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Unraveling the Multiple Biocontrol Mechanisms of *Trichoderma* spp. in the Protection of Grapevines Against *Botrytis cinerea*

Faical Aoujil [1,2], Achraf Dagha [1], Najoua Agharabi [1], Basma Tommis [1], Imane Hourmatallah [1], Hiba Yahyaoui [1], Imane Karkach [3], Houda ElYacoubi [3], Aziz Aziz [4], Ilyass Maafa [5], Majida Hafidi [2] and Khaoula Habbadi [1,*]

1 Phytobacteriolgy and Biological Control Laboratory, Regional Center of Agricultural Research of Meknes, National Institute of Agricultural Research, Avenue Ennasr, BP 415 Rabat Principal, Rabat 10090, Morocco; faical.aoujil@gmail.com (F.A.); daghaachraff@gmail.com (A.D.); najouaagharabi@gmail.com (N.A.); tommisbasma31@gmail.com (B.T.); imane.hourmatallah@gmail.com (I.H.); hib.yahyaoui@edu.umi.ac.ma (H.Y.)
2 Laboratoire de Biotechnologie Végétale et Valorisation des Bio-Ressources, Faculté des Sciences, Université Moulay Ismail, B.P. 11201, Meknès 50050, Morocco; hafidimaj@yahoo.fr
3 Laboratoire des Ressources Naturelles et Développement Durable, Unité de Recherche AgroPhysiologie, Biotechnologie et Environnement, Faculté des Sciences, Université Ibn Tofaïl, B.P. 242, Kénitra 14000, Morocco
4 Induced Resistance and Plant Bioprotection (RIBP), USC 1488, INRAE, University of Reims Champagne-Ardenne, 51100 Reims, France; aziz.aziz@univ-reims.fr
5 International Center for Agricultural Research in the Dry Areas (ICARDA), Rabat 10090, Morocco; i.maafa@cgiar.org
* Correspondence: khaoula.habbadi@inra.ma

Abstract

Botrytis cinerea, the causal agent of grey mold in grapevine, remains one of the most economically important pathogens in viticulture and a key target for sustainable biocontrol strategies. This study evaluated the antagonistic potential of seven *Trichoderma* isolates (T1–T7), collected from the rhizosphere of grapevine in Morocco, using a combination of in vitro and *in planta* assays designed to capture multiple direct and indirect modes of action. The isolates exhibited variable levels of antagonism through competition, volatile organic compounds, extracellular metabolites, and elicitation responses. Preliminary *in planta* assays on detached grape berries further demonstrated that all selected isolates reduced lesion development, with preventive applications yielding the strongest protection. Overall, the study highlights the complementary and strain-specific mechanisms underlying *Trichoderma* & *B. cinerea* interactions and underscores the importance of isolate selection and application timing for the development of effective and environmentally friendly grey mold management strategies. These findings provide a mechanistic basis for the future evaluation of promising isolates under vineyard conditions.

Keywords: *Trichoderma* spp.; *Botrytis cinerea*; *Vitis vinifera*; mechanisms of action; biological control

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1. Introduction

Synthetic pesticides have become a cornerstone of modern agriculture, widely adopted to sustain crop productivity and support global food security [1]. Their widespread application is largely attributed to their high efficacy, affordability, and ease of use [2]. According to the Food and Agriculture Organization (FAO), global pesticide consumption reached approximately 3.73 million tons of active ingredients in 2023, reflecting a consistent upward

trend over the past decade [3]. However, this widespread reliance on chemical control poses serious challenges, notably disrupting ecosystem balance and raising increasing concerns about long-term impacts on environmental sustainability and human health [4,5].

Viticulture is no strange to unsustainable practices, and Mediterranean viticulture is highly dependent on the use of agrochemicals [6,7]. Grapevines (*Vitis vinifera*) are highly dependent on fungicides, as they are susceptible to numerous pests and diseases, and vineyards provide a favorable environment for their development. Consequently, intensive pesticide applications are often required to meet both qualitative and quantitative production standards. Fungicides constitute the majority of pesticide applications in vineyards, averaging around 19 treatments per season and reaching up to 27 under severe disease pressure, as reported in regions such as Morocco [6].

Among these, *B. cinerea* represents a major threat, responsible for grey mold disease that can lead to yield losses ranging from 20% to 50% under favorable environmental conditions [8]. *B. cinerea* is a necrotrophic fungus that infects over 200 dicotyledonous plant species by colonizing tissues such as stems, leaves, and fruits [9]. It poses a major threat to a wide range of economically valuable crops, including vegetables, ornamentals, bulbous plants, and fruits. Fungicides used strictly to control *B. cinerea* cover 10% of the global fungicide market [10]. Its remarkable adaptability, combined with its ability to develop resistance to multiple classes of fungicides, together with increasingly stringent pesticide regulations [11,12], is rapidly complicating control strategies. These challenges, reinforced by growing concerns among producers and consumers, underscore the urgent need to develop biofungicides and biological control methods as part of more sustainable, integrated disease management approaches [10,13].

The use of microbial antagonists has emerged as one of the most effective and sustainable biocontrol strategies. Several genera, including *Bacillus*, *Pseudomonas*, and *Aureobasidium*, have demonstrated efficacy in reducing *B. cinerea* incidence through mechanisms such as competition, antibiosis, and induced systemic resistance [14]. Among them, filamentous fungi of the genus *Trichoderma* have attracted particular attention due to their unique biological properties. More than 60% of registered fungal-based biofungicides are derived from *Trichoderma* species, with approximately 77 commercial products currently approved in the European Union [15,16].

Trichoderma spp. exhibit antagonistic activity against fungal pathogens such as *B. cinerea* through a combination of complementary mechanisms that enhance their efficacy across diverse agroecological contexts [17]. Their biocontrol strategies involve competition with phytopathogenic fungi for limited resources such as nutrients and space, as well as direct mycoparasitism [18,19]. Mycoparasitism involves the physical attack of pathogen hyphae, mediated by the secretion of a complex arsenal of cell wall-degrading enzymes (CWDE), including chitinases, β-1,3-glucanases, and proteases, which collectively degrade fungal cell wall integrity [20]. In addition, *Trichoderma* spp. produce a wide array of secondary metabolites, including antibiotics and volatile organic compounds (VOCs), which inhibit spore germination and mycelial growth. These bioactive compounds also contribute to shaping the rhizosphere microbiome, fostering beneficial microbial interactions and enhancing overall plant health [21].

Recent studies highlight the potential of *Trichoderma* species as effective and versatile biological control agents against *B. cinerea*, acting through multiple synergistic mechanisms [22–24]. This evidence underscores their suitability for integration into sustainable viticulture disease management strategies aimed at reducing the reliance on chemical fungicides. In this context, the present study aimed to investigate the antagonistic potential of indigenous *Trichoderma* strains through in vitro assays (competition, antibiosis, and optimization of culture conditions for metabolite production) and evaluate their efficacy

in suppressing *B. cinerea* infection in grape berries under *in planta* conditions. This work provides new insights into the multifunctional mechanisms of local *Trichoderma* isolates, highlighting their potential for sustainable integration into vineyard disease management.

2. Results

2.1. Isolation of Trichoderma spp.

A total of seven fungal isolates were obtained from grapevine rhizosphere soils following serial dilution plating on the different culture media tested. All isolates displayed morphological traits consistent with the genus *Trichoderma*. Among the media evaluated, Rose Bengal Chloramphenicol (RBC) and modified Potato Dextrose Agar (PDAm) were the most effective, each yielding three *Trichoderma* isolates, corresponding to 43% of the total number of isolates (n = 7). Trichoderma Selective Medium (TSM) yielded one isolate (14%), while no *Trichoderma* isolates were recovered from standard PDA under the conditions of this study. The specific grapevine cultivars from which each isolate originated are provided in Supplementary Table S1.

2.2. Morphological Characterization of Trichoderma Strains

2.2.1. Macroscopic Observations

Macroscopic characteristics of seven *Trichoderma* isolates were observed over a period of 6 days. Colony development showed distinct variations in color, texture, and pigmentation pattern among the isolates. Early growth began with light or white shades, gradually shifting to green, dark green, or yellowish tones by the 6th day. All isolates developed a floccose to velvety texture, though the density and surface appearance varied across isolates.

Concentric ring patterns were observed in six of the seven isolates, either as clearly defined growth zones or pigment-based rings, indicating radial growth. Some isolates displayed a dense green pigmentation concentrated at the margins (e.g., isolates T1, T4, and T5), while others had sporulation focused at the center with lighter peripheral zones (notably T3 and T7). Isolate T6 showed distinct black pigmentation around the edges with a white center, forming sharp contrasts, whereas isolate T2 presented the most regular concentric growth with a dark green surface and structured patterns (Figure 1A).

Despite the variation, all isolates showed consistent mycelial coverage of the medium by day 6, with colony morphologies ranging from cottony to felt-like textures and pigmentation ranging from pale yellow to dark green.

2.2.2. Microscopic Observations

Microscopic characterization of the seven *Trichoderma* isolates (T1 to T7) was carried out based on hyphal structure, conidiophores, phialides, and conidia, following the morphological criteria described by Watanabe [25]. All isolates showed hyaline, septate, and branched hyphae, forming dense and organized mycelial networks. The conidiophores were upright and branched, supporting short and thick phialides. The conidia were generally globose to subglobose, occasionally oval, with thick walls and a green pigmentation typical of *Trichoderma* species.

Isolates T3 and T4 exhibited hyphal arrangements forming cross-like (X-shaped) patterns, with conidia aggregated along these structures. T1 and T2 showed dense conidial clusters concentrated toward the colony margins, contributing to a floccose texture. T5 and T7 presented a felt-like appearance, with profuse spherical conidia distributed more uniformly across the mycelium. T6 showed both cross-shaped hyphal structures and dense conidial zones, indicating a highly organized sporulation pattern (Figure 1B). While all isolates shared core *Trichoderma*-like features, such as erect conidiophores, short thick

phialides, and pigmented conidia, precise species delimitation was not possible based solely on morphology and would require molecular confirmation.

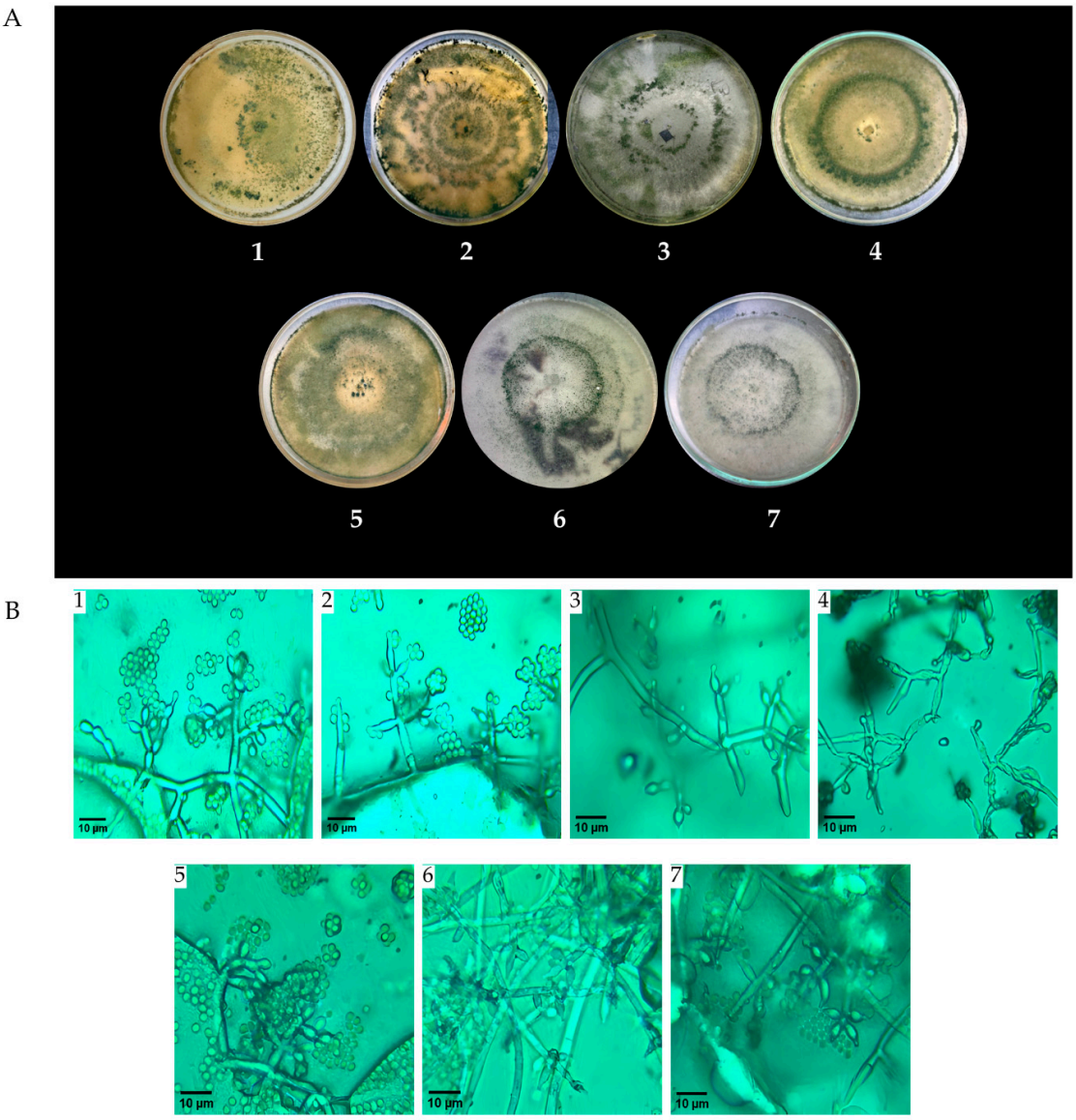


Figure 1. Macroscopic and microscopic characterization of Trichoderma isolates. (**A**) Colony morphology of seven *Trichoderma* isolates (T1 to T7), corresponding to labels 1 to 7, grown on PDA medium for 7 days at 25 ± 1 °C. Isolates showed variations in colony color, texture, and sporulation pattern. (**B**) Microscopic structures of the same isolates (labels 1 to 7 = T1 to T7) observed under light microscopy at 400× magnification. Typical *Trichoderma* morphological features were visible, including branched conidiophores, ampulliform or lageniform phialides, and subglobose to ellipsoidal conidia.

2.3. Dual Culture Assay for Direct Confrontation

The inhibitory activity of seven *Trichoderma* isolates (T1–T7) against *B. cinerea* was assessed using a dual culture assay. Analysis of variance revealed significant differences among isolates (ANOVA: $F(6, 70) = 10.36$, $p < 0.001$), indicating that antagonistic capacity is strongly strain-dependent.

Mean inhibition ranged from 51.9% (T7) to 71.4% (T5). Isolates T1, T3, T4, and T5 achieved the highest inhibition (>66%), whereas T6 and T7 displayed significantly lower activity. Post hoc Tukey's HSD tests confirmed significant differences between several isolates, most notably between T5 and T7 ($p < 0.001$), and between T6 and the more inhibitory strains (T1, T3, T4, T5).

Model assumptions were satisfied, as indicated by residual analysis (Shapiro–Wilk test: W = 0.995, p = 0.995), confirming the robustness of the ANOVA. The results are illustrated in Figure 2, which presents the mean inhibition values with standard errors and statistically distinct groups. These findings demonstrate considerable variation in the direct antagonistic ability of *Trichoderma* isolates against *B. cinerea*, reinforcing the importance of selecting strains with the highest inhibitory performance for biocontrol applications.

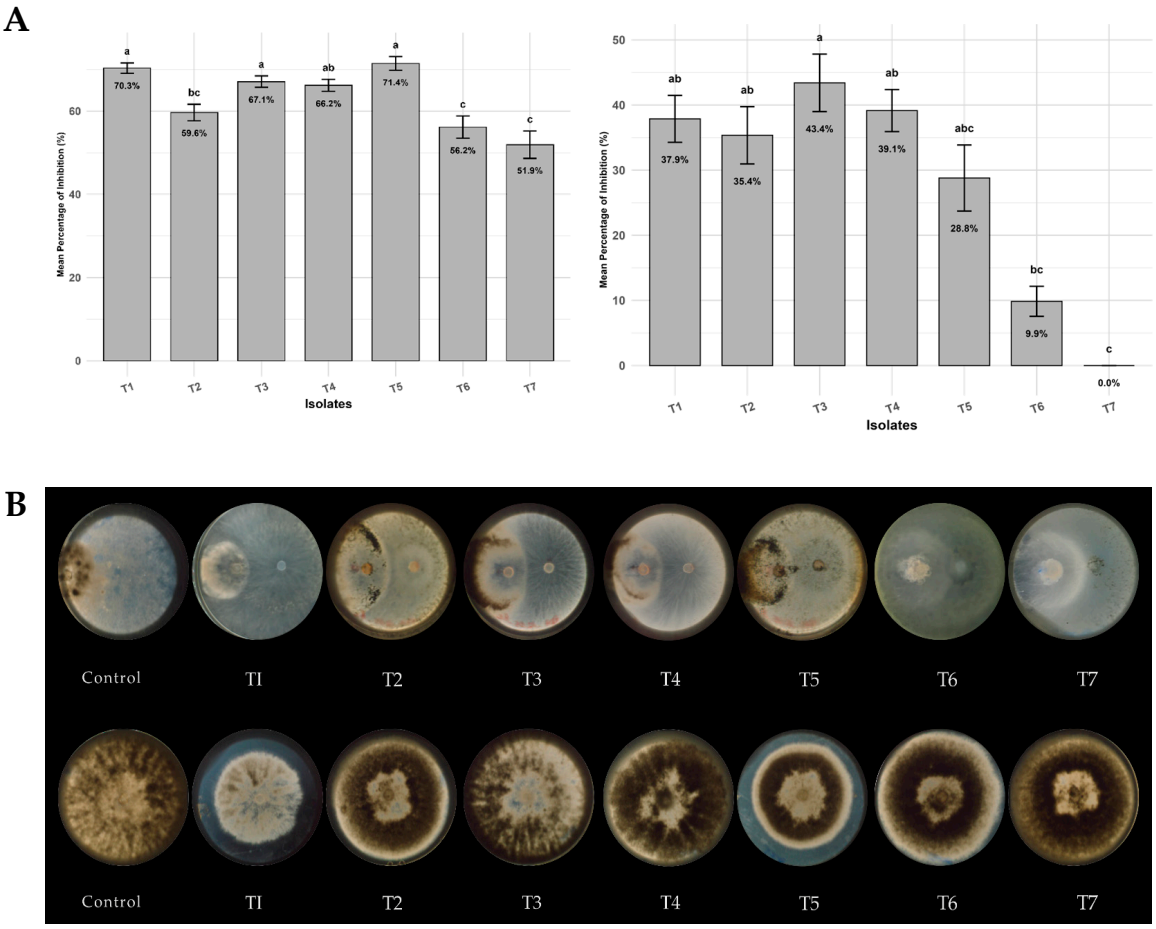


Figure 2. (**A**) Mean percentage inhibition of *B. cinerea* mycelial growth by seven *Trichoderma* isolates under direct confrontation (**left**) and VOC-mediated indirect interaction (**right**). Bars represent means ± standard deviation. Different letters above bars indicate statistically significant differences among treatments according to Tukey's HSD test at $p < 0.05$. (**B**) Representative Petri dish images illustrating fungal inhibition: (**top panel**) shows dual culture assays (direct contact), with *Trichoderma* inoculated on the right side and *B. cinerea* on the left; (**bottom panel**) depicts VOC-mediated inhibition assays (indirect contact), where physical separation prevents direct hyphal interaction.

2.4. VOCs Assay for Indirect Inhibition

The ability of seven *Trichoderma* isolates (T1–T7) to inhibit *B. cinerea* via volatile organic compounds (VOCs) was evaluated using a sealed double-plate system. Most isolates exhibited measurable levels of inhibition under indirect contact conditions, although overall inhibition was generally lower and more variable compared to the direct confrontation assay. Notably, isolate T7 did not exhibit any inhibitory effect in this assay.

Mean inhibition rates ranged from 0% (T7) to 43.4% (T3). Isolates T1, T2, T3, and T4 demonstrated moderate inhibitory effects (≥35%), while T5 showed lower activity (28.8%). T6 displayed minimal inhibition (9.9%), and T7 showed none (Figure 2). Analysis of variance confirmed significant differences among isolates ($F(6, 57) = 6.66$, $p < 0.001$), indicating that the emission of VOCs with antifungal activity is highly isolate-dependent.

Tukey's HSD post hoc test revealed that T3 and T4 were significantly more effective than T7 ($p < 0.001$), and T3 also differed significantly from T6 ($p = 0.034$). No other pairwise comparisons showed statistically significant differences. These findings indicate that VOC-mediated antagonism varies substantially among Trichoderma isolates, with certain strains (notably T3) showing promising indirect inhibitory potential against *B. cinerea*.

2.5. Molecular Confirmation of Trichoderma Strains

The molecular identification of the five fungal isolates (T1–T5) chosen for their superior antagonistic performance was performed using BLASTn analysis of the ITS region sequences. The sequences were compared against the NCBI database, and the closest matches, along with their accession numbers and similarity statistics, are presented in Table 1.

Table 1. Molecular identification of *Trichoderma* isolates (T1–T5) based on ITS sequence analysis using BLASTn. The table shows the closest BLAST hit with the corresponding GenBank accession number, percentage of identity, query cover, maximum score, and E-value.

Isolate Code	Closest BLAST Hit (Species/Strain)	Accession No.	Identity (%)	Query Cover (%)	Max Score	E-Value
T1	*Trichoderma harzianum*	PQ774982.1	97.67%	100%	147	3×10^{-31}
T2	*Trichoderma compactum*	NR_138435.1	97.73%	98%	148	3×10^{-36}
T3	*Trichoderma harzianum*	OQ789692.1	100.00%	100%	100	1×10^{-17}
T4	*Trichoderma longipile*	JF303878.1	100.00%	100%	91.6	4×10^{-15}
T5	*Trichoderma longibrachiatum*	PP837621.1	100.00%	100%	102	3×10^{-18}

All isolates were affiliated with the genus *Trichoderma*. Isolate T1 showed 97.67% identity and 100% query cover with *T. harzianum* (PQ774982.1), while isolate T2 was closely related to *T. compactum* (NR_138435.1) with 97.73% identity and 98% query cover. Isolates T3, T4, and T5 displayed 100% identity with *T. harzianum* (OQ789692.1), *T. longipile* (JF303878.1), and *T. longibrachiatum* (PP837621.1), respectively, with complete query coverage.

Overall, these results confirm that all five isolates belong to the genus *Trichoderma*, with species-level resolution achieved for three isolates (T3–T5), while T1 and T2 showed high similarity to *T. harzianum* and *T. compactum* but below the conventional species-level threshold of $\geq$98.7% identity.

2.6. Filtrate Assay for the Effect of Extracellular Metabolites

The antifungal activity of extracellular metabolites secreted by five *Trichoderma* isolates (T1 to T5) was assessed using a culture filtrate assay in microplate format. The corrected Area Under the Disease Progress Curve (AUC), expressed as a percentage relative to the untreated *B. cinerea* control (C+), was used to quantify fungal growth over a 48-h period. Two isolates (T6 and T7) were excluded from this test due to their previously observed low inhibition levels.

Significant differences were detected among treatments (Kruskal–Wallis test, $\chi^2(6) = 82.2$, $p < 0.001$), confirming differential efficacy of the secreted compounds (Figure 3). The positive control (C+), which contained only *B. cinerea*, exhibited a corrected AUC of 100%, representing full fungal growth. In contrast, the negative control (C−), which received no *B. cinerea* inoculum, showed a corrected AUC of 0%, confirming the absence of background noise or contamination in the assay.

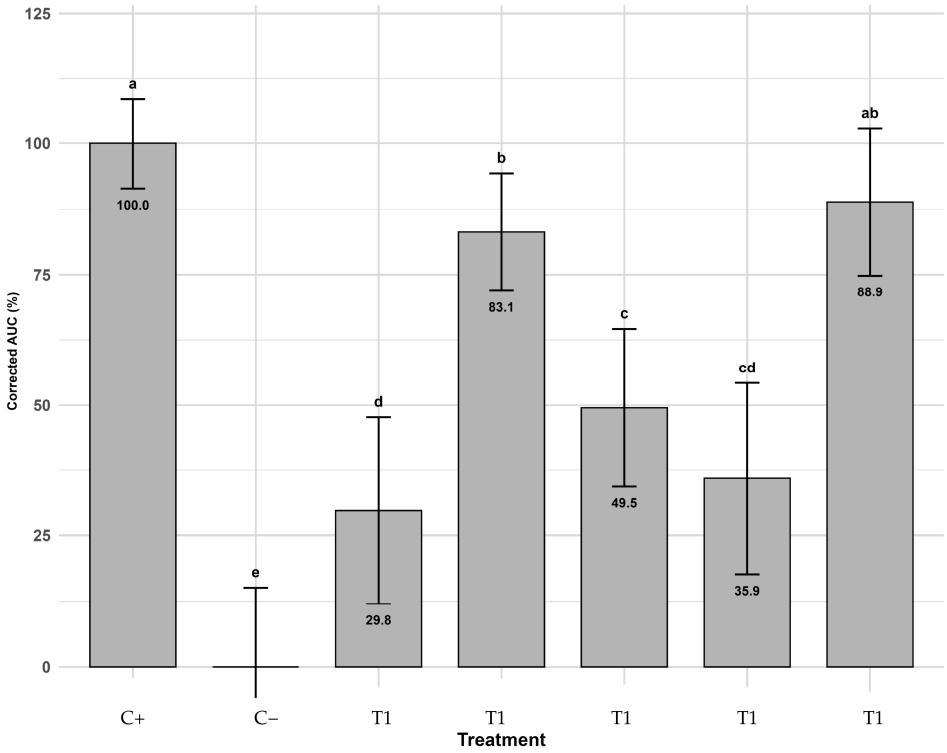


Figure 3. Mean corrected area under the curve (AUC%) representing the in vitro inhibition of *B. cinerea* growth by culture filtrates of five selected *Trichoderma* isolates. Bars indicate means ± standard deviation. Statistical analysis was performed using the non-parametric Kruskal–Wallis test, followed by a post hoc Dunn's test for pairwise comparisons. Different letters above the bars denote statistically significant differences at $p < 0.05$.

Among the *Trichoderma* filtrates, T1 (29.8%), T3 (49.5%), and T4 (35.9%) significantly reduced *B. cinerea* development compared to C+, forming statistically distinct groups based on Dunn's post hoc test (adjusted $p < 0.05$). In contrast, T2 (83.1%) and T5 (88.9%) were not significantly different from the positive control, suggesting that they exhibit low or no inhibitory activity via extracellular metabolites.

2.7. Elicitation Assay Using B. cinerea Cell Wall Fragments

To evaluate the elicitor potential of *Botrytis cinerea* mycelial fragments in reducing the in vitro growth of the fungal pathogen, microplate assays was conducted using five *Trichoderma* isolates (T1 to T5) in combination with increasing concentrations of elicitor (0, 0.25, 0.5, and 1 g/50 mL). Disease progression was quantified by measuring the corrected Area Under the Disease Progress Curve (AUC), expressed as a percentage relative to the untreated *B. cinerea* control (C+).

A total of 21 treatment groups (C+ and four concentrations per isolate) were tested (Figure 4). The results revealed substantial differences in AUC values depending on both the *Trichoderma* isolate and the elicitor concentration applied. Mean AUC percentages ranged from ~100% (C+), reflecting full disease progression, to <40% in several elicitor-treated conditions, indicating the strong growth inhibition of *B. cinerea*.

Statistical analysis using one-way ANOVA confirmed a highly significant effect of treatment on fungal growth ($F(20, 42) = 56.52$, $p < 2 \times 10^{-16}$). Tukey's HSD post hoc test revealed multiple significant pairwise differences between treatments. All *Trichoderma* isolates significantly reduced the growth of *Botrytis cinerea*.

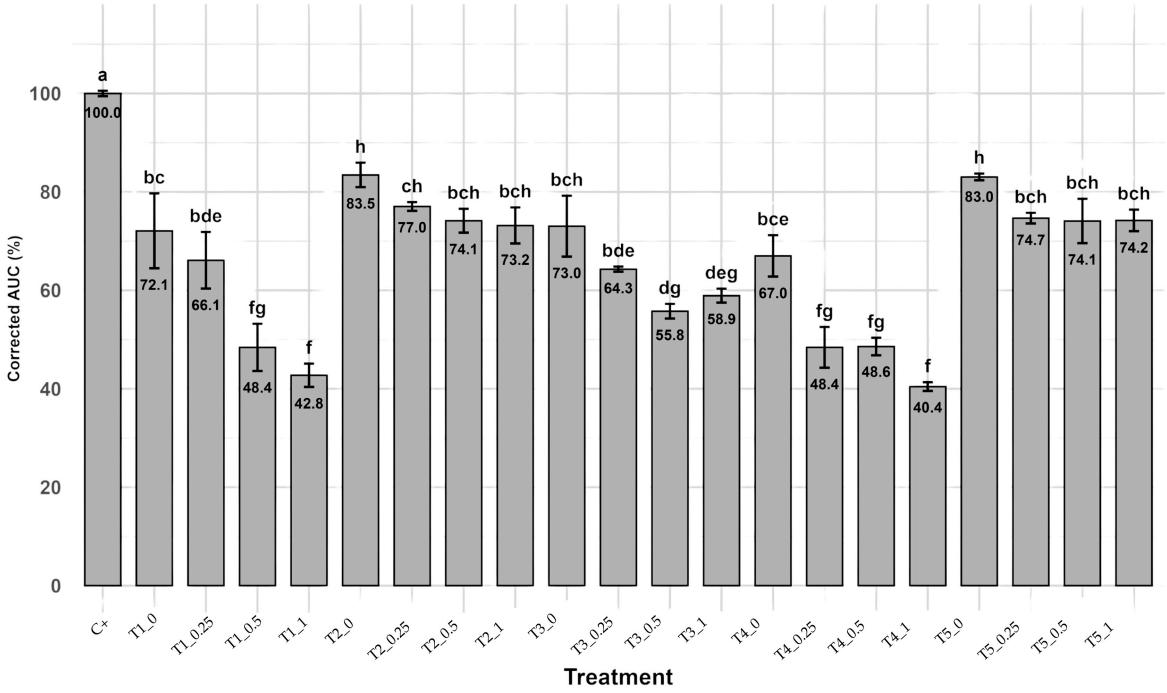


Figure 4. Effect of elicitor addition (ground and autoclaved *B. cinerea* mycelium) at increasing concentrations (0, 0.25, 0.5, and 1 g per 50 mL) on the biocontrol activity of culture filtrates from five Trichoderma isolates (T1–T5). The histogram shows the corrected mean area under the curve (AUC%) of *B. cinerea* growth inhibition for each treatment. Bars represent means ± standard deviation. Statistical analysis was performed using one-way ANOVA, followed by Tukey's HSD post hoc test. Different letters indicate statistically significant differences among treatments at $p < 0.05$.

Among them, isolates T1 and T4 showed the most consistent inhibitory activity, with enhanced performance at higher elicitor concentrations. In the case of isolate T3, inhibition improved slightly with increasing elicitor dose; however, the dose effect was not statistically significant. Conversely, isolates T2 and T5 did not exhibit consistent elicitation, as not all concentrations tested led to significant inhibition. The progressive reduction in AUC observed for several isolates with increasing elicitor concentration supports a dose-dependent elicitation effect.

These findings suggest that pre-treatment with *B. cinerea* mycelial fragments can enhance the antifungal activity of specific *Trichoderma* isolates; it can be hypothesized that this effect is linked to the induction of defense responses or priming mechanisms. However, not all isolates responded equally, emphasizing the importance of isolate-specific elicitation capacity in biocontrol strategies.

2.8. In Planta Biocontrol Assay on Grape Berries

To assess the biocontrol efficacy of *Trichoderma* isolates under *in planta* conditions, five selected strains (T1–T5) were tested on grape berries using three application strategies: preventive (TrBc24), simultaneous (TrBc), and curative (Tr24Bc). Two additional controls were included: negative control (Tr_only) and positive control (Bc_only).

Statistical analysis using the non-parametric Kruskal–Wallis test revealed a highly significant effect of treatment combinations on lesion area ($p < 0.001$). Post hoc comparisons classified the treatments into statistically distinct groups, with considerable variation observed between isolates and application modes (Figure 5).

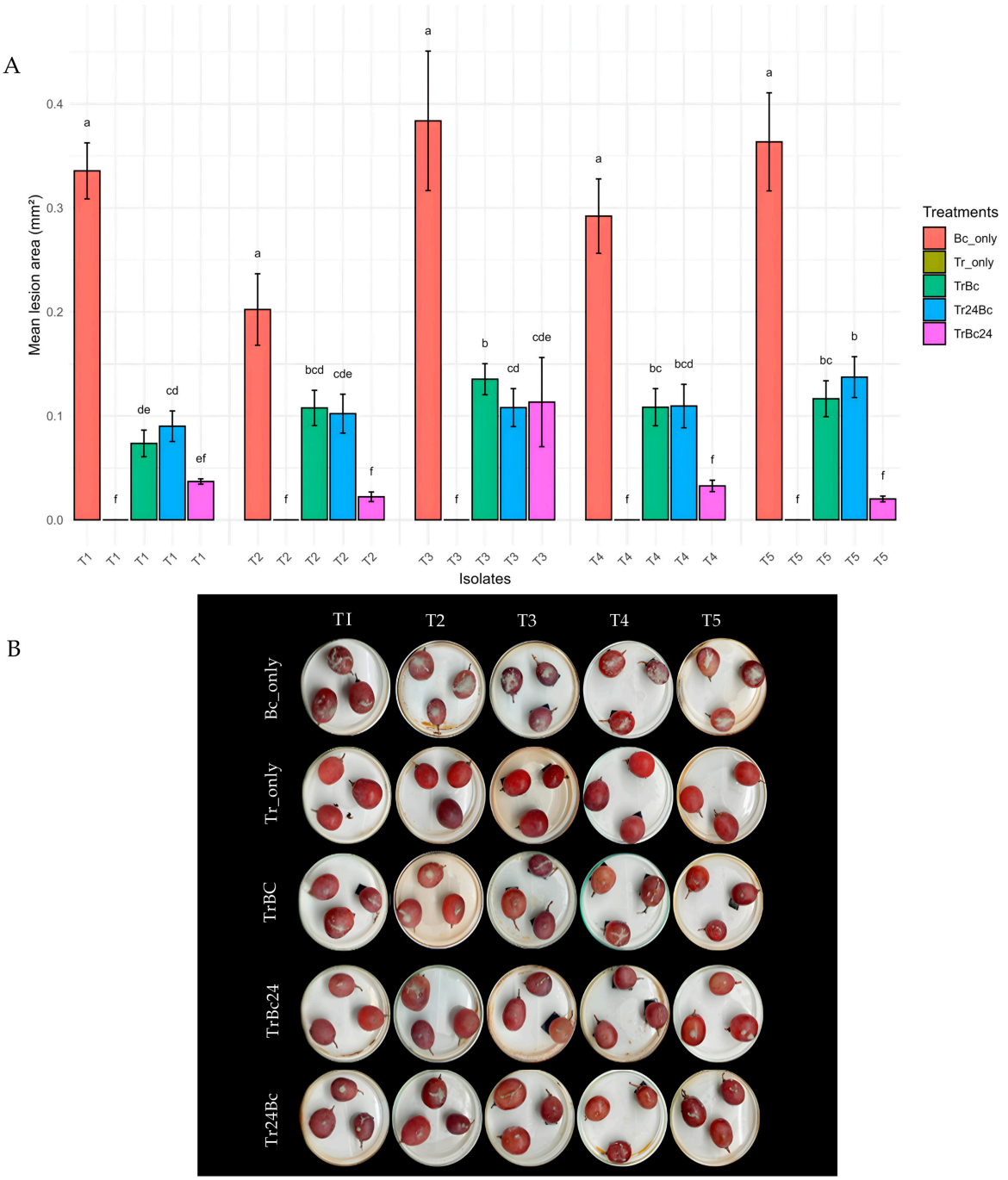


Figure 5. In planta biocontrol efficacy of five *Trichoderma* isolates (T1–T5) against *Botrytis cinerea* on *Vitis vinifera* cv. Red *Globe berries*. (**A**) Histogram representing the mean percentage of infection severity under different treatment modes: preventive, simultaneous, and curative, for each Trichoderma isolate. Negative (C−) and positive (C+) controls were included for comparison. Data were analyzed using the non-parametric Kruskal–Wallis test, followed by Dunn's post hoc test for multiple comparisons. Different letters above the bars indicate statistically significant differences between treatments at $p < 0.05$. (**B**) Representative images of infected grape berries following the three treatment modes and controls for each of the five antagonistic isolates. Disease symptoms and the extent of infection were visually compared to illustrate the efficacy of each biocontrol strategy.

As expected, the positive control (Bc_only) resulted in the largest lesion areas, with mean values ranging from 0.202 to 0.384 mm^2 depending on the isolate background. In contrast, no lesions were observed in any of the Tr_only treatments, confirming the non-

pathogenicity and innocuity of the *Trichoderma* isolates toward grape berries under the tested conditions.

Among the biocontrol strategies, preventive application (TrBc24), in which *Trichoderma* was applied 24 h prior to *B. cinerea* inoculation, proved highly effective for isolates T2, T4, and T5, reducing lesion areas to 0.0222, 0.0327, and 0.0201 mm^2, respectively. These treatments were among the most protective, statistically grouped with the negative control (group f). Simultaneous application (TrBc) also resulted in significant protection for all isolates. Notably, T1 again performed best (0.0735 mm^2), while T2–T5 ranged from 0.108 to 0.135 mm^2. These values, while higher than in preventive mode for some isolates, still represented a strong reduction compared to the positive control. The curative application (Tr24Bc), in which *Trichoderma* was applied 24 h after *B. cinerea*, showed more variable results. For T5 and T3, curative efficacy was lower than in other modes (e.g., T5: 0.137 mm^2), while T1 maintained moderate control (0.090 mm^2).

In summary, the results indicate that all tested *Trichoderma* isolates significantly reduced disease development on grape berries. However, the level of protection was dependent on both the isolate and the timing of application, with preventive strategies generally offering the highest efficacy, particularly for T2, T4, and T5. These findings suggest that application timing and strain selection represent key factors influencing the performance of *Trichoderma*-based biocontrol strategies.

3. Discussion

Trichoderma species are recognized among the most effective fungal biocontrol agents, widely employed in agriculture due to their ability to suppress a broad range of phytopathogens through mechanisms such as mycoparasitism, competition for resources, and the production of hydrolytic enzymes and secondary metabolites [26–28]. Their antagonism is often strain-specific, which highlights the importance of selecting isolates with the greatest inhibitory potential [29,30]. In the present study, *Trichoderma* isolates were recovered from the rhizosphere of grapevine plants cultivated under semi-arid, non-irrigated conditions. Such environments exert strong selective pressure that likely favors the persistence of microbial strains with enhanced ecological fitness, including superior abilities for root colonization, survival under drought stress, and competitive resource acquisition [31,32]. Sampling across multiple grapevine cultivars allowed us to capture a broad spectrum of native rhizospheric diversity [33]. Phenotypic and preliminary molecular characterizations revealed substantial variability among the collected isolates, suggesting functional divergence and potential differences in their biocontrol efficacy.

We demonstrated significant variability in the inhibitory capacity of seven *Trichoderma* isolates against *Botrytis cinerea* in dual culture assays. Mean inhibition ranged from 51.9% (T7) to 71.4% (T5), with isolates T1, T3, T4, and T5 showing the strongest antagonism (>66%), while T6 and T7 were significantly less effective. Our results are comparable to those reported in other studies. For instance, Cuban indigenous *T. harzianum* and *T. asperellum* strains achieved up to 80% inhibition in dual cultures [34], while, among the 52 isolates in another investigation, identified mainly as *T. atroviride* and *T. harzianum*, inhibition ranged from 45% to 78% [35]. A large screening in Inner Mongolia similarly reported inhibition levels of 44–83% against *B. cinerea*, with many isolates belonging to *T. harzianum* and *T. asperellum* exceeding 50% antagonism [36]. Beyond quantitative differences in inhibition, several studies have also investigated the mechanisms underlying the superior performance of specific isolates in direct confrontation with pathogens. Competition for space and nutrients has been identified as a key factor in their biocontrol efficiency when grown in dual culture with pathogenic fungi [37].

Volatile organic compounds (VOCs) emitted by *Trichoderma* spp. are recognized as crucial, albeit minor, components of their metabolome, originating from both primary and secondary metabolism. Characterized by their low molecular weight, high vapor pressure, and volatility, these compounds facilitate complex ecological interactions by diffusing through the air or soil, thus enabling communication between organisms across trophic levels [38]. In our study, the VOC-mediated inhibition of *Botrytis cinerea* varied significantly among the tested isolates, with some (e.g., T3) demonstrating moderate suppressive effects, while others (e.g., T7) exhibited no inhibition. This variability is consistent with previous findings that highlight pronounced differences in the VOC profiles, or volatilomes, across *Trichoderma* species and strains [39], which can directly influence antifungal activity.

Moreover, the antagonistic effect of *Trichoderma* VOCs is known to be pathogen-specific, acting effectively against some phytopathogens while remaining inactive against others [40]. For instance, exposure to VOCs emitted by *T. atroviride* LZ42 has been shown to reduce *Fusarium oxysporum* growth by 54.57% [41]. Similarly, a broader screening of nine *Trichoderma* isolates from three species (*T. atroviride*, *T. harzianum*, and *T. asperellum*) revealed inhibitory effects on fungal mycelial growth ranging from 13.9% to 63.0% [42], which aligns with the moderate inhibition levels (up to ~43%) observed in our experiment.

In addition to their biocontrol potential, VOCs from *Trichoderma* spp. have also been implicated in the modulation of plant physiological processes, influencing root architecture, seed germination, and overall development [43,44]. This dual role reinforces the ecological importance of *Trichoderma*-derived volatiles and supports their inclusion in integrated disease management strategies. From a chemical standpoint, *Trichoderma* VOCs include alcohols, ketones, esters, sesquiterpenes, and particularly the lactone 6-pentyl-α-pyrone (6PP), a compound frequently associated with antifungal activity [45,46]. Mechanistic studies have shown that certain VOCs can inhibit fungal pathogens by damaging the integrity of fungal cell membranes [47]. While some progress has been made, the precise mechanisms by which VOCs exert their antifungal effects remain unclear [48]. Given their volatile nature, these compounds offer promising avenues for non-contact biocontrol strategies, particularly in controlled environments like greenhouses, where they can be integrated into fumigation systems or slow-release formulations to manage aerial pathogens.

The antifungal activity observed in both the culture filtrate and elicitor-based assays highlights the critical role of *Trichoderma*'s extracellular metabolites in suppressing *Botrytis cinerea*. These secreted compounds include an array of hydrolytic enzymes and low-molecular-weight bioactive molecules that are involved in pathogen degradation. Analyses by LC-MS/MS identified a broad spectrum of proteins in *Trichoderma* exudates, including glycoside hydrolases, chitinase, mutanase, α-1,3-glucanase, α-1,2-mannosidase, glucan 1,3-β-glucosidase, α-galactosidase, and neutral protease 2 [49]. These enzymes directly contribute to the degradation of fungal cell walls and to pathogen suppression.

Interestingly, in our study, elicitor treatments using ground and autoclaved mycelium of *B. cinerea* resulted in the stronger inhibition of fungal growth than culture filtrates alone, suggesting that contact with pathogen-derived molecules may amplify *Trichoderma*'s antifungal response. This enhanced inhibition could be explained by the perception of oligomers released from the *Botrytis* cell wall during elicitor preparation. These pathogen-associated molecular patterns (PAMPs) are likely recognized by *Trichoderma* receptors, triggering signaling cascades that promote the expression of defense genes and the secretion of cell wall-degrading enzymes [50,51].

Such elicitation of secondary metabolism has been demonstrated in previous studies, where exposure to fungal elicitors induced the production of antifungal metabolites by *Trichoderma* [52]. Moreover, proteomic studies have shown that *T. harzianum* can modulate its extracellular protein profile in response to fungal cell wall components, notably inducing

specific enzymes such as an aspartic protease [53]. This finding supports the concept of an inducible secretome triggered by biotic stimuli.

Together, our findings, aligned with the literature, emphasize that *Trichoderma*'s extracellular arsenal is both constitutive and inducible, capable of being modulated by pathogen-derived signals. This adaptability may represent a key mechanism in its effectiveness as a biological control agent.

In planta assays confirmed that all tested *Trichoderma* spp. significantly reduced disease development on grape berries, although the degree of protection varied depending on the strain and the application strategy. Preventive treatments, particularly with isolates T2, T4, and T5, were the most effective, reducing lesion areas to levels statistically comparable to the negative control. These outcomes align well with earlier reports, as previous studies have shown that the early colonization of host tissues by *Trichoderma* provides a competitive advantage against *B. cinerea* and prevents pathogen establishment [54]. Likewise, pre-colonization of grape berries with *T. atroviride* provided high protection against *B. cinerea*, with relative control values reaching approximately 90% under favorable environmental conditions, such as high humidity and optimal temperature [55]. This protective effect is mainly explained by competitive exclusion, in which *Trichoderma* deprives *B. cinerea* of space and nutrients, along with the production of cell-wall-degrading enzymes and secondary metabolites [26,27].

Taken together, our results provide a comprehensive view of the multifaceted antagonistic mechanisms employed by *Trichoderma* spp. against *Botrytis cinerea*, from direct mycelial inhibition and volatile-mediated suppression to the inducible production of hydrolytic enzymes and bioactive metabolites. This stepwise approach, spanning ecological isolation, strain selection, functional screening, and *in planta* validation, highlights both the potential and the complexity of native *Trichoderma* isolates as biocontrol agents. Nevertheless, several limitations must be acknowledged. The *in planta* assays were conducted under controlled conditions, which do not fully capture the environmental variability and microbial complexity of vineyard ecosystems. Additionally, the molecular basis of strain-dependent differences in antifungal efficacy remains to be elucidated. Future work should integrate transcriptomic, metabolomic, and field-level evaluations to better characterize the regulatory networks driving biocontrol activity and to validate the most promising isolates under real agronomic conditions. Ultimately, such advances will contribute to the development of targeted, climate-adapted biocontrol strategies for sustainable disease management in viticulture.

4. Materials and Methods

4.1. Soil Sampling and Fungal Collection

In July 2024, rhizosphere soil samples were collected from healthy grapevine (*Vitis vinifera* L.) plants at the INRA experimental station in Aïn Taoujdate, Morocco (Fez-Meknes; 550 m; 33.931031 N, −5.274508 W). The vineyard is managed under supplemental irrigation with a spacing of 4 × 2.5 m between vines and trained using the double Guyot pruning system. Vines were 23 years old at the time of sampling. Each soil sample was collected from a different cultivar, with the experimental plot comprising 45 grapevine cultivars. At each site, four subsamples (10 cm depth) were collected using a sterile hand auger, pooled into composite samples, stored at 4 °C, and processed within 24 h. Fungi were isolated using the soil dilution plate method on *Trichoderma* Selective Medium (TSM), Potato Dextrose Agar (PDA), modified PDA (mPDA), and Rose Bengal Chloramphenicol Agar (RBC) whose detailed compositions are provided in Supplementary Table S2. Plates were incubated at 27 °C in the dark for 5 days. Colonies exhibiting morphological traits

typical of *Trichoderma* were purified on PDA, microscopically examined for confirmation, and preserved at −80 °C in 30% glycerol.

The *Botrytis cinerea* strain used for pathogenicity assays corresponds to the isolate BC53, previously isolated from grapevine and characterized as the highly virulent strain [56]. The fungal strain was preserved in PDB supplemented with 30% glycerol, then revived, grown, and maintained on Potato Dextrose Agar (PDA) plates.

4.2. Dual Culture Assay

The antagonistic activity of *Trichoderma* strains against *B. cinerea* was evaluated using the dual culture method described by Olowe et al. (2022) [57]. Mycelial plugs (5 mm diameter) were excised from the actively growing margin of *Trichoderma* colonies cultured on PDA and placed at one edge of the Petri dish, opposite to a 5-mm plug from a 5-day-old *B. cinerea* colony, positioned 7 cm apart.

To assess the effect of volatile organic compounds (VOCs) produced by *Trichoderma* isolates, paired plate assays were conducted following the method of Ruangwong et al. [58]. In this setup, the bottom halves of two Petri dishes, one containing *B. cinerea* and the other a *Trichoderma* isolate, both centrally inoculated on PDA, were superimposed and sealed to allow gaseous exchange without physical contact.

All plates were incubated at 25 ± 2 °C, and radial mycelial growth was recorded daily, with observations terminated until the pathogen, in the control treatment, had grown to cover the plate's surface. Each assay was performed in triplicate for every *Trichoderma* isolate. Controls consisted of *B. cinerea* cultured alone under identical conditions. Antagonistic activity was quantified by calculating the percentage reduction in the radial growth of *B. cinerea* relative to the control using the following formula:

$$R\ (\%) = ((R1 - R2)/R1) \times 100\ (\%)$$

where R_1 is the radial growth of the pathogen in the control, and R_2 is the radial growth of the pathogen in the presence of the *Trichoderma* isolate.

4.3. Molecular Identification

Genomic DNA was extracted from fungal isolates using a bead-beating and automated purification protocol. Following a screening based on the dual culture assay, the isolates showing the strongest antagonistic activity were selected for molecular identification. Each sample was mixed with 200 µL of BL2B buffer (Zinexts Life Science Corp., New Taipei City, Taiwan), 10 µL of RNase A, and two grinding beads, homogenized with a Tissue Lyser, and incubated overnight at 65 °C. Lysates were pre-filtered, centrifuged at 6000× *g*, and 200 µL of the supernatant were then processed using the MagPurix automated extraction system (Zinexts Life Science Corp., Taiwan) together with the Plant DNA Extraction Kit from the same manufacturer.

PCR amplification of the internal transcribed spacer (ITS) region was performed for five representative DNA samples using the universal primers ITS-F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-R (5′-TCCTCCGCTTATTGATATGC-3′), yielding an amplicon of approximately 500 bp. Each 25 µL reaction contained 5 µL of 5× buffer, 1 µL of ITS-F (10 µM), 1 µL of ITS-R (10 µM), 0.2 µL of MyTaq DNA polymerase (5 U/µL, Bioline), 100 ng of template DNA, and nuclease-free water. Amplification was carried out in a Verity thermal cycler (Applied Biosystems, USA) under the following program: 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 3 min.

PCR products were purified and sequenced in a single direction using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Obtained DNA sequences

were compared to sequences available in the NCBI database using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify closely related sequences and assess sequence similarity.

4.4. Preparation of Culture Filtrates and Mycelial Growth Inhibition

Culture filtrates of *Trichoderma* strains were prepared by transferring five mycelial discs (5 mm diameter, 7-day-old PDA cultures) into 250 mL sterile Erlenmeyer flasks containing 50 mL of Potato Dextrose Broth (PDB). Flasks were incubated at 22 °C on a rotary shaker (100 rpm) for 7 days, and the resulting cultures were filtered through 0.2 µm syringe filters to obtain sterile culture filtrates.

A microplate-based assay was performed to evaluate the inhibitory activity of *Trichoderma* culture filtrates on the in vitro growth of *B. cinerea*. Sterile 96-well microplates (ref. 00F-100) were prepared with 160 µL of Potato Dextrose Broth (PDB), 20 µL of culture filtrate (10% of the final volume), and 20 µL of a standardized *B. cinerea* spore suspension, for a total of 200 µL per well. Wells containing PDB and spore suspension served as positive controls (C+), while wells with PDB and sterile distilled water served as negative controls (C−).

Fungal growth was monitored by measuring the optical density (OD) at 635 nm [51] using a microplate reader (ELx800, BioTek Instruments, Inc., Winooski, VT, USA) operated with Gen5 software (v2.0). Each treatment consisted of 12 biological replicates, and the experiment was independently repeated twice for reproducibility. Microplates were incubated at 25 °C for 48 h, with OD measurements recorded every hour to track *B. cinerea* growth dynamics.

4.5. Elicitation of Trichoderma Cultures Using B. cinerea Mycelia

Mycelium of *B. cinerea* was collected from a 14-day old culture grown on PDA. Defined amounts of 0.25, 0.5, or 1 g of mycelium were transferred into 50 mL of PDB, homogenized at 16,000 rpm for 10 s, and subsequently autoclaved at 120 °C for 20 min to obtain an inactivated mycelial extract.

For each antagonistic strain, 250 mL Erlenmeyer flasks containing 50 mL of PDB (with or without the inactivated *B. cinerea* mycelial extract, used as a control) were inoculated with five mycelial plugs (5 mm diameter) excised from actively growing cultures of the antagonist.

Microplate assays were then performed as previously described for the culture filtrate tests to evaluate the *in vitro* growth of *B. cinerea* under the different treatments. Each treatment was replicated 4 times, and the experiment was independently repeated twice.

4.6. In Planta Antagonism of Trichoderma Against B. cinerea in Grapes

The *in planta* biocontrol efficacy of *Trichoderma* strains against *B. cinerea* was evaluated using grape berries, following the methodology described in [59] with slight modifications. Experiments were conducted in disinfected plastic boxes lined with sterile filter paper moistened with distilled water to maintain adequate humidity. Within each box, sterile Petri dishes were placed, with their lids and bases separated to serve as supports, on which six berries of the cultivar Red Globe were positioned.

Five treatments were tested: (i) simultaneous inoculation of both fungi (TrBc), (ii) sequential inoculation of *B. cinerea* followed by *Trichoderma* after 24 h (Tr24Bc), (iii) sequential inoculation of *Trichoderma* followed by *B. cinerea* after 24 h (TrBc24.), (iv) control berries inoculated with *B. cinerea* alone (10^6 spores/mL) (Bc_only), and (v) control berries treated solely with *Trichoderma* (10^6 spores/mL) (Tr_only). Each treatment was applied to six berries per replicate, and the entire experiment was independently repeated three times. The efficacy of each treatment was assessed by measuring lesion

diameters (mm) using ImageJ software (Version 1.52r), based on data collected 5 days post-inoculation from all replicates.

5. Conclusions

This study provides a comprehensive evaluation of the antagonistic potential of seven *Trichoderma* isolates against *Botrytis cinerea*, incorporating multiple modes of action including direct mycoparasitism, volatile organic compound (VOC) emissions, extracellular metabolite production, and elicitor-induced responses. All isolates exhibited inhibitory activity to varying extents, with strains T1, T4, and T5 showing consistently high levels of efficacy across assays. Culture filtrates demonstrated the strongest inhibitory effect, likely due to the presence of bioactive extracellular compounds involved in cell wall degradation and/or interference with pathogen metabolism. Elicitor-based assays further revealed dose-dependent responses in several isolates, supporting the presence of inducible defense mechanisms. VOC-mediated inhibition varied significantly among strains, reflecting the chemical diversity of the *Trichoderma* volatilome.

Overall, these findings highlight the multifaceted biocontrol potential of *Trichoderma* and emphasize the importance of selecting isolates with complementary mechanisms of action. The integration of such strains into a functional consortium could enhance robustness and field-level efficacy, particularly within integrated pest management (IPM) frameworks. Future work should focus on in vivo validation of these results and the molecular identification of the bioactive compounds involved to support the development of sustainable and reliable disease control strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants15040627/s1, Table S1: Origin of the seven *Trichoderma* isolates and culture media used for isolation. Table S2: Detailed composition of the media used for fungal isolation and assays (quantities expressed per 1 L of medium).

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