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Effective Control of *Neofusicoccum parvum* in Grapevines: Combining *Trichoderma* spp. with Chemical Fungicides

Aranzazu Gomez-Garay [1,*], Sergio Astudillo Calderón [1], Mª Luisa Tello Mariscal [2] and Beatriz Pintos López [1]

[1] Research Group FiVe-A, Department of Genetics, Physiology and Microbiology, Faculty of Biology, Complutense University of Madrid, 28040 Madrid, Spain; bpintos@ucm.es (B.P.L.)
[2] Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria—Spanish National Research Council, Ministry of Science, Innovation and Universities, 28040 Madrid, Spain; marisa.tello@inia.csic.es
* Correspondence: magom02@bio.ucm.es

Abstract: *Vitis vinifera* is highly susceptible to grapevine trunk diseases, with *Neofusicoccum parvum* recognized as a highly destructive pathogen. This study investigates the biocontrol potential of five *Trichoderma* species (*T. harzianum*, *T. viride*, *T. asperellum*, and *T. virens*) against *N. parvum*, evaluating multiple biocontrol mechanisms (mycoparasitism, competition for nutrients, production of volatile organic compounds (VOCs), and antibiosis) as well as their compatibility with the fungicides copper oxychloride and sulfur. Results demonstrated that (1) *Trichoderma harzianum* effectively suppressed *N. parvum* through VOC production, mycoparasitism, and nutrient competition, significantly reducing pathogen growth *in planta* while showing compatibility with both fungicides, highlighting its suitability for integrated disease management; (2) *Trichoderma viride* showed high inhibition of *N. parvum* in vitro, but its phytotoxicity *in planta* limits its field application. These findings support *T. harzianum* as a promising agent within integrated disease management strategies, offering a sustainable alternative to reduce chemical fungicide reliance in controlling grapevine trunk pathogens.

Keywords: biocontrol; grapevine trunk disease; *N. parvum*; *Trichoderma* spp.; integrated disease management

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1. Introduction

Grapevine trunk diseases (GTDs) are among the most destructive fungal diseases affecting *Vitis vinifera*, becoming increasingly prevalent due to factors such as climate change [1,2]. GTD pathogens represent the largest group of grapevine pathogens, with up to 133 fungal species across 34 genera identified globally [3]. These diseases include Petri disease, Black foot, Eutypa dieback, Phomopsis dieback, Esca, grapevine leaf stripe disease, and Botryosphaeria dieback.

Among the pathogens responsible for GTDs, *Botryosphaeria* dieback is particularly concerning, with 26 species from the Botryosphaeriaceae family linked to this disease. *Neofusicoccum* species, particularly *N. parvum*, are recognized as the most virulent and fast-colonizing wood pathogens within this group [4–6]. Studies have shown that *N. parvum* causes significant grapevine damage, including dieback, cankers, and vascular colonization, which affect grapevine health and yield in regions such as Chile and Croatia [7–12]. Prevalence of *N. parvum* in affected vineyards ranges from 2% to 15% [10], emphasizing the need for effective management strategies.

The pathogenicity of *N. parvum* is attributed to its ability to colonize wood, produce phytotoxins, and degrade cell walls [13,14]. Its genome contains clusters of virulence-related genes, including those involved in secondary metabolite production and carbohydrate-active enzymes (CAZymes), which are expressed differentially during infection [15,16]. Additionally, *N. parvum* harbors a diverse mycovirome, potentially influencing its biological properties [17]. Experimental models demonstrate that *N. parvum* causes more severe

lesions and foliar symptoms than other Botryosphaeriaceae species, correlating with plant stress responses [18]. Unlike other pathogens that may be exacerbated by abiotic stress factors like water deficit, *N. parvum* acts as a primary pathogen, independent of such stressors [19]. Its ability to infect multiple plant species in the same habitat, including alternative hosts, further facilitates pathogen persistence [19]. Timely detection and management of *N. parvum* are critical for maintaining grapevine health and productivity in affected regions.

GTD pathogens, including those responsible for Botryosphaeria dieback, are primarily spread by airborne spores and infected cuttings. Wounds and cuts incurred during propagation, especially in nurseries, create entry points for these fungal pathogens [13]. Currently, no curative measures exist for managing Botryosphaeria dieback in nurseries and young vineyards, highlighting the urgent need for alternative control strategies.

Integrated disease management (IDM) strategies, combining physical, chemical, and biological control measures, are essential for reducing fungal infections in nurseries [14]. Biocontrol agents, especially microbial antagonists, offer several advantages over chemical fungicides by being more cost-effective, safer, and environmentally friendly [15,16]. Among these agents, *Trichoderma* spp. have been extensively studied [20] and used in agriculture, with commercial formulations of species such as *T. virens*, *T. harzianum*, *T. atroviride*, and *T. viride* widely available [21].

Research on chemical and biological control agents for managing *N. parvum*, which also causes stem canker in blueberries, has produced mixed results. Chemical fungicides, including Luna Sensation (fluopyram and trifloxystrobin), thiophanate-methyl + myclobutanil, benomyl, tebuconazole, and iprodione, have shown efficacy in controlling *N. parvum* [22]. Biological control agents, particularly *Trichoderma* based treatments, are effective in protecting pruning wounds from *N. parvum* infection, as are biofungicides like *Xenorhabdus szentirmaii* [12,23]. However, inconsistencies in control outcomes, as seen with *Bacillus subtilis*, highlight the need for further research to improve biological control methods [22].

Trichoderma species utilize various mechanisms for pathogen suppression, including mycoparasitism, competition for nutrients, antibiosis, and induction of plant resistance [24,25]. Mycoparasitism involves the production of cell-wall-degrading enzymes like cellulases, glucanases, and chitinases, which enable *Trichoderma* to parasitize and degrade other fungi [26]. Additionally, *Trichoderma* strains produce antimicrobial secondary metabolites, siderophores, and plant growth factors that enhance biocontrol efficacy [24].

Trichoderma species also produce volatile organic compounds (VOCs) with antifungal properties that inhibit pathogen growth without direct contact, offering a sustainable tool for integrated pest management [27,28]. These VOCs contribute to the success of *Trichoderma* in suppressing pathogens and promoting plant growth [29].

The multifaceted mechanisms employed by *Trichoderma* provide a significant advantage over chemical fungicides, offering enhanced and longer-lasting pathogen protection under varying environmental conditions [30,31]. In addition, *Trichoderma* species act as biofertilizers, benefiting agricultural practices [26]. Studies indicate that *Trichoderma* applications in nurseries can reduce fungal pathogens in rootstock cuttings and infected young plants [12,14,30,32–34].

This study investigates the compatibility of *Trichoderma* with chemical fungicides, an essential step for optimizing integrated disease management strategies. *Trichoderma* species show limited compatibility with some fungicides, particularly triazoles, but demonstrate better compatibility with copper-based fungicides and mancozeb [35,36]. Copper oxychloride and sulfur are widely used in viticulture, with contrasting properties: copper oxychloride is effective for fungal control, while sulfur is favored for its lower environmental impact. By evaluating these fungicides at their recommended concentrations, it is possible to assess their efficacy and compatibility with *Trichoderma*, which is known for its disease-suppressive potential. Furthermore, it is critical to evaluate lower fungicide concentrations, as they are typically more compatible with *Trichoderma*, whereas higher concentrations may reduce its efficacy [37]. Striking the right balance in fungicide use can minimize environmental impact, reduce fungicide resistance, and improve overall

disease management strategies, particularly in combination with biocontrol agents like *Trichoderma* [38].

By investigating both the biocontrol potential and compatibility with chemical fungicides, this study aims to contribute to developing effective integrated strategies for managing Botryosphaeria dieback and other GTDs in grapevines.

2. Materials and Methods

2.1. Strain Collection and Culture Conditions

Trichoderma strains T1, T2, T3, T4, and T5 were used for both in vitro and *in planta* experiments. These strains were sourced from the microbial collection of the FiVe-A Research Group at Complutense University of Madrid, from soil samples collected in agricultural fields in Spain. The soil samples used in this study were collected from agricultural fields in a region where grapevine cultivation is prominent and *Neofusicoccum parvum* is known to be prevalent. Due to confidentiality agreements, the precise location cannot be disclosed. However, the selection of these fields was based on their representativeness and relevance to the study objectives, ensuring the reliability of the results. Two strains of the phytopathogenic fungus *Neofusicoccum parvum* (JL396 and JL445 from the microbial collection of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Barcelona, Spain) were also included, originally from infected wood of *Vitis vinifera* vines, as detailed in [7]. The fungi were maintained on Potato Dextrose Agar (PDA, Pronadisa; Condalab, Madrid, Spain). Cultures on PDA were grown for 5 days at 22 °C ± 2 °C in the dark for assays and inoculum preparation.

To assess conidia production for each *Trichoderma* strain, 8 mL of sterile distilled water was added to a PDA plate with one week old cultures. Plates were gently agitated on an orbital shaker at 30 rpm for 5 min to dislodge conidia. The water containing conidia was passed through a sterile filtration process using a 40 µm filter to remove mycelial fragments. The filtrate was then centrifuged at 3000 rpm for 5 min to collect conidia, which were resuspended in 3 mL of sterile distilled water. Conidia concentration was determined using a Neubauer Chamber Cell Counting method to ensure accuracy. The final suspension achieved a concentration of 1×10^6 conidia/mL, suitable for experimental inoculations. Each experiment was performed in triplicate to ensure reproducibility.

2.2. Species Identification and Strain Differentiation by Nuclear Ribosomal Internal Tran-Scribed Spacer (ITS) Region

DNA was extracted following Cenis, 1992 [39]. Briefly, strains were cultured in 500 µL of liquid Potato-Dextrose medium, started by inoculating some hyphal threads from five-day-old PDA plates, and allowed to grow for 72 h at 25 °C. The mycelial mat was pelleted by centrifugation for 5 min at 13,000 rpm in a microfuge, washed with 500 µL of TE buffer (Tris (tris(hydroxymethyl)aminomethane) and EDTA (ethylenediaminetetraacetic acid), and pelleted again. The TE was decanted and 300 µL of extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) added. The mycelium was crushed with a conical grinder. Then, 150 µL of 3 M sodium acetate, pH 5.2, were added, and tubes placed at −20 °C for about 20 min. Tubes were centrifuged (5 min at 12,000 rpm) and the supernatant transferred to another tube for isopropanol precipitation at room temperature. The precipitated DNA was washed twice with 70% ethanol, air dried, and resuspended in 50 µL of TE.

Two oligonucleotide fungal primers (ITS1: TCCGTAGGTGAACCTGCGC and ITS4: TCCTCCGCTTATTGATATTGC) described by White et al. [40] were used for amplification. The PCR assay was performed with 20 ng of DNA in a total reaction volume of 25 µL consisting of PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl; 0.1 mM each of dATP, dGTP, dCTP, and dTTP; 1.5 mM $MgCl_2$; 50 ng each primer; and 1 U of NZYTaq DNA Polymerase (NZYtech, Lisboa, Portugal). Forty cycles of amplification were performed in a Mastercycler Gradient thermocycler (Eppendorf) after initial denaturation of DNA at 95 °C for 5 min. Each cycle consisted of a denaturation step at 95 °C for 30 s, an annealing step

at 55 °C for 30 s, and an extension step at 72 °C for 1 min, with a final extension at 72 °C for 3 min following the last cycle. After amplification, the products were stored at 4 °C until used.

DNA sequencing was performed at the Genomic Unit in the Complutense University of Madrid. PCR products were directly sequenced using the ITS1 and ITS4 PCR primers. Sequence analysis of the amplicons was performed by BLASTN comparison using the National Center for Biotechnology Information (NCBI) database's best hit to confirm the identities of the strains. All fungal sequences were at least 98% identical to the best hit in the NCBI database. This value was considered sufficiently robust for species identification [41].

2.3. In Vitro Growth Rates

Five-millimeter diameter discs of each *Trichoderma* strain from five-day-old PDA plates were transferred to the center of PDA Petri dishes. The Petri dishes were then incubated for 7 days at 25 ± 1 °C, and colony growth of each fungus was recorded daily for a week. Growth rate was calculated by the formula:

$$\varphi_t = \varphi_0 + \mu t$$

where φ_t is the radial length at measurement point t; φ_0 is the radial length in the measurement point 0 and μ is the growth rate. Three repeats and three replicates were performed.

2.4. Antagonism Assays on PDA Plates

Confrontation assays (dual cultures tests) were conducted on PDA to evaluate the antagonistic activity of *Trichoderma* strains against *Neofusicoccum parvum*. Five mm diameter mycelial plugs from 7-day-old cultures of both fungi were placed 3 cm apart on PDA plates, with *N. parvum* alone serving as the control. Plates were incubated at 22 °C ± 2 °C and 80% humidity in the dark for 5 days. Fungal growth was measured daily, and inhibition was calculated relative to control plates.

The degree of parasitism was calculated according to the scale of Ezziyyani et al. [42]. Results were expressed as percentage of colony growth inhibition (PCGI). Each experiment was replicated three times.

2.5. Inhibitory Effects of Volatile Compounds Produced by Trichoderma spp. *on N. Parvum*

The effect of volatile organic compounds (VOCs) produced by *Trichoderma* strains T0, T1, T2, T3, and T4 was evaluated following Schubert et al. [43] with minor modifications. *Trichoderma* strains were centrally inoculated by placing 5-mm discs on PDA media taken from the margin of 7-day-old cultures and incubated at 22 ± 2 °C and 80% relative humidity for 3 weeks. The top of each Petri dish was replaced with the bottom of the PDA plates inoculated centrally (5-mm discs) with each *N. parvum* strain. Plates without *Trichoderma* spp. were used as controls. Three replicates were maintained for each treatment. The pairs of Petri dishes were sealed together with paraffin tape and incubated at 22 ± 2 °C and 80% relative humidity. Colony diameter of *N. parvum* was measured daily for a week, and the inhibition of mycelial growth (PCGI) was calculated.

2.6. Compatibility Estimation of Trichoderma spp. *with Fungicides*

Stock solutions of agrochemicals were prepared by dissolving the required quantities of each into sterile distilled water. Increasing concentrations (1/8 R.D. (Recommended Dose), 1/4 R.D., 1/2 R.D., and; R.D. = 4 g/L) of Copper oxychloride (Cheminova Agro S.A.) and increasing concentrations (1/8 R.D., 1/4 R.D., 1/2 R.D., and Recommended Dose; R.D. = 2 g/L) of Sulfur (Fertiberia, S.A.) were then prepared and incorporated into the PDA culture medium kept molten at 50 °C and mixed thoroughly by gentle shaking. The mixture was then poured into Petri plates. PDA plates without any added compounds served as controls.

After solidification, the plates were inoculated with 5-mm discs of 5-day-old *Trichoderma* spp. strain cultures. Three replicates were used for each concentration of every tested

fungicide. The inoculated plates were incubated at 25 ± 2 °C in the dark, and radial colony diameter was recorded daily for one week post-inoculation. Results were expressed as percentage of colony growth inhibition. Each experiment was replicated three times.

2.7. Plant Material and Experimental Design

Disease-free cuttings of a Garnacha cultivar of grapevine were used in all bioassays. They were processed and prepared as described by Amponsah et al. [44]. For incubation, the plants were grown in a closed greenhouse.

2.7.1. Cutting Bioassay

The principal treatments corresponded to the *Trichoderma* strains co-inoculated with the pathogen, and the control treatments consisted of (i) an uninoculated, untreated control (UUC), where samples were not inoculated with the fungus and not treated with *Trichoderma*, and (ii) an untreated control (UC), where samples were inoculated only with the fungus *N. parvum*.

2.7.2. Evaluation of Stem Necrosis

The incubation period was 21 ± 1 days post-inoculation (dpi), after which the stem of each plant was cut longitudinally in the middle, and the length of the internal vascular lesions (or necroses) in the cutting wood just under the bark was visually evaluated by measuring the necrotic lesions upwards and downwards from the wound-inoculation hole. Each experiment was replicated three times.

Similarly to the percentage of colony growth inhibition (PCGI), results were expressed as the percentage of lesion length reduction (PLLR):

$$PLLR = \frac{L1 - L2}{L1} \times 100$$

where $L1$ is the length of the lesion in the positive control (pathogen only) and $L2$ is the length of the lesion in the presence of the antagonist.

For quantitative results, lesions were measured in millimeters (mm) using a millimeter scale ruler with an accuracy of ±1 mm. A damage threshold of 5 mm lesion length was established to distinguish between minor lesions and more significant ones, which were considered indicative of severe pathogenic infection.

To confirm that the lesions were caused by the trunk pathogens, small pieces of wood from the margin of each lesion were surface sterilized for 1 min in 0.6% sodium hypochlorite solution, rinsed in three successive baths of sterile distilled water, and incubated on PDA. Koch's postulates were satisfied according to the procedure described above for shoot inoculations.

2.8. Statistical Analysis

Data from in vitro assays were analyzed by analysis of variance (ANOVA) to test the effects of variation factors (different samples) on each variable. If significant effects were found at a 95% confidence interval, ANOVA was followed by Tukey's Honestly Significant Difference (HSD) and Duncan's post-hoc test with a 5% level for rejection of the null hypothesis to identify differences among groups.

3. Results

3.1. Antagonistic and Pathogenic Fungi: Species Identification and Strain Differentiation by Nuclear Ribosomal Internal Transcribed Spacer (ITS) Region

Species differentiation within the genus *Trichoderma* and *Neofusicoccum parvum* was achieved using the nuclear ribosomal internal transcribed spacer (ITS) region as a molecular marker. The ITS regions amplified from *Trichoderma spp.* and *Neofusicoccum parvum* were approximately 520 bp and 560 bp, respectively. The following identifications were obtained, along with their respective GenBank accession numbers corresponding to sequences with

the highest similarity: *Trichoderma harzianum* (T1: HQ149776.1 and T3: KC819133.1), *Trichoderma viride* (T2: KP763495.1), *Trichoderma asperellum* (T4: JX677934.1), and *Trichoderma virens* (T5: KF150223.1). Both pathogen strains were confirmed as *Neofusicoccum parvum* (JL396: JN222970.1 and JL445: KP140964.1).

3.2. In Vitro Growth Rates

The growth rate of *Trichoderma* is directly linked to its ability to compete with pathogens. Faster-growing strains can colonize the plant surface more quickly, outcompeting harmful fungi for space and resources. This enhanced growth allows *Trichoderma* to more effectively produce antifungal metabolites and enzymes that inhibit pathogen development. Thus, measuring colony diameter helps identify *Trichoderma* strains with superior competitive abilities, which are essential for effective biocontrol.

Among the five *Trichoderma* strains tested, T5 (*T. virens*) exhibited the fastest growth rate, averaging 1 ± 0.09 cm per day (Figure 1). The growth rates of *Trichoderma* strains T1, T3, and T4 were similar, measuring 0.90 ± 0.1 cm per day, 0.89 ± 0.04 cm per day, and 0.86 ± 0.1 cm per day, respectively. In contrast, T2 (*T. viride*) showed the slowest growth rate, with a mean of $\leq 0.7 \pm 0.05$ cm per day.

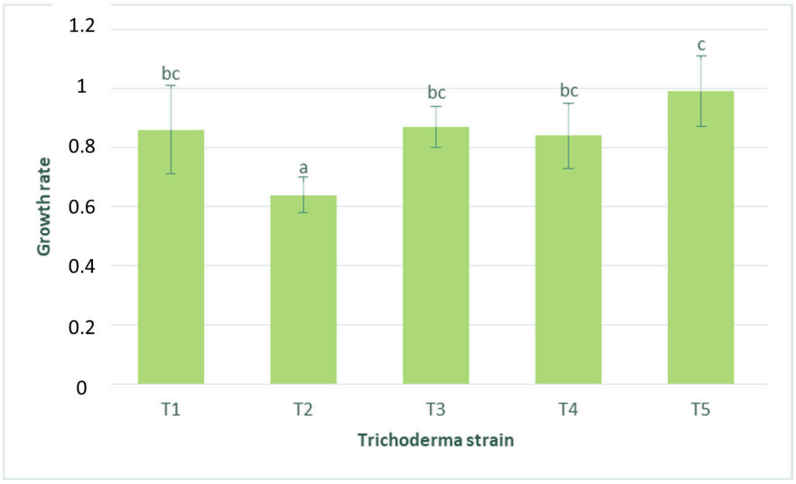

Figure 1. Growth rate of *Trichoderma* strains on PDA at 22 ± 2 °C. Values presented are the mean of three replicates, with error bars showing the standard deviation. Different letters indicate significant differences between groups according to the Duncan Test ($p < 0.05$).

Spore production varied significantly among the species. T5 produced the highest number of spores, reaching 9.21×10^7 spores per ml, which correlates with its faster growth rate. This was followed by T4, which produced 2.14×10^7 spores per ml, and T2, which produced 6.04×10^6 spores per ml. notably, no sporulation was observed under the tested conditions for T1 and T3, both identified as *Trichoderma harzianum*. These results suggest that faster-growing *Trichoderma* strains, like T5, may be more prolific in spore production, which could contribute to their biocontrol efficacy.

3.3. Antagonism Assay: Dual Plate Assay Results

All five *Trichoderma* strains effectively reduced the growth rate of *Neofusicoccum parvum* in the dual plate assay (Figure 2). Among them, T5 (*T. virens*) exhibited the highest suppression, reducing *N. parvum* growth by 48.0%. This suppression rate was significantly greater than that observed for the other strains: T1, T2, T3, and T4 reduced *N. parvum* growth by 9.4%, 17.1%, 22.2%, and 16.1%, respectively.

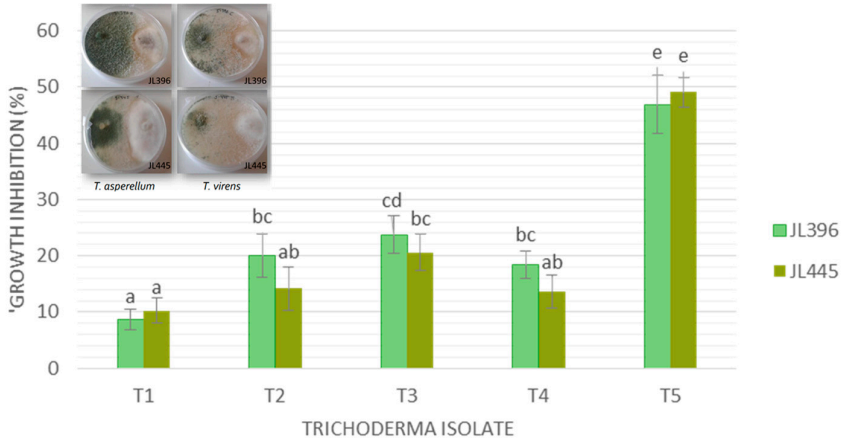

Figure 2. PCGI (Percentage of Colony Growth Inhibition) of *Neofusicoccum parvum* produced by antagonistic *Trichoderma* fungi based on the dual plate assay. Values presented are the mean of three replicates, with error bars showing the standard deviation. Different letters indicate significant differences between groups according to the Duncan Test ($p < 0.05$). The inset shows the dual plate assay between JL396 and JL445 of *Neofusicoccum parvum* with *Trichoderma asperellum* (T4) and *Trichoderma virens* (T5).

Additionally, the degree of parasitism for all *Trichoderma* strains against the two *N. parvum* strains was rated as 4 on the scale of Ezziyyani et al. [42], indicating a total invasion of the pathogenic fungal colony surface and sporulation on it.

3.4. Inhibitory Effects of Volatile Compounds Produced by Trichoderma spp.

Colony growth of *Neofusicoccum parvum* was inhibited when exposed to the trapped atmosphere from cultures of *Trichoderma* spp. After three and four days of co-cultivation, the mycelial radial growth of *N. parvum* was reduced by all the *Trichoderma* strains tested (Figure 3). However, the volatile compounds produced by T1 (*T. harzianum*) resulted in a PCGI of only 8.6%. In contrast, fungal growth rates for both JL396 and JL445 strains of *N. parvum* were significantly inhibited by exposure to volatile compounds from the T5 strain (*T. virens*), achieving a PCGI of 48.1%.

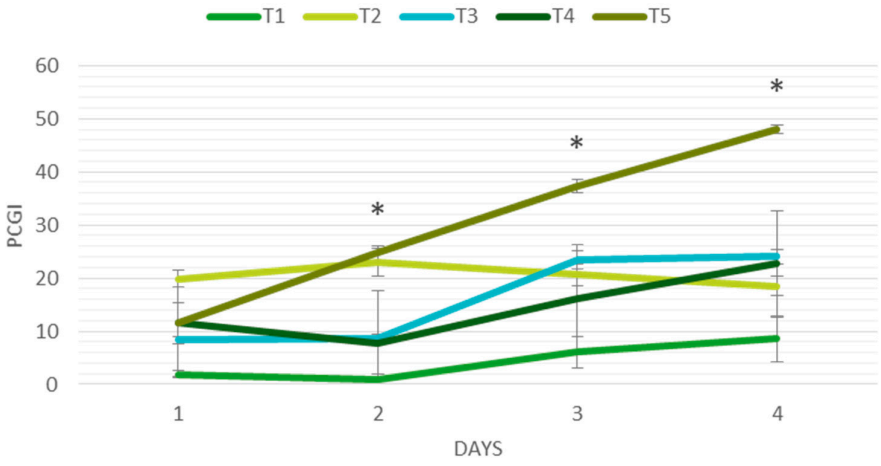

Figure 3. Percentage of Colony Growth Inhibition (PCGI) of *Neofusicoccum parvum* produced by antagonistic *Trichoderma* fungi based on the volatile compounds assay. In this assay, *Neofusicoccum parvum* strains were exposed to the volatiles emitted by *Trichoderma* strains in a co-cultivation setup for five days. Values presented are the mean of three replicates, with error bars showing the standard deviation. Asterisks denote significance at $p < 0.05$ (Duncan Test).

Based on these results, it can be concluded that volatile compounds released by all the tested *Trichoderma* species (*T. harzianum*, *T. viride*, *T. asperellum*, and especially *T. virens*) are capable of restricting the growth of the pathogen.

3.5. Compatibility of Trichoderma Strains with Chemical Fungicides

The effects of fungicides on the growth of *Trichoderma* strains are summarized in Table 1. Among the fungicides tested, Sulfur was found to be compatible with the *Trichoderma* antagonists up to the recommended dose of 2 g/L, with growth inhibition ranging from 3% to 9%. In contrast, Copper oxychloride exhibited varying levels of incompatibility. At the recommended dose of 4 g/L, Copper oxychloride caused complete suppression of vegetative growth in *Trichoderma* strains T1 and T2, and inhibited growth in T2 and T3 strains by 60% to 90%, respectively. However, Copper oxychloride allowed growth of the T5 (*T. virens*) strain. Overall, mycelial growth was reduced in all treatments compared to the untreated control, except for T5, where Copper oxychloride did not affect fungal growth.

Table 1. Effect of fungicides (Sulfur and Copper oxychloride) on the radial growth of *Trichoderma* strains (T1, T2, T3, T4, and T5).

Trichoderma Strain	Control (Radial Growth; cm)	Agrochemical (Radial Growth; cm)							
		Sulfur R.D.				Copper Oxychloride R.D.			
		1/8	1/4	1/2	1	1/8	1/4	1/2	1
T1	3.7 ± 0.3	3.7 ± 0.1	3.7 ± 0	3.7 ± 0.1	3.6 ± 0.4	3.7 ± 0.1	NG [a]	NG	NG
T2	3.6 ± 0.5	3.6 ± 0.3	3.6 ± 0.4	3.5 ± 0.4	3.3 ± 0.5	3.7 ± 0.3	2.6 ± 0.2	2.2 ± 0.3	1.6 ± 0.2 *
T3	3.9 ± 0.3	3.9 ± 0.3	3.9 ± 0.3	3.9 ± 0.1	3.8 ± 0.3	3.7 ± 0.1	1.8 ± 0.3	1.6 ± 0.2	0.4 ± 0.1
T4	3.8 ± 0.4	3.8 ± 0.1	3.8 ± 0.3	3.8 ± 0.3	3.7 ± 0.4	3.8 ± 0.2	NG	NG	NG
T5	3.9 ± 0.2	3.9 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	3.9 ± 0.2	3.8 ± 0.2	3.4 ± 0.2	3.2 ± 0.3 **

[a] NG: No growth. Mean ± SD in centimeters. Four doses were tested: 1/8, 1/4, 1/2, and 1 × Recommended Dose (R.D.). * and ** denote significance at $p < 0.05$ (Duncan Test).

3.6. Stem Disease Bioassay

Three weeks post-inoculation, cuttings inoculated with *Neofusicoccum parvum* strains JL396 and JL445 developed distinct brown external lesions that extended both upward and downward from the inoculation point. In contrast, negative control plants exhibited only a narrow necrotic area around the inoculation point. The percentage of lesion length reduction (PLLR) varied significantly between different inoculation treatments ($p < 0.05$; Figure 4).

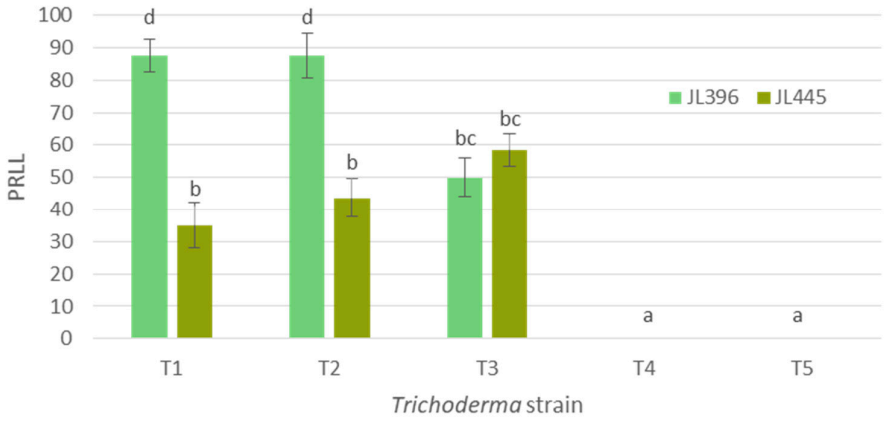

Figure 4. Percentage of lesion length reduction (PLLR) of *Neofusicoccum parvum* produced by antagonistic *Trichoderma* fungi in an *in planta* assay. Values are the mean of three replicates for two different *N. parvum* strains co-cultivated with each *Trichoderma* strain for three weeks. The control treatment consisted of healthy, non-inoculated plants, which did not develop lesions. Different letters indicate significant differences between groups ($p < 0.05$; Duncan's test).

Among the treatments, *Trichoderma viride* (T2) achieved the highest mean PLLR of 65.6%, showing a significant increase compared to other treatments. This PLLR was significantly greater than the 0% PLLR observed with *Trichoderma asperellum* (T4), which showed no reduction in lesion length. An exception was noted with *Trichoderma virens* (T5), where all inoculated shoots broke at the node above the inoculation site, preventing lesion measurement (Figure 5).

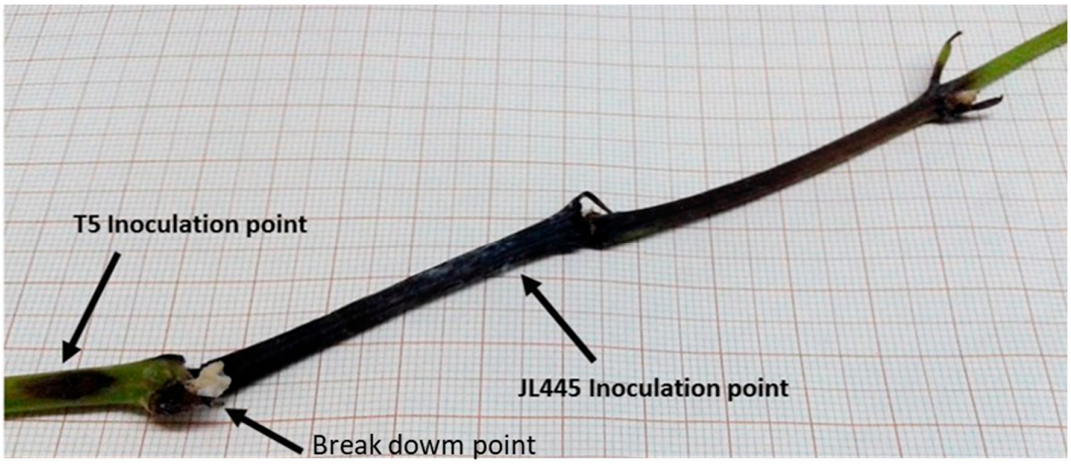

Figure 5. Lesions observed in the shoots of *Vitis vinifera* cv. Garnacha co-inoculated with *Neofusicoccum parvum* strain JL445 and *Trichoderma virens* (T5). The figure shows the development of brown external lesions extending both upward and downward from the inoculation point.

Re-isolation from plants inoculated solely with JL396 and JL445 resulted in the growth of white colonies with radially distributed aerial hyphae on PDA. For plants subjected to dual inoculation, re-isolation also yielded white colonies with radially distributed aerial hyphae, in addition to green colonies with aerial growth on the PDA surface. No pathogens were re-straind from negative control plants or from plants inoculated with *T. viride* (T2).

4. Discussion

The genus *Trichoderma* is renowned for its biocontrol capabilities, particularly in combating soil-borne and wood-decaying pathogens. This study focused on five *Trichoderma* strains, namely *T. harzianum* (T1, T3), *T. viride* (T2), *T. asperellum* (T4), and *T. virens* (T5), and their effectiveness against the grapevine pathogen *Neofusicoccum parvum*. The identification of these strains was confirmed through ITS sequencing, revealing that T1 and T3 are *T. harzianum*, T2 is *T. viride*, T4 is *T. asperellum*, and T5 is *T. virens*. The Internal Transcribed Spacer (ITS) region is a popular genetic marker (or "DNA barcode") for identifying fungal species because it tends to vary significantly between species. This variability often allows to distinguish one species from another based on differences in their ITS sequences. However, the ITS marker has limitations depending on the fungal group being studied. For most fungi, ITS alone is enough to identify species, but for some groups, additional genetic markers are needed to make accurate identifications [45].

The Internal Transcribed Spacer consists of two sub-regions, ITS1 and ITS2, which perform similarly in distinguishing many species, especially within the Basidiomycota. However, some Basidiomycota genera (11 out of 113 studied) cannot be reliably identified with ITS alone, revealing the marker's limits [45]. Furthermore, the high variability within the ITS region, while helpful for distinguishing species, can create challenges in data analysis and accurately defining species boundaries. Despite these challenges, ITS remains the most widely used marker for fungal identification, although research is ongoing to find additional or alternative markers that could improve accuracy for specific groups [46,47].

This species-level identification is crucial for accurately interpreting their biocontrol potential, as different *Trichoderma* species may exhibit varied mechanisms of action and

efficacy against pathogens [48,49]. The internal transcribed spacer (ITS) region has proven to be a reliable molecular marker for identifying species within the *Trichoderma* genera. Espinoza et al. [50] successfully identified *N. arbuti*, *N. australe*, and *N. parvum* as causal agents of stem canker in blueberries through ITS sequencing. Similarly, ITS sequencing has been effective in distinguishing various *Trichoderma* species, including *T. harzianum*, *T. asperellum*, and *T. virens* [51,52]. Sánchez-García et al. [53] demonstrated high precision in identifying *Trichoderma* species using the ITS1 and ITS2 regions, emphasizing the role of accurate species identification for biotechnological applications. These findings highlight the robustness of ITS-based molecular identification for species in *Trichoderma* genera.

Different *Trichoderma* species exhibit varying levels of antagonistic activity against plant pathogens; for example, *T. asperellum* has shown superior antagonistic effectiveness compared to *T. harzianum* against root pathogens in beans [53]. Our findings emphasize the relevance of species-specific characteristics in *Trichoderma* biocontrol efficacy, corroborating the view that not all *Trichoderma* strains exhibit the same biocontrol potential. These differences may arise from inherent genetic and phenotypic variations that influence growth rate, competition, mycoparasitic abilities, and production of antifungal compounds. In particular, the faster-growing strains, like T5 (*T. virens*), showed substantial antagonistic capabilities. Faster growth rates allow certain *Trichoderma* species to outcompete pathogens through resource monopolization, a phenomenon highlighted in studies demonstrating a correlation between rapid colony expansion and biocontrol efficacy [54]. This advantage in rapid growth contributes to their effectiveness as biological control agents, especially in environments where timely pathogen suppression is crucial for crop protection [54,55]. This result aligns with Harman et al. [56], who observed that the ability to colonize and dominate the substrate quickly contributes significantly to the effectiveness of *Trichoderma* as a biocontrol agent.

The results of this study show significant variability in spore production among the *Trichoderma* strains tested. Strain T5, with its faster growth rate, produced the highest number of spores, which aligns with previous studies demonstrating a positive relationship between faster growth and higher spore production in *Trichoderma* species [48]. This suggests that faster-growing strains, such as T5, may offer enhanced biocontrol potential due to their ability to produce larger quantities of conidia, which are crucial for pathogen dispersal and infection. In contrast, strains T1 and T3, both identified as *Trichoderma harzianum*, did not exhibit sporulation under the tested conditions. This may indicate lower adaptability or efficiency under specific growth parameters, thus potentially limiting their effectiveness as biocontrol agents in certain environments.

These findings highlight the importance of considering both growth rate and spore production when selecting *Trichoderma* strains for biocontrol applications. Strains like T5, which are more prolific in spore production, may be preferred for specific disease management contexts. In support of this, various studies have explored optimal conditions for spore production in different *Trichoderma* species. For instance, maize husk has been identified as the best substrate for maximizing spore production in *T. viride* and *T. harzianum* [57], while broken corn seed medium yielded the highest spore count for *T. atroviride* [58]. Moreover, factors such as the carbon-to-nitrogen ratio and glucose concentration significantly influence spore production and viability in *T. harzianum*, with optimal conditions identified for these parameters [59].

The dual culture assays provided insights into the competitive interactions between *Trichoderma* and *N. parvum*. *T. virens* (T5) was notably effective in suppressing the growth of *N. parvum*, achieving a 48.1% reduction. This strain's superior performance could be attributed to its rapid growth rate and efficient colonization ability, which enhances its competitive edge over the pathogen. The high mycoparasitism grade (4) observed for all *Trichoderma* strains confirms their potential for direct antagonistic interactions with *N. parvum* [60].

The role of volatile compounds (VOCs) in Trichoderma's antagonistic activity was further explored through the volatile compound assay. The results suggest that VOCs

are a significant factor in the biocontrol mechanism, particularly for strains like *T. viride* (T2), which had a high PCGI despite its slower growth rate. VOCs such as 6-pentyl-α-pyrone, which has been previously associated with antifungal activity, may contribute to this phenomenon [61–63].

Endophytic fungi-derived VOCs have shown significant effectiveness in controlling postharvest diseases in fruits and vegetables, providing a safe and environmentally friendly alternative to traditional chemical fungicides [64]. However, despite the promising potential of VOCs as biocontrol agents, there are still challenges in scaling up these applications to an industrial level. More research is required to optimize the production, stability, and efficacy of VOCs, as well as to establish practical protocols for their widespread use. Additionally, fostering collaboration between researchers and farmers is crucial for the successful adoption of VOC-based biocontrol strategies in agricultural practice [29].

The *Trichoderma* species employed in this study exhibit diverse modes of action in biocontrol, including competition for resources, mycoparasitism, and production of antifungal compounds. The observed in vitro growth rates revealed significant variation among the strains. *T. virens* (T5) demonstrated the highest growth rate, which aligns with its potential for competitive exclusion in agricultural settings. Conversely, *T. viride* (T2) showed the lowest growth rate but still managed to produce a substantial percentage of colony growth inhibition (PCGI) in the volatile compound assay. This suggests that *T. viride* may rely more heavily on VOCs for its biocontrol activity [65].

The compatibility of *Trichoderma* strains with agrochemicals was assessed to ensure their practical applicability in agricultural settings. Sulfur, a commonly used fungicide, was found to be compatible with all *Trichoderma* strains at recommended doses, showing minimal growth inhibition. This supports its use in conjunction with *Trichoderma* for integrated disease management. In contrast, copper oxychloride showed varying degrees of inhibition across the strains, with some strains like *T. virens* (T5) exhibiting greater tolerance than others. This differential tolerance to copper oxychloride may be attributed to strain-specific responses, which could influence their effectiveness in field conditions [66–68].

Our findings align with the varied fungicide tolerance observed in *Trichoderma* strains. While some strains develop resistance when exposed to gradually increasing fungicide concentrations [69], others show inhibited growth in the presence of certain fungicides. Conversely, compounds like sulfur may even promote *Trichoderma* growth [70]. Additionally, some *Trichoderma* strains can successfully germinate in fungicide-contaminated environments, though their sensitivity can differ widely [71]. Notably, *Trichoderma* strains from wild plant endospheres demonstrate remarkable fungicide tolerance and bioremediation capabilities, with some strains removing up to 89% of specific fungicides from liquid media [72]. However, conventional pesticides may negatively impact indigenous *Trichoderma* strains in soil ecosystems [70]. These findings suggest that while some *Trichoderma* strains can adapt to fungicide-contaminated environments, their integration into pest management strategies should consider the compatibility and potential effects of agrochemicals to maximize their biocontrol efficacy, particularly in systems transitioning toward organic practices.

Research on *Trichoderma* species has shown promising results for controlling *N. parvum* and other grapevine trunk disease pathogens. For instance, other in vitro studies demonstrated that *Trichoderma* strains could inhibit *N. parvum* mycelial growth by up to 97.5% [12]. Greenhouse and field trials have also highlighted the efficacy of *T. asperellum* and *T. gamsii* in offering up to 100% disease control against *N. parvum* via pruning wound protection [73]. Native *Trichoderma* strains have shown similar potential, effectively controlling *N. parvum* in young grapevine plants [74]. In British Columbia, *T. asperelloides*, *T. atroviride*, and *T. canadense* demonstrated 70–100% control of Botryosphaeria dieback fungi in pruning wounds for up to 21 days [75]. These studies underscore *Trichoderma* species as effective biocontrol agents for *N. parvum* and other grapevine pathogens, offering a sustainable alternative to chemical fungicides.

The *in planta* bioassay provided a real-world assessment of *Trichoderma's* biocontrol efficacy. The reduction in lesion length in plants inoculated with *T. viride* (T2) was significantly higher compared to other strains, aligning with its performance in in vitro assays. However, the negative impact observed with *T. virens* (T5) in the cutting bioassay, where shoots broke at the inoculation site, indicates a potential adverse effect that warrants further investigation. This unexpected outcome underscores the complexity of biocontrol interactions and highlights the need for comprehensive field trials to validate laboratory findings.

In the following summary table (Table 2), an overview of the biocontrol strategies and compatibility of various *Trichoderma* species with fungicides is provided. The table highlights the key biocontrol mechanisms of each species, such as the production of volatile organic compounds (VOCs), nutrient and space competition, and pathogen growth inhibition. Additionally, it presents the compatibility of these species with commonly used fungicides, including sulfur and copper oxychloride, and offers a brief evaluation of their effectiveness and applicability. While was chosen to include *T. harzianum* T3 in this summary due to its promising performance, it is important to note that *T. harzianum* T1 was not included. The specific strain plays a significant role in the biocontrol potential, and in this case, T3 was selected because it demonstrated superior effectiveness in the tests. While some species, like *T. harzianum* and *T. viride*, show high efficacy in *in planta* tests and are compatible with certain fungicides, others, such as *T. asperellum*, exhibit limited or no efficacy in field trials, and compatibility issues may hinder their practical application. Further studies are necessary to better understand the integration of *T. viride* into biocontrol strategies.

Table 2. Summary table of biocontrol strategies and compatibility for *Trichoderma* species.

Trichoderma Species	Biocontrol Mechanisms	Compatibility with Fungicides	Effectiveness and Applicability Comments
***T. harzianum* (T3)**	Highest VOCs effect, Nutrient and space competition, high pathogen growth inhibition	Sulfur compatible; Copper oxychloride limited compatibility	High efficacy *in planta*, compatible with fungicides; promising for integrated management
T. viride	Moderate nutrient and space competition	Sulfur compatible; Copper oxychloride limited compatibility	High efficacy *in planta*, further studies are needed for integrated management
T. asperellum	Nutrient and space competition, moderate VOCs effect	Sulfur compatible; Copper oxychloride incompatibility	No efficacy *in planta*, not suitable for further field trials
T. virens	Highest Nutrient and space competition, moderate VOCs effects, high pathogen growth inhibition effect, high pathogen growth inhibition	Sulfur compatible; Copper oxychloride partially compatible	High in vitro inhibition, but phytotoxicity *in planta* limits field use

Legend: in vitro: Laboratory test results on pathogen growth inhibition. *in planta*: Tests performed on *Vitis vinifera* plants. VOCs: Volatile Organic Compounds. Compatible: No significant reduction in *Trichoderma* growth at recommended dose. Partially compatible: Moderate reduction in *Trichoderma* growth at recommended dose. Limited compatibility: Significant reduction in *Trichoderma* growth at recommended dose. Incompatibility: No *Trichoderma* growth at recommended dose.

Future research should focus on elucidating the exact mechanisms behind the adverse effects observed with *T. virens* and exploring potential optimization strategies for its use. Additionally, investigating the synergistic effects of *Trichoderma* with other biocontrol agents and assessing long-term field efficacy will be crucial for developing effective integrated pest management strategies.

5. Conclusions

This study highlights *Trichoderma harzianum* T3 as a potent biocontrol agent against *Neofusicoccum parvum*, demonstrating significant potential for managing this pathogen in

grapevines. Among the five *Trichoderma* strains tested, *T. harzianum* T3 showed particular promise due to its effective suppression of *N. parvum* through rapid growth, mycoparasitism, and the production of volatile organic compounds (VOCs). Additionally, *T. virens* T5 displayed strong inhibitory effects, although its potential phytotoxicity suggests a need for selective application. The study reinforces *Trichoderma's* value in viticulture disease management, particularly as part of integrated pest management (IPM) strategies when compatible with certain fungicides. Future research should focus on optimizing VOC stability and efficacy in field conditions and exploring environmentally influenced interactions to enhance *Trichoderma's* practical application, ensuring optimal field performance with minimal adverse effects on host plants.

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