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In Vitro Compatibility of Three Native Isolates of *Trichoderma* with the Insecticide Chlorpyrifos

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Abstract: The compatibility between biocontrol agents and pesticides seems to be a sustainable control strategy in agriculture. Therefore, the in vitro compatibility of three native isolates of *Trichoderma* was evaluated in three concentrations of chlorpyrifos (960, 1200, and 1440 mg/L), by determining the effect on spore germination, mycelial growth, and the antagonistic capacity. The isolates correspond to *Trichoderma asperellum* TCA 3, *Trichoderma asperellum* TCA 21 and *Trichoderma harzianum* TCA 23. Both spore germination and mycelial growth were performed using the poisoned medium method, while the antagonistic capacity was evaluated against *Botrytis* sp. in a dual culture. The results showed that TCA 21 strain had a higher germination percentage (79.46, 59.79, and 37.43%) than the TCA 3 and TCA 23 strains, in the three concentrations of chlorpyrifos. Regarding the mycelial growth of the three native strains in chlorpyrifos are affected when concentration of chlorpyrifos increase ($p < 0.05$). Finally, the antagonistic capacity of the three strains was not affected by any concentration of chlorpyrifos, where strains TCA 21 and TCA 23 presented a degree of antagonism of one, while TCA 3 presented a degree of two, according to the scale used by Bell. In conclusion, *T. asperellum* TCA 21 was the one that presented the best in vitro compatibility with chlorpyrifos at concentrations of 960 and 1200 mg/L, compared to *T. asperellum* TCA 3 and TCA 23. These results are favorable for field application since these native strains can also have the ability to degrade the insecticide, representing a sustainable and eco-friendly alternative to the environment.

Keywords: *Trichoderma asperellum*; *Trichoderma harzianum*; biocontrol agent; pesticides; *Botrytis* sp.

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1. Introduction

Agriculture is the backbone of a nation [1]. However, the quality and productivity of cultivars are affected by different microorganisms known as phytopathogens, which lead to economic losses [2–4]. For this reason, pesticides have been used against these plagues, where the unbalanced application of pesticides leads to unwanted effects, for example, pesticide resistance in plant pathogens, environmental persistence, and the contamination of soils and groundwater [5,6]. In addition, in recent years, it has generated various controversies due to its effects on public health, which have been well documented [7,8].

Organophosphates are part of modern agriculture; however, their indiscriminate use has caused serious effects on the environment and public health [9]. Chlorpyrifos (C 9H11Cl3NO3PS) or (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate)) is an organophosphate insecticide used against a wide spectrum of pest insects that attack crops, such as citrus fruits, bananas, vegetables, potatoes, coffee, cocoa, tea, cotton, wheat, and rice, among others [10,11]. This insecticide is poorly soluble in water (2 mg/L) but has strong adsorption to organic matter and soil particles. This insecticide can persist in the

soil for 60–120 days or up to a year, depending on climate, dose, and other environmental conditions [11,12]. Chlorpyrifos is known to inhibit acetylcholinesterase [13]. Trasande [14] mentions that there is strong evidence of fetal brain damage and cognitive and behavioral dysfunction through multiple mechanisms, including thyroid disruption. A request in order to seek bean chlorpyrifos was denied by the United States Environmental Protection Agency (EPA) in 2017 [15].

Chlorpyrifos is listed as an unauthorized pesticide in developed countries. In contrast, it is still used in countries such as Peru in spite of its negative effects on people's health [16,17]. For this reason, there is a double need to address this issue; on the one hand, farmers need to be aware of the danger that the use of chlorpyrifos attracts. On the other hand, there is an urgency to decontaminate environments of chlorpyrifos residues [18]. An alternative strategy in the control of pests and plant disease is integrated pest management (IPM) [18]. One of the IPM strategies is biological control (BC). BC involves using microorganisms or biocontrol agents (BA), mainly bacteria and fungi, to combat phytopathogens through a series of strategies. In addition, BC does not require the use of pesticides, which makes it a green technology with low production costs [19–25].

One of the most widely applied BA is the filamentous fungi of the genus *Trichoderma* (Ascomycota: Hypocreaceae), which are cosmopolitan and very versatile. They represent between 50 and 60% of all BA. In the global biopesticides market, a variety of commercial products based on *Trichoderma* can be found due to their ability to produce enzymes and secondary metabolites. This fungus can behave as an antagonist against a wide range of fungal phytopathogens, such as pathogenic bacteria *Ralstonia solanacearum*, Lepidoptera insect pests, and nematodes of the genus *Meloidogyne*. On the other hand, they can be potential biofertilizers found in symbiotic associations of the plant, giving them certain nutritional advantages. Based on all these characteristics, *Trichoderma* constitutes a promising alternative for use in sustainable agriculture [25–32]. Despite the remarkable characteristics of BA, modern agriculture still needs to depend on pesticides; therefore, the compatibility of use between these two control alternatives is being investigated as part of an IPM strategy [33]. Kumar et al. [34] mentioned that both pesticides and biopesticides are necessary for the current scenario of agriculture.

The metabolic diversity of the *Trichoderma* species allows them to use diverse xenobiotic compounds as carbon and energy sources [35]. The compatibility between fungus and pesticides is known in other BA, for example, *Beauveria bassiana* and *Metarrhizium anisopliae* [36,37]. Barberis et al. [38] showed that non-toxigenic *Aspergillus* section Flavi strains could grow in soils contaminated with chlorpyrifos. Widmer showed that *T. asperellum* is compatible with some fungicides [33]. Likewise, Silva et al. [39] indicated that in the biological control of lettuce, *T. asperellum* IBLF 914 could be used together with phytosanitary treatments. Ramanagouda and Naik [40] showed that Chlorpyrifos is compatible with indigenous *Trichoderma* isolates. On the other hand, Ríos et al. [41] demonstrated that *Trichoderma* is capable of using the pesticides chlorpyrifos and cypermethrin as the only carbon source, which demonstrates the versatility of these filamentous fungi when found in polluted environments.

Finally, it is important to investigate the compatibility between BA and pesticides because, in this way, they can be integrated as sustainable strategies for the pest control of economically important crops. Based on this, the objective of the present investigation was to evaluate the in vitro compatibility of three native isolates of *Trichoderma* with the insecticide chlorpyrifos, for which it was necessary to determine the effect of chlorpyrifos on spore germination, the mycelial growth, and in the antagonistic capacity of the three native *Trichoderma* strains, isolated from agricultural soils.

2. Materials and Methods

2.1. Culture of Native Trichoderma Isolates

Pure cultures of *T. asperellum* TCA 3, *T. asperellum* TCA 21, and *T. harzianum* TCA 23 (pigments the medium yellow) were used, which were reactivated in inclined Potato Sucrose

Agar (APS) medium. These three strains of *Trichoderma* were provided by the Phytopathology Laboratory of the Academic Department of Microbiology and Parasitology of the Faculty of Biological Sciences (National University of Trujillo). These strains were isolated from crop fields and identified using molecular biology techniques by Villacorta et al. [31].

2.2. Insecticide

The commercial name of the insecticide was DORSAN® 48 EC (Nº 624-98-AG-SENASA), which is a non-systemic organophosphate, and its active ingredient is Chlorpyrifos, which is classified as moderately dangerous. This insecticide is recommended for the control of various species of Diptera, Hemiptera, and Lepidoptera. The action mechanism of the active ingredient is acetylcholinesterase inhibition [42]. Three high concentrations (960, 1200, and 1440 mg/L) compared to field doses (480 g/L) were used to evaluate the in vitro compatibility of each *Trichoderma* strain.

2.3. Effect of the Insecticide Chlorpyrifos on the Germination of Trichoderma

Regarding the insecticide, concentrated double solutions were prepared in Potato Sucrose broth until they reached 1920, 2400, and 2880 mg/L from the original product (480 g/L). On the other hand, an inoculum of 10^5 spores/mL for each strain (TCA 3, TCA 21, and TCA 23) was obtained.

Subsequently, three treatments were prepared for each strain of *Trichoderma* and were evaluated, which was carried out in test tubes, where the inoculum of spores and double concentrated chlorpyrifos were added in a 1:1 ratio, resulting in final concentrations of 960, 1200, and 1440 mg/L. The control tube consisted of inoculum in CPS without chlorpyrifos (1:1). The procedure was performed in triplicate for each strain. Then, the treatments and the control were incubated at 25 °C ± 2 °C for 24 h. Finally, the reading was expressed as an average percentage of the germinated spores (%G) compared to the spore count of the control (100%).

$$\%G = G1/G2 \times 100 \quad (1)$$

where:

G1: average germination of the strain of *Trichoderma*.
G2: average germination of the control.

2.4. Effect of the Insecticide Chlorpyrifos on the Growth of Trichoderma

To evaluate growth, a potato sucrose agar (PSA) medium poisoned with final concentrations of 960, 1200, and 1440 mg/L was used. From the pure cultures of the *Trichoderma* strains, it was seeded by a puncture in the central part of the Petri dish. The control consisted of APS agar without chlorpyrifos. The plates were incubated at 25 ± 2 °C until the fungus grew to occupy the entire surface of the control plate. This procedure was performed in triplicate for each treatment of each strain.

Measurements were made from day 1 of sowing until the day that the fungus covered the entire surface of the control plate. The mycelial growth diameter was measured in millimeters (mm) in different directions.

2.5. Effect of the Insecticide Chlorpyrifos on the Antagonistic Capacity of Trichoderma

The antagonistic capacity of *Trichoderma* was performed using the dual culture technique and the phytopathogenic fungus *Botrytis* sp., which is considered the second most dangerous plant pathogen [43].

First, at one end of each Petri dish with PSA, *Botrytis* sp. was seeded by a puncture before being incubated at 25 ± 2 °C for three days. After this time, it was sown at the opposite end to *Trichoderma*, which had previously been subjected to growth at different concentrations of chlorpyrifos (960, 1200, and 1440 mg/L), and incubated at 25 ± 2 °C. for five more days. Subsequently, readings were made every 24 h, and the degree of

antagonism was evaluated after seven days of incubation according to the Bell's scale [44] (Table 1).

Table 1. Antagonism degree scale according to Bell's scale (1982) [44].

Degree of Antagonism	Criterion
1	*Trichoderma* completely outgrows the pathogen colony and covers the surface of the culture medium.
2	*Trichoderma* grows on at least two-thirds of the surface of the culture medium.
3	*Trichoderma* and the pathogen grow on approximately half the surface of the culture medium (neither seems to dominate the other).
4	The pathogen grows in at least two-thirds of the culture medium, limiting the growth of *Trichoderma*.
5	The pathogen grows on the *Trichoderma* colony, occupying the culture medium's entire surface.

2.6. Analysis of Data

The data obtained from the parameters to evaluate the effect of chlorpyrifos on *Trichoderma* were processed through a one-way analysis of variance (ANOVA) using the IBM SPSS Statistics 22 software. The mean values of spore germination (%) and the final mycelial growth were used in data analysis.

3. Results

3.1. In Vitro Effect of Chlorpyrifos on the Spore Germination of Native Strains of Trichoderma

Figure 1 shows that as the concentration of chlorpyrifos increased, spore germination decreased in the three strains of *Trichoderma* (TCA 3, 21, 23). The spore germination percentages in the TCA 3 strain were 54.60% (960 mg/L), 27.63% (1200 mg/L), and 11.14% (1440 mg/L), while 67.23% (960 mg/L), 29.18% (1200 mg/L), and 11.52% (1440 mg/L) corresponding to TCA 23 strain. The spore germination, both TCA 3 and TCA 23 strains were significantly affected at 960, 1200, and 1440 mg/L chlorpyrifos concentrations ($p < 0.05$) (Figure 1a,c). By contrast, spore germination of the TCA 21 did not present a statistically significant difference between the 960 mg/L concentration (79.46%) and the control group (100%) ($p = 0.161$). Similarly, this occurred at 1200 mg/L (59.79%) and 1400 mg/L (37.49%) as shown ($p = 0.111$) (Figure 1b).

3.2. In Vitro Effect of Chlorpyrifos on the Mycelial Growth of Native Strains of Trichoderma

Concerning Figure 2, the maximum values of the diameter of the fungal colony are shown on the fourth day of incubation after being treated with three concentrations of chlorpyrifos. In the case of *T. asperellum* TCA 3, it reached mean diameters of 21.13 mm (960 mg/L), 17.93 mm (1200 mg/L), and 16.00 mm (1440 mg/L). While for *T. asperellum* TCA 21, the diameters measured were 26.43 mm (960 mg/L), 23.50 mm (1200 mg/L), and 21.87 mm (1400 mg/L). Finally, the values of 30.93 mm (960 mg/L), 26.03 mm (1200 mg/L), and 21.60 mm (1400 mg/L) corresponded to *T. harzianum* TCA 23, with slightly higher values than the other strains (Figure 2a). All the means of mycelial growth of each strain of native *Trichoderma* evaluated were different statistically ($p < 0.05$).

3.3. In Vitro Effect of Chlorpyrifos on the Antagonistic Capacity of the Three Native Strains of Trichoderma

In Figure 3, it is observed that there is a degree of two for the antagonism of *T. asperellum* TCA 3, while in *T. asperellum* (TCA 21) and *T. harzianum* (TCA 23), they present a degree of antagonism of one, according to Bell's scale. In other words, the biocontroller occupies two-thirds of the growth of the pathogenic fungus in the first case, while in the other two cases, *Trichoderma* grows on the entire colony of the pathogen. These results indicate that

after *Trichoderma* strains TCA 3, 21, and 23 grew in the media poisoned with different concentrations of chlorpyrifos, no effect was observed on their antagonistic capacity towards *Botrytis* sp. when compared to the controls.

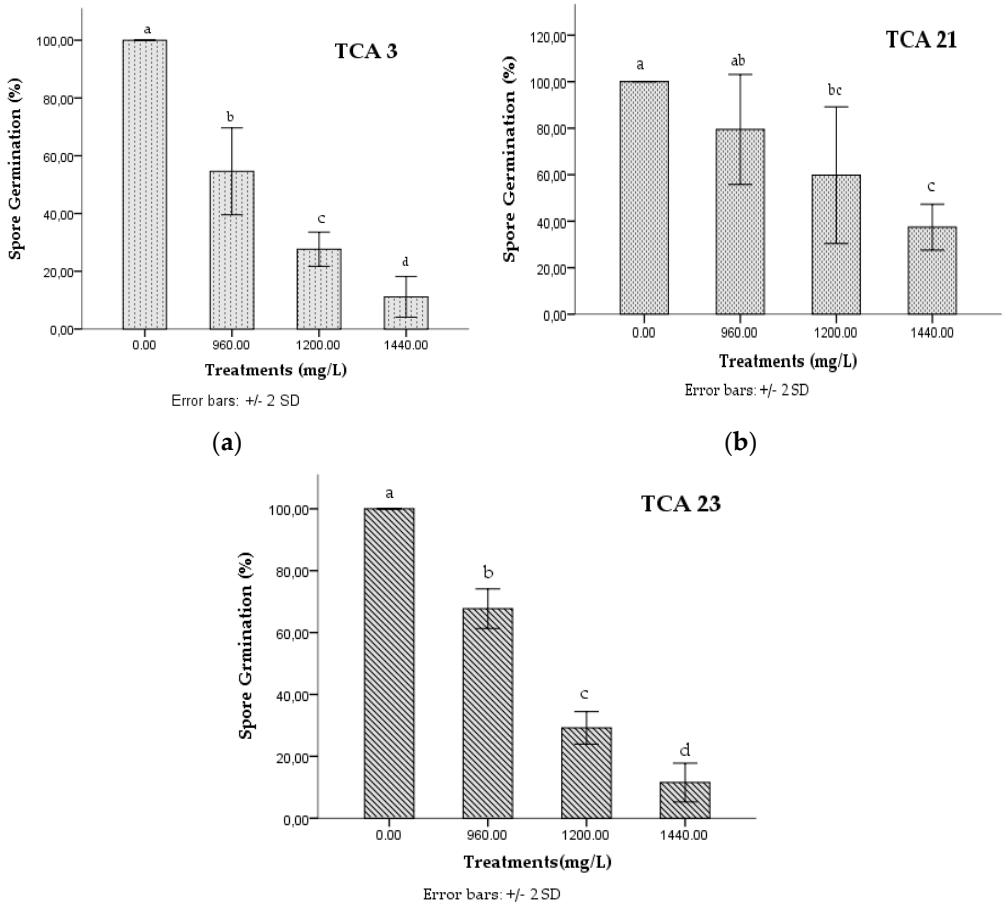


Figure 1. Effect of three concentrations of chlorpyrifos on spore germination of *T. asperellum* TCA 3 (**a**), *T. asperellum* TCA 21 (**b**), and *T. harzianum* TCA 23 (**c**). According to Tukey's test, different letters mean significant differences ($p < 0.05$). Error bars represent the standard deviation (SD) of the mean values ± SD of 2 readings per 3 replicates.

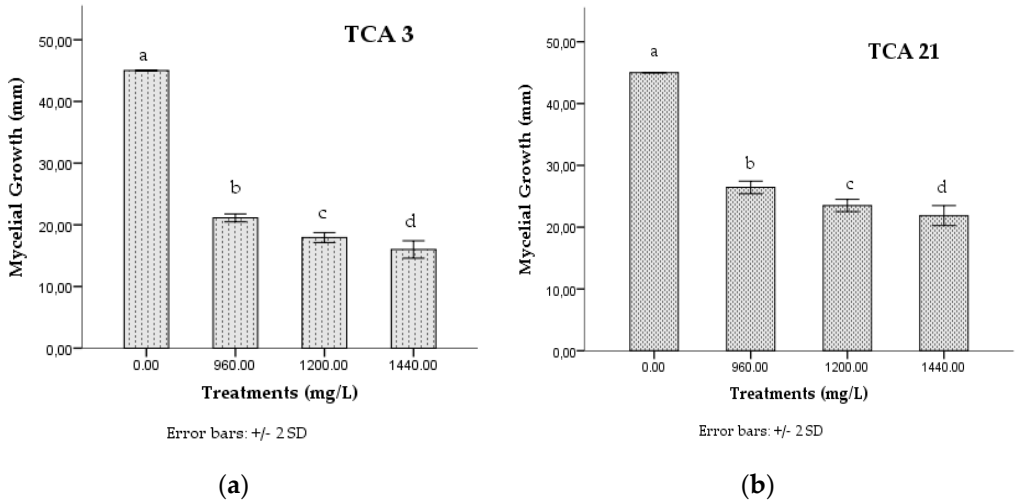
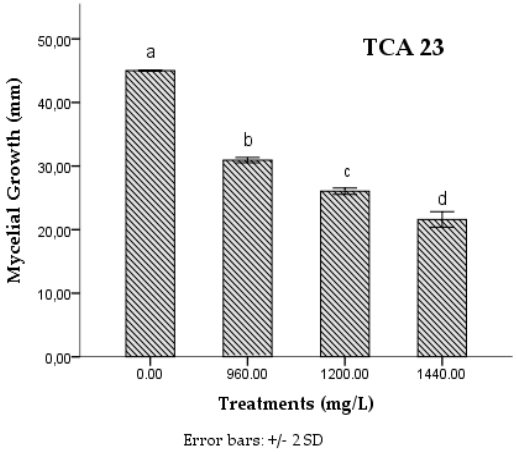


Figure 2. *Cont*.



(**c**)

Figure 2. Effect of three concentrations of chlorpyrifos on the mycelial growth (mm) of *T. asperellum* TCA 3 (**a**), *T. asperellum* TCA 21 (**b**), and *T. harzianum* TCA 23 (**c**). Maximum diameter of the three strains at 96 hours of incubation. The same letters mean that there are no significant differences according to Tukey's test ($p < 0.05$). Error bars represent the standard deviation (SD) of the mean values ± SD of 2 readings per 3 replicates.

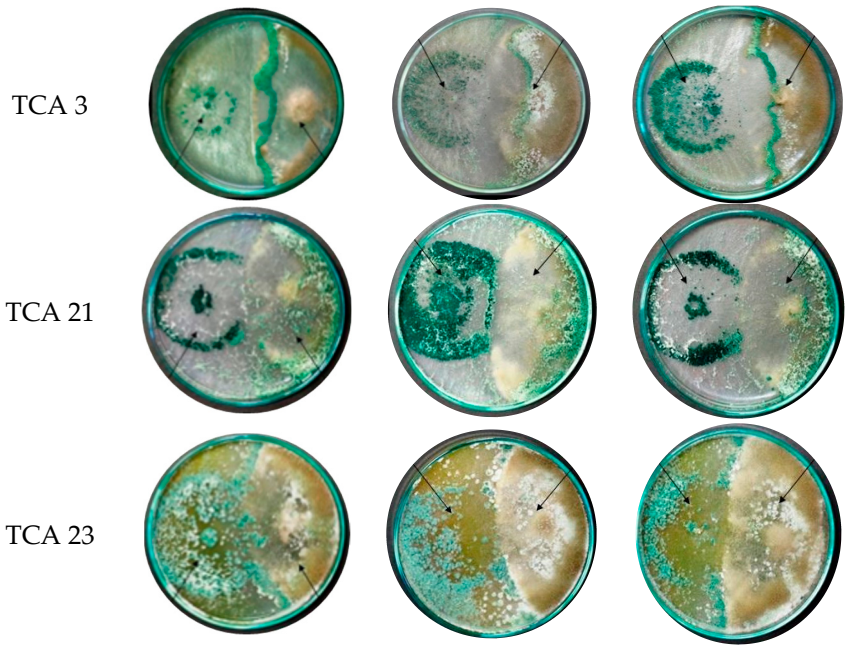


Figure 3. In vitro antagonism of the three native *Trichoderma* strains (TCA 3, 21, 23) against *Botrytis* sp. in dual culture. Each *Trichoderma* previously grew in different concentrations of chlorpyrifos: 960 mg/L (**a**), 1200 mg/L (**b**), and 1440 mg/L (**c**).

4. Discussion

The results of the spore germination of the three native *Trichoderma* strains (Figure 1) can be compared with other studies. Mohammadi [45] reported 41.33 and 14% germination of the *T. harzianum* spores in a medium with chlorpyrifos and at a concentration of 2000 mg/L and 2500 mg/L, respectively, while at 3000 mg/L, germinations were inhibited. In another investigation, the spore germination of eight *Trichoderma* isolates reached 100% after 24–48 h of incubation when treated with chlorpyrifos (500 mg/L) [46]. It is evident that the control group and the potato sucrose broth represent a favorable medium to induce

the germination of the spores of the three strains of *Trichoderma*, which went through a series of stages such as the isotropic phase or swelling, the polarization of the cells, the emergence of the hyphae, and finally its elongation [47]. However, when the medium was mixed with chlorpyrifos, spore germination was affected according to the concentration of the insecticide. This could be related to the dormancy stage of the spores and their concentration [48]. In this sense, a large number of spores within the inoculum could have maintained the latency stage due to the accumulation of germination auto-inhibitors [47]. Additionally, the nutrients of the potato sucrose broth may have stimulated the germination of the spores in the three concentrations. However, high chlorpyrifos concentrations may have limited the germination percentage, which would explain different values in each *Trichoderma* strain evaluated. The participation of other germination induction mechanisms is not ruled out.

Figure 2 shows the effect of chlorpyrifos on the mycelial growth of native strains. The values reached were similar to those reported in other investigations, such as that of Mohammadi [45], where *T. harzianum* reached a maximum diameter of 43 mm when the medium had a concentration of 2000 mg/L of chlorpyrifos. On the other hand, Mayo-Prieto et al. [49] used 48% chlorpyrifos, which affected the in vitro growth of autochthonous *Trichoderma* isolates, which reached colony diameters of less than 30 mm. Likewise, Prathibha et al. [50] showed that a species of *T. harzianum* was affected in its growth when it was treated with doses higher than 5 mg/L of chlorpyrifos. In another study, Bose et al. [51] found that when the concentration of chlorpyrifos exceeded a threshold level, there was a negative effect on microbial populations. To explain the different values of the mycelial growth of the *Trichoderma* strains (Figure 2), it is important to consider the formation of the hypha.

Nutrients are uptaken at the apical part of the hypha and transferred to the interior regions of the fungus for the development and formation of the next generation of spores [52]. In this apical zone of the hypha, the enzymes (exoenzymes) responsible for the degradation of chlorpyrifos can be found. On the other hand, the uptake of nutrients and chlorpyrifos can be explained by mediating cometabolism, which occurs when there is the presence of another carbon source in the medium, such as sugars, etc. [41], as occurs in the media used in this investigation. Likewise, it is known that some microorganisms, including *Trichoderma*, are capable of using organophosphorus compounds metabolically to meet their energy requirements [53]. The radial growth of native *Trichoderma* strains in media poisoned with different concentrations of chlorpyrifos can support the compatibility (Figure 2). However, the radial growth of three *Trichoderma* is low when the chlorpyrifos concentration is 1400 mg/L. Amaresh et al. [54] mentioned that the maximum inhibition of mycelial growth is related to increasing pesticide concentrations. On the other hand, chlorpyrifos compatibility can be explained by two mechanisms of tolerance. The first mentions that pesticides do not affect the viability of *Trichoderma* due to structural changes in the target sites of pesticides. The second hypothesis refers to the degradation of chlorpyrifos [55]. Chlorpyrifos degradation has also been studied in bacteria such as *Bacillus* sp. [53] and *Arthrobacter* sp. HM01 [56], etc. While in the fungi, species of *Trichoderma* spp., *Fusarium* spp., and *Cladosporium cladosporioides* Hu-01 have been reported [57,58].

However, concerning the genus *Trichoderma*, works, such as that of Ríos et al. [41], demonstrated in vitro that *T. asperellum* could use chlorpyrifos at 250 mg/L as the only carbon source when it is placed in a medium minimum of salts without any other carbon source. Likewise, Bisht et al. [59] demonstrated that *T. harzianum* and *T. virens* had a better degrading effect on chlorpyrifos (25, 50, 75, and 100 mg/L) than endosulfan (organochlorine). This degradation could be attributed to the presence of the *opd* gene (organophosphate degradation), which encodes the enzyme phosphotriesterase and can hydrolyze bonds such as P–O, P–F, P–NC, and P–S [51,60,61]. This gene has been identified in bacteria such as *Agrobacterium radiobacter* P230 [62], *Flavobacterium* ATCC 27551, and *Pseudomonas diminuta* [60]. On the other hand, according to the Scopus database search,

only *Trichoderma virens* (GvT6) have been reported as carrying the *opd* gene [63]. Based on this information, three native strains (TCA 3, 21, and 23) could have expressed the *opd* gene to encode the enzyme capable of hydrolyzing the chlorpyrifos in the culture medium, which is evidenced in the growth (Figure 2b). On the other hand, it is known that in biodegradation, one of the primary products is TPC (3,5,6-trichloro-2-pyridinyl), which is resistant to microbial degradation due to its three chlorine groups, causing the degradation of chlorpyrifos, which may explain why on the fourth day of evaluation the radial growth of the three strains stopped compared to the control [33].

The antagonism of *Botrytis* sp. by the three native strains (TCA 3, 21, and 23) of *Trichoderma* is an important characteristic of the biological controller. This antagonistic capacity can be affected by the type of pesticide and its high concentration [64]. On the other hand, the inhibition of *Botrytis* sp. growth is due to the capacity of hyperparasitism, antibiosis, and competition for nutrients and space by *Trichoderma* [29,65]. Vos et al. [65] mentioned that different *Trichoderma* species show the potential to parasitize *Botrytis* sp. both in the soil and the surface of plants. In the same way, cell wall degrading enzymes (β-1,3-glucanases) play a key role in the antagonism of *Botrytis* sp [65]. Likewise, a ceratoplatanin protein (Ep1-1) produced for *T. harzianum* can affect the expression of virulence genes of *Botrytis cinerea* [66]. Many studies have reported the participation of volatile organic compounds in the antibiosis of *B. cinerea*, such as 6-pentyl-α-pyrone (6PP), which has been identified as a metabolite of *T. atroviride* and *T. asperellum* [67–70].

A recent study has shown a new perspective of the antagonism of *Trichoderma afroharzianum* towards *Botrytis* sp. at the transcriptional level, where it has been shown that this species can degrade oxalic acid (the virulence factor of *Botrytis* sp.) at the same time as it is mycoparasitic, meaning that when this species of *Trichoderma* is treated with oxalic acid (20 mmol/L), an increase in *Toxdc* genes (of oxalate decarboxylase) and three genes *Tchit*, *Tpro*, and *Tglu* (translate enzymes responsible for cell wall degradation) can be detected [71–73]. Based on the above, it follows that the three strains evaluated may have used some of these antagonism mechanisms against *Botrytis* sp. In addition, it can be stated that, even though *Trichoderma* spores were exposed to different concentrations of chlorpyrifos when they were moved to another favorable environment, they could germinate and maintain their antagonistic capacities.

These results suggest that three native *Trichoderma* show potential to be applied together with chlorpyrifos, as long as the concentration of the insecticide is equal to or less than the field dose. *T. asperellum* TCA 21 shows greater potential because it tolerates high concentrations of chlorpyrifos. In future investigations, it might be possible to evaluate the compatibility of this native *Trichoderma* with a mixture of other pesticides, as demonstrated in different investigations [72,73].

Despite some native *Trichoderma* that can be used as biocontrol agents, current agriculture still depends on pesticides. For this reason, it is necessary to carry out more investigations in the field of pesticides to improve the results of biological control. This view is supported by Kumar et al. [34], who mentioned that the use of pesticides might be completely replaced if both private companies and research institutes work together.

5. Conclusions

The three species of *Trichoderma* are compatible in vitro with chlorpyrifos in 960, 1200 mg/L, and 1440 mg/L. *T. asperellum* TCA 21 showed better results with respect to germination compared to *T. asperellum* TCA 3 and *T. harzianum* TCA 23. In addition, spore germination and mycelial growth are affected when the chlorpyrifos concentration increases. On the other hand, the antagonistic capacity against the phytopathogen *Botrytis* sp. is apparently unaffected. These findings are important because bring scientific support for the use of native *Trichoderma* strains together with chlorpyrifos in a MIP. In addition, these results represent a sustainable and eco-friendly alternative to the environment. However, more research on this topic needs to understand the compatibility between *Trichoderma* and

pesticides, for example, the influence of environmental factors, gene characterization, and pathway degradation of the chlorpyrifos and biodegradation by-products.

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