nees) [9]. In previous studies, AM fungi were shown to suppress the effects of BFD [10] and white root rot [8] in grapevine rootstocks.

There are several proposed mechanisms for how AM fungi may provide pathogen protection. There is evidence that when AM fungi colonize roots, infection by other fungi is limited [11]. Thus, colonization by AM fungi within vine roots may occlude pathogen infection via competition for space [9,11,12]. Another mechanism is via activating systemic host defense responses. AM fungi may suppress pathogen infection and symptoms throughout a plant by stimulating host immune response [9], otherwise known as induced systemic resistance. In this mechanism, AM fungal colonization results in an array of defense compounds produced by the plant's natural defense pathways [9,13] but also in early-defense mechanisms including stomatal closure, reactive oxygen species accumulation, and cell wall reinforcement [14]. Through these means it is possible that inoculating young grapevines with AM fungi could reduce BFD in newly planted vineyards.

Regardless of the mechanism, the ability of AM fungi to mediate pathogen susceptibility may be determined by the timing of infection by AM fungi. For example, if AM fungi inhibit pathogen establishment via competition for root space [11], then it would be advantageous for AM fungi to be introduced to grapevine rootstocks in advance of pathogen exposure, such as at the nursery. Similarly, if AM fungal disease suppression is via induced systemic resistance, then exposure to AM fungi before pathogens would be necessary to active these defences. In a greenhouse study by Petit and Gubler [10], it was found that pre-inoculating grapevines could prevent BFD symptoms in *V. rupestris* cv. St. George rootstocks. However, in practice, commercial AM fungal inoculum (biofertilizer) is typically applied in the field at the time of planting as per manufacturers' instructions. This approach is likely suboptimal for preventing pathogen infection and suppressing disease progression. To improve grapevine rootstock resistance to fungal pathogens it may be more effective to introduce AM fungi early [10,15], shortly after rooting in the greenhouse or nursery, prior to transplanting into the field.

In this study, we set out to determine whether inoculation by AM fungi reduces fungal infection in a grapevine rootstock. Further, we ask whether the timing of AM fungal addition determines the strength of this effect. We hypothesized that grapevine rootstocks pre-colonized with AM fungi will have reduced BFD infection and symptom severity compared to rootstocks inoculated at the time of transplant.

2. Methods

2.1. Experimental Design

The experiment was conducted in a greenhouse at the University of British Columbia Okanagan Campus in Kelowna, BC (49.9410° N, 119.3969° W). Potted 'Riparia gloire' rootstocks (*Vitis riparia*) were inoculated with the BFD causal agents *Ilyonectria liriodendra* (PARC60, PARC72, PARC340, and PARC393), previously isolated from BFD symptomatic vines in the Okanagan Valley, BC [1]. These isolates were applied either alone, or with AM applied as a pre-inoculant or a co-inoculant with the pathogens (Table 1). The AM inoculant was a common commercial biofertilizer (AGTIV® wettable powder for specialty crops, 12,000 spores per g) containing only the AM fungi isolate *Rhizophagus irregularis.* Each treatment was applied to eight potted rootstocks, and treatments were arranged in a completely randomized design on a greenhouse bench.

Table 1. Inoculation treatments applied to 'Riparia gloire' grapevine rootstocks. The fungal inoculants consisted of an arbuscular mycorrhizal (AM) fungus *R. irregularis*, and black foot pathogens *Ilyonectria liriodendra*. Treatment codes are as follows: Ctrl = no microbial addition, AMF = Arbuscular mycorrhizal fungi addition, Ily = *Ilyonectria liriodendra* addition. The slack "/" indicates the separation between two different treatment dates.

Treatment	May 2018	August 2018	Description
Ctrl/Ctrl	No addition	No addition	Non-microbial control, receiving neither AM fungi nor *Ilyonectria*
Ctrl/Ily	No addition	Pathogen	Pathogen positive control, receiving only *Ilyonectria* in August
AMF/Ctrl	AM fungi	No addition	Mycorrhizal pre-inoculation control, receiving AM fungi at the first time point, but no pathogen
AMF/Ily	AM fungi	Pathogen	Pre-inoculated pathogen treatment, first receiving AM fungi in May, then exposed to *Ilyonectria* in August.
Ctrl/AMF + Ily	No addition	Pathogen + AM fungi	Co-inoculation treatment, applying both AM fungi and *Ilyonectria* concurrently in August.

2.2. Rootstock Preparation

Dormant 'Riparia gloire' rootstock canes were collected from a 3 year-old field planting at the Summerland Research and Development Centre (Summerland, BC, Canada) in early April 2017. The canes were soaked in 10% bleach for 30 s before they were sectioned into 2-bud cuttings, which were dipped in Stimroot No. 2 (Plant-Prod Inc., Brampton, Canada) and planted to an approximate depth of 15 cm in a rooting box containing damp perlite. The root zone was kept at 21 °C to stimulate root development, while the buds were cooled at 4 °C to slow bud break. After 4 wk, root development was apparent, and the rooted cuttings received inoculation treatments in May (Table 1, and below) before transplanting into 1 L cardboard sleeves filled with a peat-based growing medium (Sunshine Mix #1, AGTIV® wettable powder for specialty crops).

2.3. Pathogen Inoculum Preparation

Six cultures of *Ilyonectria liriodendra* were obtained from the Plant Pathology fungal collection at the Summerland Research and Development Centre, Summerland BC, and activated on 4% (*w*/*w*) potato dextrose agar (PDA) on 3 April 2018. After 23 days (26 April), four isolates were chosen that had signs of sporulation, which was based on visual microscope confirmation of conidia on cultures. These were *I. liriodendra* isolates PARC60, PARC72, PARC340, and PARC393 [1]. Viable cultures were transferred to new plates of 4% (*w*/*w*) PDA containing ampicillin and maintained until the time of pathogen addition (16 weeks). At this point, conidia plus mycelia were scraped off the plates using 1% tween, with the concentration of conidia measured using a hemocytometer. All isolates were diluted to a concentration of 1.5×10^6 conidia per mL so that when combined, the total concentration of pathogen was 6×10^6 conidia per mL.

2.4. Application of Treatments, and Plant Culture

The AM pre-inoculation treatments arbuscular mycorrhizal fungi (AMF)/Ctrl and AMF/Ily (Table 1) were consisted by dipping the grapevine roots in a slurry of commercial AM fungi (12.5 g of inoculum in 25 mL reverse osmosis (RO) water) before planting. All other grapevines were dipped in RO water prior to planting.

The ambient environment in the greenhouse was set at 26 °C, with 16 h of light and 8 h of darkness. Watering was every Monday, Wednesday, and Friday to soil saturation and visible runoff. Plants were provided 5 g of low phosphorus of Miracle-Gro 24-8-16 fertilizer, (Scotts Miracle-Gro Company, Marysville, OH, USA) every two weeks, which was spread over the soil surface and watered in via hand irrigation. In mid-Aug 2018, 2 months after planting, roots from each plant were stained using the protocol from Holland et al. [16] to visually determine AM infection prior to the introduction of the pathogen. Briefly, root fragments, one cm in length, were rinsed in RO water, soaked in 10%

KOH at 80 °C for 2 h, 1 h in a 1:1 (*v*/*v*) mixture of 3% H_2O_2 and 10% KOH, and then stained overnight in 5% ink in vinegar. Roots were then de-stained in RO water for 24 h and visualized under 200× magnification for the presence of mycorrhizal hypha, vesicles, or arbuscules.

After microscopic detection of AM fungi in roots, root systems were pruned to 15 cm in length to mimic the common grower practice of root trimming at transplanting, and to increase the probability of pathogen infection. The root systems were submerged in the *Ilyonectria* fungal suspensions overnight, approximately 12 h, before planting into the peat based medium (Sunshine Mix #1). At this point the stems were pruned to four growing nodes to standardize new growth from the time of inoculation.

2.5. Data Collection

2.5.1. Vine Growth Response

At the time of plant harvest, 6 months after *Ilyonectria* treatment, leaf greenness was measured using a SPAD meter (SPAD 502 Plus, Konica Minolta, Tokyo Japan), taking an average measure of 5 leaves, which were selected evenly along the length of the vine shoots. Additional measurements were taken to determine total stem length, average internode length (total stem length divided by number of nodes), and internode width (averaged for three internodes between the fifth node from the base and fifth node from the growing tip). The base (approximately 4 cm) of each stem was removed and used for pathogen isolation and stem necrosis quantification. Isolated stem sections were stored at 4 °C until analysis. The remaining stems and leaves were dried and weighed.

Roots were removed from the pots, washed clean of soil using RO water, blotted using paper towels to remove surface water, and weighed. A 5 g sample was taken randomly from the root system and stored at 4 °C, to be used for DNA extractions and pathogen re-isolation.

2.5.2. Pathogen Isolation and Assessment of Necrosis

To determine the establishment of the pathogen, 10 1-cm long root segments and 10 1-cm long stem slices (from the plant base) were plated on 4% PDA containing 0.25% (*w*/*w*) ampicillin. The root segments were submerged in bleach (10% *v*/*v*) for 15 s, ethanol (10% *v*/*v*) for 30 s, and finally rinsed in RO water before being placed on the plates. The stem slices were dipped in ethanol and flamed to disinfect the outer surface before the outer bark was removed and slices of the inner tissue (<1 mm thick and <5 mm in width) were sectioned from the cortex region, targeting darkened areas that showed signs of necrosis. These were plated evenly around the PDA plates. Both root and stem plates were grown for up to 6 weeks with regular monitoring for the growth of colonies, which were used to confirm the presence of *Ilyonectria*.

To determine the amount of necrosis within the stem cortex, a cross-section was taken from the bottom section of the stem, where the roots emerge and necrosis is commonly most evident. A one-centimeter cross section was cut and brushed with mineral oil and scanned using an Epson Expression 11000XL scanner. Images were imported into ImageJ [17] to measure the relative proportion of the cross section with necrotic staining. To do this, first the necrotic region (indicated by darkening and browning of the tissue) was outlined and measured using the 'area' tool. The same was done for the entire cortex, excluding the pith. The necrotic region was then divided by cortex area to provide the percent of necrosis.

2.5.3. Digital Droplet PCR Quantification

To perform molecular quantification of both the AM fungal and *Ilyonectria* isolates, DNA was first extracted from a subsample of 0.1 g of roots per plant, cut into <1 cm fragments, according to the manufacturer's protocol (MPBio FastDNA™ SPIN kit (MP Biomedicals LLC, Santa Ana, CA, USA). This was followed by digital droplet (dd) PCR, which allows for quantification of the number of copy numbers within a sample.

2.5.4. Quantification of AM Fungal DNA Using ddPCR

To quantify AM fungi, we targeted a segment of the mtDNA specific to the commercial isolate using a probe-based reaction in ddPCR (197198F 5′-AGCAAATCTAAGTTCCTCAGAG-3′ [18]; Reverse 5′-ACTTCTATGGCTTTGTACAGG-3′; and probe 5′-FAM/CCCACCAGG/ZEN/GCAGATTAATCTTCCTT/3IABKFQ-3′ [19]). In short, each ddPCR reaction contained: 10 µL of 1X ddPCR SensiMix (supermix for probes by Bio-Rad Inc., Hercules, CA, USA), 1 µL of primer-probe mix (Integrated DNA Technologies, Coralville, IA, USA) 20× (500 nM primers and 250 nM probe in final reaction), and 2 µL of undiluted root DNA and 7 µL of DNase free water (MP Biomedicals LLC, Santa Ana, CA, USA), for a total volume of 20 µL. The total volume was used to generate droplets, along with 70 µL of Bio-Rad Droplet Generator Oil for Probes, using the Bio-Rad QX100 Droplet Generator producing 40 µL of droplets. Thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 59 °C for 30 s, followed by a single step at 98 °C for 10 min; the ramp rate was increased by 2 °C/s.

Droplet analysis was carried out on the BioRad QX100, and raw data was collected using Quantalife software (Version 1.7.4.0917, Bio-Rad Laboratories Inc.). For each run, three non-template controls (NTCs) were included, containing no DNA, and three environmental controls, which were collected from an area that would contain no commercial inoculum. The former to eliminate the possibility of contamination and the latter to determine the fluorescence amplitude threshold.

2.5.5. Quantification of *I. liriodendra* DNA Using ddPCR

A new primer pair and probe was developed to quantify *Ilyonectria*, targeting a portion of the beta-tubulin region; (5′-CGAGGGACATACTTGTTTCCAGAG-3′ Tm 61, GC 60%,) and (Reverse 5′-TCAACGAGGTACGCGAAATC-3′ Tm 62, GC 50%). In addition, a probe (5′-TGTCAAACTCACACCACGTAGGCC-3′ Tm 68, GC 52%) was included to increase specificity to *Ilyonectria*. To check for specificity to *Ilyonectria* we performed two tests. First the primer-probe set was used with each of the four *Ilyonectria* isolates (PARC60, PARC72, PARC340, and PARC393), along with other fungal DNA to ensure amplification (Supplementary Figure S1). The *Ilyonectria* ddPCR probe reaction was performed using the same method as the AM fungal reaction previously described, except for the following differences: the reaction thermal cycling conditions were 95°C for 10 min, followed by 60 cycles of 95 °C for 10 s and 60 °C for 10 s, followed by a single extension step at 72 °C for 30 s. Second, we used the blast and nucleotide tools from the National Center for Biotechnological Information (NCBI) to check the primer-probe specificity against the entire NCBI database. For this, a blast was initially performed with the two primers to obtain accession numbers. These were then cross-referenced with the nucleotide tool using the probe sequence to determine which species would amplify (Supplementary, Table S1).

2.6. Statistical Analysis

All statistical analyses were conducted as appropriate for a completely randomized design using R v2.8.1 [20]. Differences in stem necrosis, ddPCR copy number and vine growth responses were determined among the treatments using a one-way analysis of variance (ANOVA). Differences among the treatment means were detected using a general linear hypothesis within the multcomp R-package [21]. This was done with the multiple comparison function (mcp) defined for Tukey linear function testing, as follows: Tukey comparison = glht (aov, linfct = mcp (Treatment = "Tukey")).

Multivariate analysis of variance (MANOVA) was used to further detect overall differences in vine growth responses, with root biomass, dry stem weight, dry leaf weight, internode width, internode length, and leaf greenness included in the analysis.

A Pearson's correlation in R v2.8.1 [20] was used to detect any correlation between the AM fungal and *Ilyonectria* abundance.

3. Results

3.1. Plant Response

Inoculation by AM fungi and *Ilyonectria* isolates did not affect plant response. This was consistent both when looking at individual traits using an ANOVA (Table 2) or when looking at total overall vine response using MANOVA.

Table 2. Means for plant growth chracteristics and abundance of fungal isolates, six months after inoculations with AM fungi and Ilyonectria isolates. F-values and *p*-values are the result from ANOVA for inoculation treatment effects (n = 40). Copy numbers of mtDNA (AM fungi) and beta tubulin (*Ilyonectria*) were obtained using molecular-based quantitative digital droplet PCR.

Measure	F-Value	*p*	Average
Dry Stem Mass	1.24	0.308	11.6 g
Dry Leaf Mass	0.651	0.662	12.2 g
Fresh Root Mass	1.53	0.201	333.5 g
Internode Width	1.52	0.206	223 mm
Internode Length	0.188	0.965	2.3 mm
Leaf Greenness	0.594	0.705	23.6 spad units
% Stem Necrosis	0.577	0.717	25%
AM fungal copy number	5.15	<0.001 *	See Figure 1
Ilyonectria copy number	5.59	<0.001 *	See Figure 2

* Significant at $p = 0.05$.

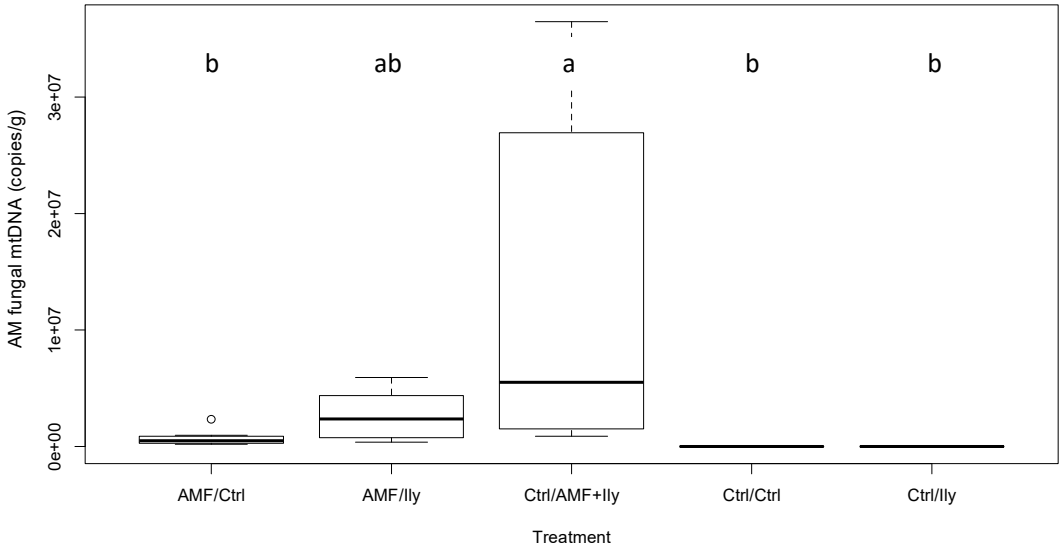

Figure 1. Abundance of AM fungi within vine roots (copies mtDNA/µL) after harvest (6 mo after the second inoculation) in response to inoculation treatments. Treatments: Ctrl/Ctrl, non-microbial control; Ctrl/Ily, pathogen control inoculated with only *Ilyonectria*; AMF/Ctrl, mycorrhizal control inoculated only with AM fungi; AMF/Ily, pre-inoculated with AM fungi followed later by *Ilyonectria* inoculation; and Ctrl/AMF + Ily, co-inoculated with AM fungi and *Ilyonectria*. Values were obtained by ddPCR and differences determined using one-way ANOVA ($F_{5,41} = 5.135$, $p < 0.001$), with differences at $p < 0.05$ indicated by letters above bars (Tukey's multiple comparison via general linear hypothesis).

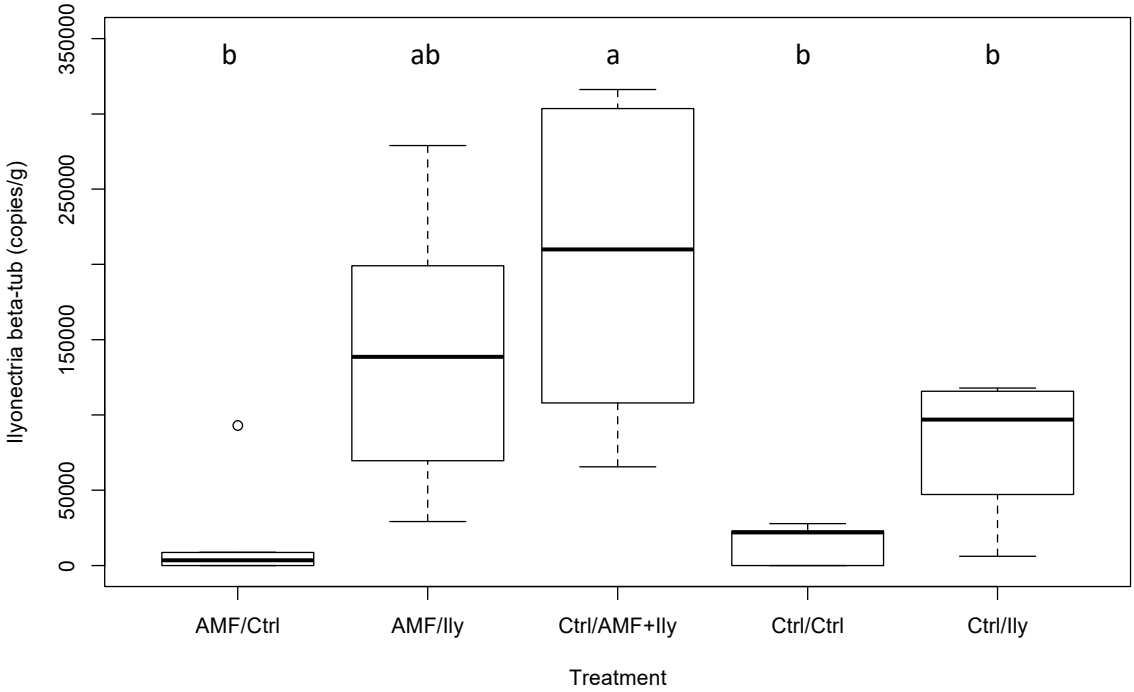

Figure 2. *Ilyonectria* beta-tubulin copy number per µL in vine roots in response to inoculation treatments. Treatments: Ctrl/Ctrl, non-microbial control; Ctrl/Ily, pathogen control inoculated with only *Ilyonectria*; AMF/Ctrl, mycorrhizal control, inoculated with only AM fungi; AMF/Ily, pre-inoculated with AM fungi followed later by *Ilyonectria* inoculation; Ctrl/AMF + Ily, co-inoculation with AM fungi and *Ilyonectria*. Values were obtained by digital droplet polymerase chain reaction and differences determined using a one-way analysis of variance (ANOVA; $F_{5,41} = 5.586$, $p < 0.001$), with differences at $p < 0.05$ indicated by letters above bars (Tukey's multiple comparison via general linear hypothesis).

3.2. Necrosis

There were no differences among the treatments in percent stem necrosis ($F_{5,41} = 0.577$, $p = 0.717$). Visually, the root systems appeared healthy with no signs of necrotic development.

3.3. Molecular Quantification

3.3.1. AMF

The presence of AM fungi was visually confirmed in all three treatments that were inoculated. However, the quantities differed among the treatments: Rootstocks that received both AM fungi and *Ilyonectria* at the same time (Ctrl/AMF + Ily) had a higher AM fungal copy number ($F_{5,41} = 5.135$, $p < 0.001$) compared with both rootstocks not inoculated with AM fungi (Ctrl/Ctrl and Ctrl/Ily) and those pre-inoculated with AM fungi but not inoculated with *Ilyonectria* (AMF/Ctrl) (Table 2, Figure 1).

3.3.2. *Ilyonectria*

Ilyonectria was isolated from all rootstocks inoculated with the pathogens, but only from a subset (4/24) of rootstocks not inoculated with *Ilyonectria*. Rootstocks inoculated with *Ilyonectria* and AM fungi at the same time had a higher number of copies of *Ilyonectria* beta-tubulin genes (($F_{5,41} = 5.586$, $p < 0.001$), Table 2, Figure 2).

4. Discussion

Contrary to our prediction, AM fungal inoculation increased the abundance of the fungal pathogen within grapevine rootstocks. This has not been previously reported, with the generally reported trend being that AM fungal pre-inoculation decreases the presence and expression of plant diseases.

This result is important as it shows that AM fungal benefits, contrary to popular thought, are not consistently good, and growers should carefully consider whether AM inoculation is warranted.

4.1. Vine Growth

Surprisingly, neither the *Ilyonectria* nor AM fungi affected vine growth during the experiment. While AM fungi are generally thought to increase vine physiological performance [5,7,15,22–24], this is not always the case. Holland et al. [25], using a similar commercial product, found no effects on growth in both greenhouse and field studies. There are many situations where AM fungal inoculation has had no influence on plant growth [26–28], or even growth depression [29,30]. Mycorrhizal benefits are more commonly realized when plants are grown under stressful conditions, such as poor soil field conditions [31].

4.2. Pathogen Isolation and Necrosis

Surprisingly, inoculation with the *Ilyonectria* pathogens did not influence vine growth. While the abundance of beta tubulin genes (Figure 2) indicates the pathogen was present, we observed no expression of disease symptoms. This lack of disease symptoms is contrary to other studies that applied similar *Ilyonectria* treatments to young grapevines, which regularly resulted in leaf chlorosis and growth reductions [1,10,32]. For instance, two of the isolates used in our study caused significant disease and growth reductions in a previous pathogenicity study with similar experimental techniques [1]. That our pathogen control did not have higher levels of pathogen and necrosis was surprising, as even rootstocks that had higher levels of *Ilyonectria* did not differ in their levels of necrotic streaking or show foliar symptoms. This indicates that infection was successful, but that the experimental conditions, such as growing parameters or the rootstock used, may not have been ideal to result in vine disease.

Ilyonectria is a weak pathogen that can live asymptomatically within grapevines without causing visual signs of disease until stress occurs [1–3]. For example, when grapevines are first transplanted into a vineyard they will be subjected to a new soil environment that could include stressors that trigger the *Ilyonectria* to become pathogenic. In particular, it is understood that BFD has a greater effect on grapevines exposed to high soil compactness, poor drainage, or low water availability [1–3]. Lack of stressors could have been a factor in our experiment, resulting in no evident vine disease. In this study we kept the medium well-watered to mimic poor drainage, which is known to enhance *Ilyonectria* proliferation. However, due to the mode of disease (vascular tissue occlusion) it is possible that once the disease had proliferated within stem tissues, limiting the available moisture would have caused more pronounced disease expression as upper plant tissues would have suffered water stress. Further, the rootstock 'Riparia gloire' is adapted to mesic soils [33] but less adapted to drought stressed soils, which may trigger disease expression. While there is mixed evidence on the susceptibility of 'Riparia gloire' to BFD [1,34], disease symptoms may have been expressed if we had used a highly susceptible rootstock such as 3309C [1], increased the water stress severity, or used a fungal pathogen that is known to be more aggressive.

4.3. Ilyonectria and AM Fungal Quantification

Contrary to our hypothesis, *Ilyonectria* abundance within grapevine rootstocks did not decrease with the addition of AM fungi but rather significantly increased. In similar greenhouse experiments, challenging annual plants with cultured pathogens, AM fungi have been found to reduce *Fusarium solani* f. sp *phaseoli* in bean [35] and *Aphanomyces euteic* in pea [36]. Similarly, in perennial systems, mycorrhizal infections in papaya (cv. Surya) [37] and banana (cv. Neypoovan) [38] led to lower levels of *Phytopthora parasitica* var. *nicotianae* and *Fusarium oxysporum* f. sp *cubense*, respectively. These studies found a reduction in pathogen abundance and also observed suppressed disease symptoms in mycorrhizal plants, neither of which occurred in the present study.

Although colonization by AM fungi is usually linked to plant pathogen protection [9,13,35], the mechanism involved is not entirely understood. There is evidence, especially in early stages of

colonization [39,40], for an increase in jasmonic acid (JA) in plant roots [41], a hormone that helps facilitate the formation of the AM [42]. JA is closely related to the induced systematic resistance (ISR) and is directly antagonistic to salicylic acid (SA) and the systemic acquired resistance (SAR). The SAR system is activated by pathogen activity after SA accumulation [43]. SAR induced defenses are usually downregulated following colonization by AM fungi since SAR have been shown to also inhibit the development of mycorrhizas [44], similar to pathogen infection [45]. It is possible that the downregulation of the SAR following AM fungal colonization could provide the opportunity for the pathogen to enter the roots in greater extent, explaining the observed increase in pathogen abundance in our study.

The lack of reduced pathogen abundance observed here may be due to the fungal isolate *Rhizoglomus irregularis*. Previous studies have shown *R irregularis* (formerly *Glomus intraradices)* to reduce both grapevine BFD symptoms [8] and pathogen abundance [36]. The lack of AM fungal prophylaxis may be due to the relatively stress free growing conditions of our experiment. However, there is growing evidence that AM fungi may not be universally beneficial against pathogens, particularly in natural settings [46].

While AM fungi generally provide disease suppression, treatments that received AM fungi in this study tended to contain more *Ilyonectria*. A synergistic effect could have occurred between the two entities, as has been described when multiple pathogens are introduced into plants [47,48]. While AM fungi are not pathogenic, they could co-exist or even provide indirect benefits to a weak pathogenic endophyte such as *Ilyonectria*. For instance, AM fungi could allow *Ilyonectria* to further proliferate as a result of the increased carbon sink [49,50] that the beneficial AM fungi often induces. It has been shown that AM fungi can create a carbon sink within roots, causing mycorrhizal roots to contain more sugar than non-mycorrhizal counterparts [49,50]. This has been seen in multi-species AM fungal systems, where low-quality AM fungi persist within roots but only due to the benefits provided by another beneficial AM fungal counterpart [51]. Similar effects could occur in AM fungal-pathogen systems as fungi depend on exogenous carbon for growth; increased carbon caused by beneficial AM fungi could subsequently lead to rootzones supporting higher fungal loads as evidenced with increased exudate levels [52]. If this were the case, the benefit that AM fungi may be providing could be negated by also allowing higher levels of pathogenic fungi to persist.

It was interesting that rootstocks only inoculated with AM fungi (AMF/Ctrl treatment) had very low, if not negligible, levels of AM fungi in roots, compared to rootstocks that received both AM fungi and *Ilyonectria*. The low levels of AM fungi in response to AMF/Ctrl may have been due to the lack of stress. In the other treatments that included the pathogen (Ctrl/AMF + Ily and AMF/Ily), this stress was likely provided by *Ilyonectria*. While the presence of *Ilyonectria* did not result in visual stress, it still could have caused limited water and nutrient status to which the AM fungi may have still provided protective benefits. This could result in carbon flow to the AM fungi, mediating fungal proliferation in the root system. In contrast, grapevines without *Ilyonectria* would have less need for the AM fungi symbionts and therefore the AM fungi would not benefit the grapevines, limiting their establishment in vine roots. This effect may be similar to the influence of phosphorus found in experimental systems in which plants exposed to adequate levels of phosphorus have lower AM fungal colonization levels [53–56].

5. Conclusions

This study found that inoculation by AM fungi may increase pathogen abundance under the conditions of this experiment, which were chosen to encourage pathogen establishment. To test the prophylactic effect of AM fungal inoculation, we chose to trim vines roots to promote conditions optimal for infection by our pathogen. Our goal was to mimic planting practices and to determine whether pre-colonized vines would receive more benefit than exposing vines to AMF at the time of transplant (pruning). It was our goal to promote pathogen establishment. Thus, it is surprising that our study did not show positive growth for AM vines. It is possible that the rootstock selected has

lower susceptibility compared to others, and that the pathogen used was not aggressive enough to cause disease expression. However, this study shows that AM fungal inoculants applied before or with pathogen exposure may not always benefit hosts. Whether there is benefit to inoculating post-pathogen exposure remains to be seen. Because vines typically experience pathogens post-planting, this study only evaluated the effect of pre- or co-inoculation on pathogen performance. AM fungi may interact differently with a pathogen that is already established within hosts.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-7524/5/3/61/s1, Figure S1: Specificity test using the newly designed primer probe in a digital droplet PCR assay. Droplets above the threshold represent positive amplification to the primer/probe. From left to right the isolates tested in each well are: A01–**PARC393**, A02–PARC349, B02-PARC100, C01-**PARC60**, C02–NTC, D01–**PARC340**, D02–*Rhizophagus irregularis* (DAOM197198), E01–**PARC72**, G01–PARC398, H01–PARC345. *Rhizophagus irregularis* DNA originated from spores that were extracted from the AGTIV®product. Table S1: A lineage report resulting from specificity testing for the new primer-probe set, using the blast tool in NCBI. First a primer blast was performed using the newly developed primer set. The generated accession numbers were subsequently nucleotide blasted using the probe sequence. The organism, score, average e-value and number of hits for each match are reported.

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