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*Article*

# In Vitro and Field Effectiveness of the Combination of Four *Trichoderma* spp. Against *Sclerotinia sclerotiorum* and Its Impact on Potato Crop Production

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**Abstract**

White mold (*Sclerotinia sclerotiorum*) affects potato production and quality in Sinaloa, Mexico. This study aimed to determine the *in vitro* efficacy of *Trichoderma azevedoi*, *T. afroharzianum*, *T. asperellum* and *T. asperelloides* in inhibiting the mycelial growth and sclerotia production of *S. sclerotiorum*. Field studies were also conducted to evaluate the effectiveness of a *Trichoderma* spp. combination in controlling the disease and reducing sclerotia production, as well as increasing crop yield in semi-commercial plots. In parallel, the alternating use of the antagonist combination with synthetic fungicides was assessed; an additional treatment involved using synthetic fungicides alone. The *in vitro* tests demonstrated the efficacy of all four *Trichoderma* species against the pathogen. The *Trichoderma* combination also significantly controlled white mold under field conditions. The alternating application of *Trichoderma* species with synthetic fungicides was similarly effective, while the fungicides-alone treatment was less effective than the two aforementioned treatments. The results highlight the potential of using a mixture of these four *Trichoderma* species to control potato white mold in Sinaloa, which could help reduce the reliance on synthetic fungicides for disease management.

**Keywords:** antagonists; biological control; inhibition; metabolites

## 1. Introduction

The potato (*Solanum tuberosum* L.) is one of the world's most in-demand foods, with a global production of 476 million tons in 2023 [1]. In Mexico, 65,275 hectares were planted, yielding 2,123,718 tons with a production value of US$ 1,102,784,121 [2].

Fungal diseases are a primary limiting factor for potato production and quality. Among the most significant are wilt and tuber dry rot (*Fusarium* spp.), potato black dot (*Colletotrichum coccodes*), verticillium wilt (*Verticillium dahliae*), black scurf (*Rhizoctonia solani*), soft rot of tubers (*Sclerotium rolfsii*) and white mold (*Sclerotinia sclerotiorum*) [3–8].

White mold management is challenging because the pathogen affects 408 species of Dicotyledonae and has also been recorded in some monocotyledonous species [9–11]. It can behave as an endophyte in rice (*Oryza sativa*), wheat (*Triticum aestivum*), corn (*Zea mays*), barley (*Hordeum vulgare*) and oat (*Avena sativa*) [12]. Economic losses associated with white mold in the United States

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of America reach US$200 million annually [13]. In Sinaloa, Mexico, it has been reported in eggplant and beans with up to 50% damage, while potato losses are estimated at around 30% [8,14,15].

The infectious cycle of *S. sclerotiorum* begins when soil-borne sclerotia germinate to produce hyphae that directly penetrate the stem, affecting leaves and fruits [16]. Alternatively, they can germinate carpogenically, forming apothecia that release ascospores. These spores require prolonged leaf wetness periods, temperatures of 15-20 °C and senescent tissues to establish infection [17]. Once inside the host, the fungus produces cellulase, pectinase and oxalic acid, which promote cell wall degradation, defense suppression and tissue necrosis [18–20]. Subsequently, colonized tissues form a mass of hyphae that gives rise to sclerotia: compact, melanized, black structures capable of remaining viable for up to 10 years, depending on environmental conditions [21,22].

Due to the low resistance levels in host crops, various chemical compounds are used for controlling white mold. In the United States, Canada, Australia, China and European countries, fungicides such as boscalid, fluazinam, fluxapyroxad, pyraclostrobin, penthiopyrad, picoxystrobin, prothioconazole, trifloxystrobin, tetraconazole and thiophanate-methyl are applied [23–25]. In soybeans, procymidone and fluazinam showed high efficacy when applied at the beginning of flowering [26]. In Sinaloa, the most used fungicides on potatoes are carbendazim, benomyl, thiophanate-methyl and fluazinam, achieving 85–90% control with preventive applications [15]. Similarly, boscalid+pyraclostrobin, carbendazim, fluazinam, fludioxonil+cyprodinil and prochloraz have shown *in vitro* inhibition of *S. sclerotiorum*. However, under field conditions, solid evidence exists only for carbendazim and fluazinam [15]. This strategy has been ineffective as the fungus has developed resistance to synthetic fungicides, leading to increased application rates and environmental contamination [27].

Biological disease control has gained relevance due to favorable results in recent years. Various microorganisms have been used to manage white mold with promising results. In Brazil, *Bacillus amyloliquefaciens*, *B. pumilus* and *Candida labiduridarum* controlled garlic white mold [28]. In Egypt, *Streptomyces griseus*, *S. rochei* and *S. sampsonii* demonstrated control capacity over the pathogen infecting beans [29]. In India, *Pseudomonas viciae*, *P. mediterranea* and *P. asplenii* reduced the disease on lettuce [30]. In the United States, *Coniothyrium minitans* and *Bacillus amyloliquefaciens* were effective in soybeans [31]. Also in India, *T. afroharzianum* and *T. lixii* were successfully used for the biological control of *S. sclerotiorum* in mustard [32].

*Trichoderma* species inhibit the mycelial growth of *S. sclerotiorum* through competition, antibiosis and mycoparasitism [33]. Furthermore, these fungi parasitize the sclerotia and apothecia of the pathogen [34], reducing the primary inoculum and consequently decreasing damage in subsequent growing seasons [35].

In Mexico, there are no *in vitro* or field studies focused on managing potato white mold using antagonistic fungi. Therefore, the aims of this work were to: a) evaluate the effectiveness of *T. azevedoi*, *T. afroharzianum*, *T. asperellum* and *T. asperelloides in vitro* and under field conditions against *S. sclerotiorum*; b) determine the effectiveness of these antagonists in alternating applications with synthetic fungicides for disease control also under field conditions; c) compare the efficacy of these treatments with the use of fungicides alone, and d) determine the tuber yield in commercial potato fields receiving these treatments.

## 2. Results

### *2.1. Molecular Identification of Sclerotinia sclerotiorum*

The maximum-likelihood tree generated with the ITS region sequences showed that the isolate SS1 (GenBank accession PX471991.1) from Sinaloa, Mexico, clusters with the *S. sclerotiorum* with reference sequence (KX671960.1) and high bootstrap support (74%), confirming its identity as *S. sclerotiorum* (Figure 1).

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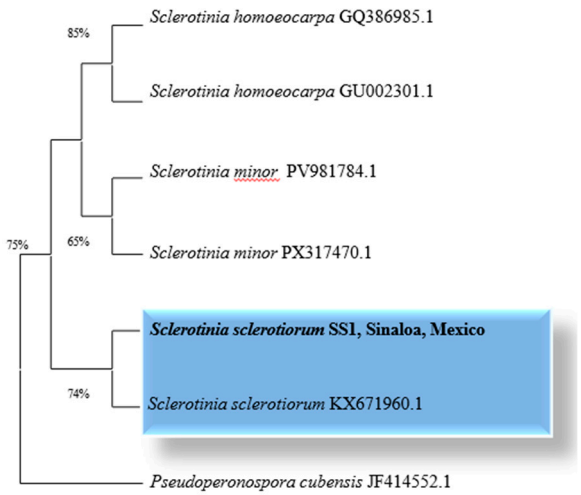


**Figure 1.** Phylogenetic analysis of the *Sclerotinia sclerotiorum* isolate from Sinaloa, Mexico. The maximum likelihood tree was constructed based on the internal transcribed spacer (ITS) region. The sequence of *Pseudoperonospora cubensis* (JF414552.1) was used as an outgroup. The isolate is shown in bold. Bootstrap values greater than 60% are shown at the nodes.

### *2.2. In Vitro Inhibition of Sclerotinia sclerotiorum by Four Trichoderma Species*

The *in vitro* antagonism assays revealed significant differences in the inhibition of *S. sclerotiorum* mycelial growth by the four *Trichoderma* species ($F = 19.38$; $P < 0.0001$). In dual cultures, the inhibition percentages ranged from 60.1% to 63.1% (Table 1). *Trichoderma asperellum* showed the highest inhibition at 63.1% (class 3), followed by *T. afroharzianum* (62.8%, class 3), *T. asperelloides* (61.8%, class 3) and *T. azevedoi* (60.1%, class 3) (Table 1).

Sclerotia production by *S. sclerotiorum* in dual cultures varied significantly among treatments ($F = 555.18$; $P < 0.0001$). The lowest numbers of sclerotia per Petri dish were observed in confrontations with *T. asperelloides*, *T. azevedoi* (both 4.0) and *T. afroharzianum* (4.3), with no significant differences among them. In contrast, the interaction with *T. asperellum* resulted in 6.8 sclerotia per dish. All antagonistic treatments differed significantly from the control (without *Trichoderma*), which produced 20.8 sclerotia per dish (Table 1).

The volatile metabolites produced by the four *Trichoderma* species also significantly inhibited the pathogen, reducing mycelial growth by 90.3% to 94.1%, with no significant differences among the species ($F = 3.8$; $P < 0.0001$). Furthermore, exposure to these volatiles completely suppressed sclerotia formation, whereas the unexposed control produced 27.0 sclerotia per Petri dish ($F = 25208.9$; $P < 0.0001$) (Table 1).

**Table 1.** In vitro antagonism of four Trichoderma spp. against Sclerotinia sclerotiorum.

| | Dual confrontation | | | Volatile metabolites | |
|---|---|---|---|---|---|
| ***Trichoderma* isolate** | **% inhibition** | **Scale** | **Number of sclerotia** | **% inhibition** | **Number of sclerotia** |
| *T. asperellum* | 63.1 a * | 3 | 6.8 b | 93.5 a | 0.0 b |
| *T. afroharzianum* | 62.8 ab | 3 | 4.3 c | 94.1 a | 0.0 b |
| *T. asperelloides* | 61.8 b | 3 | 4.0 c | 91.0 a | 0.0 b |
| *T. azevedoi* | 60.1 c | 3 | 4.0 c | 90.3 a | 0.0 b |
| Control | NA | NA | 20.8 a | NA | 27.0 a |
| CV (%) | 1.4 | | 10.9 | 2.7 | 3.9 |

* Means followed by the same letter within a column are not significantly different according to Tukey's test ($P = 0.05$).

### *2.3. Hyphal Interactions Between Four Trichoderma spp. and Sclerotinia sclerotiorum*

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The four *Trichoderma* species exhibited various antagonistic interactions with the hyphae of *S. sclerotiorum*, including adhesion (Figure 2A), coiling (Figure 2B) and granulation (Figure 2C). Specific interactions varied among species. *T. asperellum* induced vacuolization (Figure 2D), penetration (Figure 2E) and subsequent lysis of the pathogen's hyphal cells (Figure 2F). Similarly, *T. afroharzianum* caused vacuolization and penetration. In contrast, *T. asperelloides* only induced vacuolization of the cell content, while *T. azevedoi* demonstrated penetration and lysis (Table 2).

**Table 2.** *In vitro* hyphal interaction of four *Trichoderma* species and *Sclerotinia sclerotiorum.*

| | Types of hyphal interactions[z] | | | | | |
|---|---|---|---|---|---|---|
| ***Trichoderma* species** | **Adhesion** | **Coiling** | **Granulation** | **Vacuolization** | **Penetration** | **Lysis** |
| *T. asperellum* | X | X | X | X | X | X |
| *T. afroharzianum* | X | X | X | X | X | |
| *T. asperelloides* | X | X | X | X | | |
| *T. azevedoi* | X | X | X | | X | X |

[z]The morphological changes and direct physical contact that occurred during hyphal interactions were determined using lactophenol-blue to stain the interaction zone of the fungi.

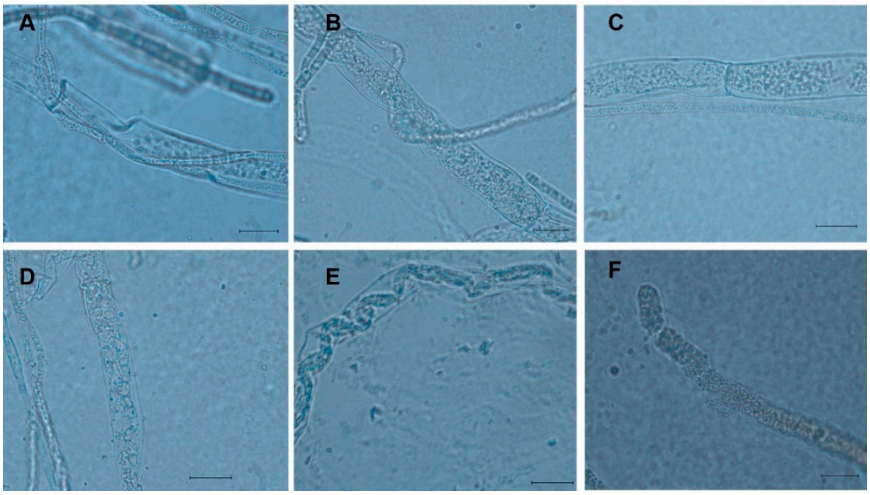


**Figure 2.** Hyphal interactions of four *Trichoderma* spp. with *S. sclerotiorum*. (A) Adhesion *T. asperellum* TAM74, (B) coiling *T. asperelloides* TES24, (C) granulation *T. afroharzianum* TAF75, (D) vacuolation *T. asperellum* TAM74, (E) penetration *T. asperellum* TAM74 (F) lysis *T. asperellum* TAM74. Scale = 10 µm.

### *2.4. Efficacy of Trichoderma spp., Synthetic Fungicides and Their Alternate Application for Controlling Potato White Mold Under Field Conditions*

In the 2021 trial, the lowest disease incidence in the 5-hectare semi-commercial plots was observed in treatments with the *Trichoderma* spp. combination or its alternate application with synthetic fungicides. No significant differences were found between these two treatments, but both differed significantly from the plots treated with synthetic fungicides alone ($F = 13.64$, $P < 0.0001$; Table 3). Disease severity in plants ranged from 27.1% to 61.7%, with significant differences among treatments ($F = 2.75$, $P < 0.0001$). The combination of the four *Trichoderma* species provided the best disease control (Table 3).

In 2022, disease incidence varied from 55.6% to 75.6%, showing significant differences among treatments ($F = 5.13$, $P = 0.0140$). The lowest incidence was recorded in plots treated with the *Trichoderma* combination or the alternate application with fungicides, with no significant differences between them ($F = 5.07$, $P = 0.0146$), but both were significantly lower than the fungicide-alone treatment (Table 3). Disease severity ranged from 18.8% to 33.8%, differing significantly among treatments ($F = 2.75$, $P < 0.0001$), with the *Trichoderma* combination again providing the best control (Table 3).

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**Table 3.** Effectiveness of four *Trichoderma* species, synthetic fungicides and their alternated use on the incidence and severity of white mold in potato plants under field conditions.

| Treatment | Experiment 2021 | | Experiment 2022 | |
|---|---|---|---|---|
| | Incidence (%) | Severity (%)[x] | Incidence (%) | Severity (%) |
| *Trichoderma* spp.[y] | 66.0 b* | 27.1 b | 55.6 b | 18.8 b |
| *Trichoderma* spp. alternating with synthetic fungicides[z] | 81.1 ab | 35.6 ab | 62.2 b | 27.0 b |
| Synthetic fungicides | 93.3 a | 61.7 a | 75.6 a | 33.8 a |
| CV (%) | 15.7 | 16.1 | 15.6 | 21.8 |

[x] The severity of the disease was determined using the Townsend and Heuberger (36) formula. [y]Trichoderma spp.: T. azevedoi, T. afroharzianum, T. asperellum and T. asperelloides. [z] Synthetic fungicides: Mancozeb 5 L ha $^{-1}$ , fluazinam 0.5 L ha $^{-1}$ and tolclofos methyl 3 L ha $^{-1}$. *Means with a common letter across columns are not significantly different (P=0.05) Tukey.

### *2.5. Effectiveness of Four Trichoderma Species, Synthetic Fungicides and Their Alternate Use on Sclerotia Production by Sclerotinia sclerotiorum in Potato Plants Under Field Conditions*

In 2021, the production of sclerotia by *S. sclerotiorum* per treatments ranged from 32.7 to 167.7. The lowest number of sclerotia was recorded in plants treated with the *Trichoderma* spp. combination, followed by the alternate application of *Trichoderma* spp. with synthetic fungicides treatment. No significant differences were observed between these two treatments ($F = 18.6$, $P < 0.0001$), but both resulted in significantly fewer sclerotia than the treatment with synthetic fungicides alone (Table 4).

In 2022, the lowest sclerotia production was also observed in plants treated with the *Trichoderma* mixture or the alternate application with fungicides. No significant differences were found between these treatments ($F = 13.91$, $P < 0.0001$), but both were significantly more effective than the fungicides-alone treatment, which yielded a significantly higher average of 93.8 sclerotia per treatments (Table 4).

**Table 4.** Effectiveness of four *Trichoderma* species, synthetic fungicides and their alternate application on sclerotia production by *Sclerotinia sclerotiorum* in potato under field conditions.

| Treatment | Sclerotia on 10 plants | |
|---|---|---|
| | Experiment 2021 | Experiment 2022 |
| *Trichoderma* spp.[y] | 32.7 b* | 14.6 b |
| *Trichoderma* spp. alternating with synthetic fungicides [z] | 40.8 b | 31.2 b |
| Synthetic fungicides | 167.7 a | 93.8 a |
| CV (%) | 36.2 | 43.1 |

[y]Trichoderma spp.: T. azevedoi, T. afroharzianum, T. asperellum and T. asperelloides. [z]Synthetic fungicides: Mancozeb (5 L ha$^{-1}$), fluazinam (0.5 L ha$^{-1}$) and tolclofos-methyl (3 L ha$^{-1}$). *Means with a common letter within a column are not significantly different according to Tukey's test (P = 0.05).

### *2.6. Effectiveness of Four Trichoderma spp., Synthetic Fungicides and Their Alternate Use in Controlling White Mold on Potato Tubers Under Field Conditions*

In 2021, the lowest disease incidence on tubers was recorded in plots treated with the *Trichoderma* combination (1.6%) or its alternate application with synthetic fungicides (4.5%). No significant differences were observed between these treatments ($F = 3.95$, $P = 0.0328$), but both showed significantly lower incidence than the fungicides-alone treatment (7.4%) (Table 5).

Disease severity on tubers followed a similar pattern in 2021. The *Trichoderma* combination (0.4%) and the alternate application (1.0%) showed the lowest severity values, with no significant differences between them ($F = 1.83$, $P = 0.1360$). However, the *Trichoderma* combination provided significantly better control than the fungicides-alone treatment (1.5%) (Table 5).

In the 2022 experiment, tuber disease incidence was lowest in plots treated with the *Trichoderma* combination (1.3%) or the alternate application with fungicides (3.3%), with no significant differences

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between them (F = 6.84, P = 0.0045). Both treatments were significantly more effective than the fungicides-alone application (6.2%) (Table 5). Similarly, disease severity was lowest in the *Trichoderma* combination (0.3%) and alternate application (0.7%) treatments, with no significant differences between them (F = 1.22, P = 0.3479), but both showed significantly better control than the fungicides-alone treatment (1.3%) (Table 5).

**Table 5.** Effectiveness of a combination of four *Trichoderma* species, synthetic fungicides and their alternate application on the incidence and severity of potato tuber white mold under field conditions.

| Treatment | Experiment 2021 | | Experiment 2022 | |
|---|---|---|---|---|
| | Incidence (%) | Severity (%) [x] | Incidence (%) | Severity (%) [x] |
| *Trichoderma* spp.[y] | 1.6 b* | 0.4 b | 1.3 b | 0.3 b |
| *Trichoderma* spp . alternating with synthetic fungicides [z] | 4.5 ab | 1.0 ab | 3.3 ab | 0.7 ab |
| Synthetic fungicides | 7.4 a | 1.5 a | 6.2 a | 1.3 a |
| CV (%) | 62.2 | 43.1 | 57.0 | 50.9 |

[x] Disease severity was determined using the formula by Townsend and Heuberger [36]. [y]Trichoderma spp.: T. azevedoi, T. afroharzianum, T. asperellum and T. asperelloides. [z] Synthetic fungicides: Mancozeb (5 L ha$^{-1}$), fluazinam (0.5 L ha$^{-1}$) and tolclofos-methyl (3 L ha$^{-1}$). *Means with a common letter within a column are not significantly different according to Tukey's test (P = 0.05).

### *2.7. Production of Tubers in Plots Sprayed with a Combination of Four Trichoderma spp., Synthetic Fungicides or the Alternate Use of These Treatments*

Potato tuber yield in 2021 ranged from 42.4 to 46.0 t ha$^{-1}$, showing significant differences among treatments (F = 4.06, P = 0.0303). The highest yield was obtained in plots treated with the *Trichoderma* combination, while the lowest yield was recorded in plots treated with synthetic fungicides alone (Table 6).

In 2022, yields were higher, ranging from 44.5 to 52.9 t ha$^{-1}$, with significant differences among treatments (F = 4.09, P = 0.0295). Plots treated with synthetic fungicides alone showed the lowest yield (44.5 t ha$^{-1}$), which was significantly lower than the yield in plots treated with the *Trichoderma* combination (52.9 t ha$^{-1}$) (Table 6).

**Table 6.** Effect of four *Trichoderma* species, synthetic fungicides and their alternate application on potato yield under field conditions.

| Treatment | Experiment 2021 | Experiment 2022 |
|---|---|---|
| | t ha $^{-1}$ | t ha $^{-1}$ |
| *Trichoderma* spp.[y] | 46.0 a* | 52.9 a |
| *Trichoderma* + Synthetic Fungicides | 44.1 ab | 48.3 ab |
| Synthetic fungicides[z] | 42.4 b | 44.5 b |
| CV (%) | 4.06 | 12.87 |

[y] Trichoderma spp.: T. azevedoi, T. afroharzianum, T. asperellum and T. asperelloides. [z] Synthetic fungicides: Mancozeb (5 L ha$^{-1}$), fluazinam (0.5 L ha$^{-1}$) and tolclofos-methyl (3 L ha$^{-1}$). *Means with a common letter within a column are not significantly different according to Tukey's test (P = 0.05).

## 3. Discussion

The molecular identification results from the present study are consistent with those reported by Kurt *et al*. [37] for *S. sclerotiorum* associated with potato white mold in Turkey. Molecular diagnosis is essential for this fungal species, given the limited resolution of morphological identification and its potential for confusion with other saprophytic or pathogenic Ascomycota [38,39]. The confirmation of the pathogen's identity in a potato production system in this study strengthens the validation of using *Trichoderma* spp. for controlling potato white mold.

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*Trichoderma* species differentially inhibited the mycelial growth of *S. sclerotiorum in vitro*. These results align with findings from India [32,40], Brazil [34], China [41], Mexico [42,43] and Serbia [44], where species including *T. afroharzianum*, *T. asperelloides*, *T. asperellum*, *T. atroviride*, *T. citrinoviride*, *T. ghanense*, *T. harzianum*, *T. inhamatum*, *T. koningiopsis*, *T. lentiforme*, *T. lixii*, *T. longibrachiatum*, *T. pseudokoningii*, *T. virens*, *T. viride* and *T. yunnanese* demonstrated potential to inhibit *S. sclerotiorum*. In our study, *in vitro* sclerotia production by *S. sclerotiorum* also varied with the *Trichoderma* species, consistent with reports from Brazil [34]. Our findings indicate a close relationship between the inhibition of sclerotia production observed *in vitro* and the results obtained under field conditions, which led to lower disease pressure and more sustainable long-term control of white mold.

Volatile metabolites from the *Trichoderma* species significantly inhibited *S. sclerotiorum* mycelial growth, corroborating studies from India [32], Brazil [33,34] and Mexico [43]. Furthermore, volatile compounds from *Trichoderma* spp. completely suppressed sclerotia production, consistent with Sridharan *et al*. [45], who reported that volatiles from *T. longibrachiatum* reduced sclerotia formation in *Sclerotium rolfsii*.

The *Trichoderma* species exhibited various hyphal interactions with *S. sclerotiorum*, including coiling, penetration and lysis, supporting findings from India [32], China [41] and Mexico [42]. Future research should identify the specific volatile and non-volatile metabolites involved and their modes of action to optimize white mold management in Sinaloa, Mexico, for potatoes and other regional crops.

The combination of *T. azevedoi*, *T. afroharzianum*, *T. asperellum* and *T. asperelloides* was the most effective treatment for managing white mold in potato plants and tubers, outperforming synthetic fungicides. These results agree with Iqbal-Faruk [46], who found combined applications of *Trichoderma*, *Bacillus* and amendments effective in mustard, bean and pea. Similarly, Zeng *et al*. [47] reported reduced disease severity in soybeans with *T. harzianum* and Geraldine *et al*. [48] observed reduced apothecia production and disease severity in beans with *T. asperellum*.

The *Trichoderma* combination also significantly reduced sclerotia production in potato plants compared to synthetic fungicides (mancozeb, fluazinam, tolclofos-methyl), aligning with Zeng *et al*. [49] in soybean. This reduction is crucial as *Trichoderma* spp. act as mycoparasites on sclerotia, degrading cell walls [50], thereby reducing primary inoculum and potentially limiting apothecia and ascospore production in subsequent growing seasons.

Plots treated with the *Trichoderma* combination or its alternation with fungicides yielded the highest production, consistent with Rubayet and Bhuiyan [51] in Bangladesh and García-Crespo *et al*. [52], in Venezuela who reported an 83% yield increase with *T. asperellum*. Conversely, some fungicides did not increase yield [53]. The yield improvement is likely due to effective disease control and extended crop longevity, mediated by competition, mycoparasitism, antibiosis, induced systemic resistance [54,55] and production of phytohormones and compounds solubilizing phosphate, $Fe_2O_3$, $MnO_2$ and Zn [56].

These results underscore *Trichoderma*'s role not only in disease suppression but also in enhancing potato yield, surpassing synthetic fungicides. The efficacy demonstrated in semi-commercial plots supports scaling the application of this *Trichoderma* combination to commercial levels in northern Sinaloa, Mexico reducing reliance on synthetic fungicides. Future research should evaluate these antagonists against other potato phytopathogens to develop sustainable, cost-effective strategies that minimize environmental and health risks.

## 4. Materials and Methods

### *4.1. Obtaining Trichoderma Isolates and Molecular Identification of Sclerotinia sclerotiorum*

Isolates of *T. asperellum* (TAM74), *T. asperelloides* (TES24), *T. afroharzianum* (TAF75) and *T. azevedoi* (TAI73), with GenBank accession numbers OR521164, OR521181, OR521183 and OR521182, respectively, were obtained from the microbiological collection of the Local Plant Health Board of the Fuerte Valley, Los Mochis, Sinaloa, Mexico. These isolates, previously characterized by Irazoqui-

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Acosta *et al*. [57], were collected in Caborca, Sonora and Ahome, Sinaloa. The *S. sclerotiorum* isolate was obtained from symptomatic potato plants in northern Sinaloa. For molecular identification, one 3 mm disk of the isolate was placed in 25 mL of nutrient broth (Becton Dickinson of Mexico). The culture was incubated at 27 ± 2 °C for five days with continuous agitation at 150 rpm (Labnet International, Inc.®, USA); afterwards, the mycelium was placed in 2 mL Eppendorf microtubes. Genomic DNA extraction was performed using the 2% CTAB protocol according to Sanger *et al*. [58], the final DNA concentration was adjusted to 50 ng/µL. The purity and concentration of DNA were determined using a NanoDrop spectrophotometer (Thermo Scientific®, USA).

The identification of *Sclerotinia sclerotiorum* isolate was carried out by polymerase chain reaction (PCR), targeting the amplification of an approximately 650 bp fragment corresponding to the Internal Transcribed Spacer (ITS) using the primers ITS1 (5′-TCC GTA GGT GAA CCT TGC GG-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), described by White *et al*. [59].

The PCR reaction contained 50 ng of DNA, 1x of reaction buffer, 1.5 mM of $MgCl_2$, 0.5 µM of each oligonucleotide, 0.2 µM dNTPs, and 0.5 U of Taq polymerase (Invitrogen® CA, EUA) in a total volume of 25 µL. The PCR amplification was performed as follows: one cycle of 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s, and a final extension at 72 °C for 5 minutes. Amplifications were performed in a thermal cycler (BIORAD®; C1000 Thermal Cycler CFX96, Singapore). The amplified product was sent to Macrogen® (Seoul, South Korea) for sequencing.

The sequence was edited in the program BioEdit version 7.2.5 [60] and compared using the software BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The sequence was aligned with reference sequences belonging to type strains of the different species within the *Sclerotinia* complex: *S. sclerotiorum*, *S. minor*, and *S. homoeocarpa*, using the MUSCLE software [61] implemented in MEGA X version 10.2.4 [62]. The sequence of *P. cubensis* (JF414552.1) was used as an outgroup in the phylogenetic analysis. Phylogenetic inference was performed using the Maximum Likelihood (ML) method in the program, applying the Tamura 3-parameter model [63]. In the ML analysis, 1,000 bootstrap replications were used. The phylograms were edited in FigTree v1.4.0 [64].

**Table 7.** GenBank accession numbers of *Sclerotinia sclerotiorum* and *Trichoderma* spp. isolates used in this study.

| Species/ isolate | Locality / georeference | Year of collection | Code in Gen Bank |
|---|---|---|---|
| *S. sclerotiorum* / SS1 | Ahome, Sinaloa/ 25.819501 -108.955445 | 2021 | PX471991.1 |
| *T. asperelloides* / TES24 | Caborca, Sonora/31.06666 -112.338333 | 2020 | OR521164 |
| *T. azevedoi* / TAI73 | Ahome, Sinaloa/25.818885 -108.956014 | 2021 | OR521181 |
| *T. afroharzianum* / TAF75 | Ahome, Sinaloa/25.491445 -108.571659 | 2021 | OR521183 |
| *T. asperellum* / TAM74 | Ahome, Sinaloa/ 25.491445 -108.571659 | 2021 | OR521182 |

### *4.2. In Vitro Antagonism of Trichoderma spp. Against Sclerotinia sclerotiorum and Sclerotia Formation*

The *in vitro* antagonistic effect of *T. asperellum*, *T. asperelloides*, *T. afroharzianum* and *T. azevedoi* against *S. sclerotiorum* was evaluated using the dual culture technique on Potato Dextrose Agar (PDA; BD, Becton Dickinson of Mexico). Mycelial discs (5 mm) from 3-day-old *Trichoderma* and 7-day-old *S. sclerotiorum* cultures were placed at opposite ends of 90 mm Petri dishes. The treatments were arranged in a completely randomized design with four replicates per combination and was conducted twice. Controls consisted of individual cultures of each fungus under identical conditions. Plates were sealed with Parafilm and incubated at 25 ± 1 °C in darkness. Mycelial growth was measured every 24 hours until the control plates were fully colonized. The inhibition percentage was calculated as $I = [(C - T)/C] \times 100$, where C is the radial growth of *S. sclerotiorum* alone and T is its growth in confrontation with *Trichoderma* spp. [65].

Antagonistic efficacy was classified using Bell *et al*. [66] scale: Class 1 = *Trichoderma* covered the entire medium; Class 2 = *Trichoderma* overgrew at least two-thirds of the medium surface; Class 3 = both organisms colonized approximately one-half of the medium surface of the medium (more than one-third and less than two-thirds); Class 4 = *S. sclerotiorum* colonized at least two-thirds of the

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medium surface and appeared to withstand encroachment by *Trichoderma*; Class 5 = *S. sclerotiorum* covered the entire medium. Sclerotia production was quantified 15 days post-inoculation. The experiment was repeated once.

### *4.3. Effect of Volatile Metabolites from Trichoderma spp. on Mycelial Growth and Sclerotia Formation*

The effect of volatile compounds was evaluated using the double plate method [67]. *Trichoderma* spp. and *S. sclerotiorum* were initially cultured on PDA for 3 days at 25 ± 2 °C. A 5-mm mycelial disc of *Trichoderma* spp. was placed in the center of a PDA plate and after 24 hours, a disc of *S. sclerotiorum* was placed in another plate. The bases of both plates were coupled and sealed with Parafilm to create a shared gaseous environment without physical contact, with *Trichoderma* in the lower base and the pathogen in the upper one. The design was completely randomized with four replicates and two independent trials were conducted. Controls included individual cultures of each organism. Antifungal activity was assessed when *S. sclerotiorum* fully colonized the control plates. Growth inhibition was calculated as described previously and sclerotia formation was evaluated 15 days after exposure.

### *4.4. Field Experiments*

Experiments were conducted in a 15-ha commercial field with clay-loam soil (pH 7.2) naturally infested with *S. sclerotiorum* (0.2 sclerotia/kg soil, based on 27 samples). Trials were established on December 5, 2021 and November 18, 2022, using sprouted tubers of variety FL2027 (PepsiCo México City, Mexico). Crop management followed Santos *et al*. [68]. Three treatments were applied to 5-ha plots: a) biological: combination of *T. azevedoi*, *T. afroharzianum*, *T. asperellum* and *T. asperelloides* applied at planting (5.0 L $ha^{-1}$, 1.6 × $10^7$ CFU $mL^{-1}$) followed by five foliar applications (2.5 L $ha^{-1}$) at 16-day intervals during the growing season . b) chemical: Fluazinam (0.5 L $ha^{-1}$, 250 g a.i. $ha^{-1}$) applied twice at flowering onset and tolclofos-methyl (3 L $ha^{-1}$, 2,250 g a.i. $ha^{-1}$) applied during tuberization and 15 days later and c) alternation: alternating applications of *Trichoderma* spp. and synthetic fungicides at the described intervals and doses. Each treatment was established on an area of 5.0 $ha^{-1}$ and carried out in 2021 and 2022.

### *4.5. Disease Incidence, Severity and Sclerotia Production in Plants*

Disease incidence was assessed at nine sampling points per treatment (10 plants each). Severity was rated on a 0–5 scale (0 = no symptoms; 1 = 1–10%; 2 = 11–20%; 3 = 21–40%; 4 = 41–59%; 5 = 60–100% affected tissue) and calculated using the Townsend and Heuberger [37] formula: Severity = $(\sum(ni \times vi)/(N \times V)) \times 100$, where ni = category value, vi = number of plants in the category, V = highest category value [5] and N = total plants. Sclerotia were counted on 10 plants per sampling point.

### *4.6. Tuber Disease Evaluation and Yield*

Tuber incidence and severity were assessed from nine sampling points per treatment (two central rows, 18 $m^2$ per point). Severity was rated on a 0–5 scale based on affected surface area and calculated using the Townsend and Heuberger [36] formula. Yield was determined by weighing harvested tubers from each sampling point.

### *4.7. Statistical Analysis*

Data on *in vitro* antagonism (dual confrontation, volatile metabolites and production of sclerotia), as well as the incidence and severity of the disease, sclerotia formation in plants and the incidence, severity and yield in tubers under field conditions, were subjected to analysis of variance (ANOVA) using the SAS 9.0 statistical package (SAS Institute Inc., Cary, NC, USA). Means were separated using the Tukey test ($P \leq 0.05$), according to the procedures previously described by Little and Hills [69].

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## 5. Conclusions

The combination of *T. afroharzianum*, *T. asperellum*, *T. asperelloides* and *T. azevedoi* demonstrated high efficacy in inhibiting *S. sclerotiorum* under both *in vitro* and field conditions. This treatment significantly reduced the incidence and severity of white mold, decreased sclerotia production in potato plants and also reduced disease incidence and severity in tubers, while increasing overall crop yield. The alternation of these antagonists with synthetic fungicides provided improved disease control compared to the exclusive use of fungicides, suggesting that integrating these biological controls into management programs may decrease chemical dependence and promote more sustainable production practices. These results validate the potential of combined *Trichoderma* species applications as an effective and environmentally safe biotechnological strategy for managing white mold in potato cultivation in Sinaloa, Mexico.

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