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Effect and Compatibility of Phosphite with *Trichoderma* sp. Isolates in the Control of the *Fusarium* Species Complex Causing Pokkah Boeng in Sugarcane

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Abstract: Sugarcane, a highly productive crop, is frequently challenged by different biotic agents, such as the pokkah boeng disease that can cause drastic yield losses of up to 40%. This airborne fungal disease is caused by various *Fusarium* species integrated into a complex. Integrating novel compounds and biological control agents is of paramount importance to cope with these fungi in sustainable systems. In this study, we aimed to evaluate the effect and compatibility of phosphite (Phi) and *Trichoderma* sp. in the control of *Fusarium* sp., in vitro and in planta. Using in vitro tests, we evaluated the effects of Phi (0, 500, 1000, 2000, 4000, and 8000 μg mL^{-1}) and the compatibility of Phi + *Trichoderma* (isolates Taz 001, 013, and 016) on the pathogen complex. Using *in planta* tests, we evaluated the compatibility of Phi + *Trichoderma* (Taz-016) in the control of *Fusarium* in sugarcane plants under greenhouse conditions. A synergistic effect of Phi + *Trichoderma* was observed in vitro on the control of the pathogen, especially when combining 1000 μg mL^{-1} + *Trichoderma* Taz-016. In the *in planta trial*, combining 4000 μg mL^{-1} Phi + *Trichoderma* Taz-016 showed the best control of *Fusarium* infection, improving plant height, culm length and leaf dry weight.

Keywords: *Saccharum* spp.; distorted top; biocontrol; biostimulation; phosphorous acid; integrated disease management

Citation: Solis-Palacios, R.; Hernández-Ramírez, G.; Salinas-Ruiz, J.; Hidalgo-Contreras, J.V.; Gómez-Merino, F.C. Effect and Compatibility of Phosphite with *Trichoderma* sp. Isolates in the Control of the *Fusarium* Species Complex Causing Pokkah Boeng in Sugarcane. *Agronomy* **2021**, *11*, 1099. https://doi.org/10.3390/agronomy11061099

Academic Editor: Essaid Ait Barka

Received: 11 April 2021
Accepted: 20 May 2021
Published: 28 May 2021

1. Introduction

Due to its high photosynthetic capacity and culm sucrose storage ability, sugarcane (*Saccharum* spp.) is one of the most productive crops globally [1]. Its cultivation covers more than 26.2 million hectares in more than 130 countries and territories worldwide [2], producing more than 1907 million tons of milling canes and 174 million tons of sugar for human and industrial consumption [3]. In addition to its contribution to sugar production, this crop is a main source of intermediaries and raw materials for producing alcohol, acetic acid, butanol, paper, industrial enzymes, and bioethanol as a gasoline alternative biofuel [4,5].

The growth and yield of this crop is affected by various abiotic factors, such as low soil fertility, water deficit, metal toxicity, prolonged drought, and salinity, among others [6], as well as factors of a biotic nature, such as diseases caused by viruses, bacteria, and fungi [7].

One of the biotic factors involved in the deterioration of sugarcane roots and culms is various species of phytopathogenic fungi of the *Fusarium* genus, which are common associates of higher plants and are among the most ubiquitous fungi in soil ecosystems [8,9]. In sugarcane, several species of *Fusarium* (i.e., *F. sacchari*, *F. moniliformae*, *F. verticillioides*, and *F. moniliforme* var. *subglutinans*) [10] form a complex that causes pokkah boeng. This

disease induces deformation of the apex in sugarcane plants. This disease can decrease crop yield by 10–40% [10] and sucrose content by 40.8 to 64.5% [11].

Conventional control of this disease is through applying benzimidazole fungicides like methyl N-(1H-benzimidazol-2-yl) carbamate and methyl N-[1-(butylcarbamoyl)benzimidazol-2-yl] carbamate. However, the indiscriminate use of these products has induced microbial resistance mechanisms [12–14], which, together with the consequences of climate change, facilitate the proliferation of these diseases and make their control more difficult. The search for new biorational alternatives is highly prioritized in formulating sustainable management strategies for plant diseases. Alternative disease management includes using biological control through antagonistic microorganisms or disease resistance induced with inorganic salts [15,16].

The *Trichoderma* fungus is commonly used in the biocontrol of soil pathogens. It has been reported as an effective antagonist of phytopathogenic fungi and a stimulator of plants' natural defense systems [17]. Phosphite (Phi) salts, which are derived from phosphorous acid (phosphonate), have been reported as an option to conventional fungicides to control plant pathogens [18,19], such as *Phytophthora infestans*, *Fusarium circitatum*, and *Rhizoctonia* sp., among others [15,20–22].

Trichoderma has different activities during the control of pathogens, including competition for the substrate, mycoparasitism, antibiosis, and deactivation of pathogenic enzymes [23]. Phosphite exerts a direct effect on phytopathogenic fungi, causing decreased conidiogenesis and inhibition of mycelial growth [22,24,25]. Additionally, Phi can act indirectly, stimulating the natural defense mechanisms of plants [16]. Phosphite salts are biocompatible chemical compounds that improve the control of various diseases in plants by stimulating induced resistance (IR) mechanisms. Their effectiveness has been proven in various horticultural crops [15]. However, using Phi may prove less effective for the control of some pathogens than chemically synthesized fungicides [26], so applying it in an integrated management strategy, combined with *Trichoderma*, could increase its effectiveness against plant pathogens. To integrate any beneficial fungus with an inorganic compound, knowledge of the compatibility between the two agents is required [27,28].

Although in vitro compatibility between *Trichoderma* species and fungicides like potassium phosphonate, fosetyl aluminum, and a mixture of cymoxanil and mancozeb has shown good results [29], this relationship has not been determined between Phi and *Trichoderma* in sugarcane. The evaluation of combinations of Phi and native *Trichoderma* isolates provides basic information to help guide field studies for the control of *Fusarium* sp. in sugarcane. The objective of this research was to determine the effect and compatibility of *Trichoderma* sp., with different Phi concentrations in the control of *Fusarium* sp., which causes pokkah boeng in sugarcane, through in vitro and in planta approaches.

2. Materials and Methods

The experiment was carried out in the Laboratory of Applied Microbiology of the National Institute of Technology of Mexico Campus Tierra Blanca (Tecnológico Nacional de México/ ITS Tierra Blanca) located on Av. Veracruz, at the corner with Héroes de Puebla, Pemex Section, Tierra Blanca, Veracruz, Mexico. The experimental site is located between coordinates 18°44′ North Latitude and 96°45′ West Longitude, at 53 masl, with a dry tropical climate, type Aw1 and Aw2 according to the Köppen climate classification.

2.1. Microbiological Material

The *Fusarium* species complex used in this study was isolated from the rhizosphere of a sugarcane plantation cultivated with the variety CP 72-2086, which exhibited pokkah boeng symptoms. The sugarcane plot was located at coordinates 18°35′31″ N and 96°29′20″ W, at approximately 50 masl. The isolate was cultivated on potato dextrose agar (PDA; Bioxon; Cuautitlán Izcalli, State of Mexico, Mexico) using the hyphal tip method until its purification and taxonomic catalog [30]. Once cataloged in the laboratory, the isolate was grown in a Binder incubator (KB 115; Tuttlingen, Germany) at 28 °C for 7 d. Finally,

the isolate was stored in 10% glycerol at 4 °C until in vitro inoculation. For in planta inoculation, spores were collected using a sterile brush and kept in suspension at 4 °C in a Tween-20 solution (Thermo Fisher; Santa Clara, CA, USA) at 0.03%. For the in vitro tests, three isolates of the genus *Trichoderma*, Taz-001, Taz-013, and Taz-016, from the ITSTB strain bank, were selected for evaluation. The *Trichoderma* isolates were obtained from the rhizosphere of sugarcane varieties CP 72-2086 (18°35′30″ N; 96°29′51″ W), Mex 69–290 (18°18′09″ N; 96°25′28″ W), and CP 70-1527 (18°22′22″ N; 96°28′24″ W), respectively. The isolates were grown, cataloged, and preserved until their inoculation as described above. For the in planta tests, only the Taz-016 isolate was evaluated, which was found to be the most effective in the in vitro tests among all strains evaluated.

2.2. Plant Material

For the in planta evaluations, 5 cm long cuttings of sugarcane variety CP 72–2086 were used. This variety was derived from the La Margarita Sugar Mill experimental field located in Vicente, Oaxaca, Mexico (18°35′ N; 96°33′ W, 100 masl). Each cutting consisted of a mature culm node with a healthy, well-developed bud.

2.3. Effect of Phi on In Vitro *Fusarium* sp. Growth

To evaluate the effect of Phi on the development and conidiogenesis of *Fusarium* sp., a biological response window was established with five Phi concentrations: 500, 1000, 2000, 4000, and 8000 μg mL^{-1}, from phosphorous acid (Sigma-Aldrich®; St. Louis, MO, USA), and control without Phi, in PDA culture medium. The pH of the growth medium was adjusted to 5.7 with NaOH 1 N. The *Fusarium* sp. inoculum was adjusted to a concentration of 1×10^6 spores mL^{-1} with 0.03% Tween-20; 5 μL of the fungus suspension was inoculated in the center of the Petri dishes. All treatments were incubated at 28 °C for 7 d. Mycelial growth was measured every 24 h with a digital caliper until the control covered the whole surface of the Petri dish. To calculate the percentage of mycelial growth inhibition (MGI (%)), the formula proposed by Pandey et al. (1982) [31] was used.

$$\text{MGI (\%)} = (Dc - Dt)/Dc \times 100$$

where Dc is the mean diameter of the control fungus colonies, and Dt is the mean diameter of the treatment fungus colonies.

To evaluate conidiogenesis after the incubation period, spores were collected using a sterile brush and kept in suspension 0.03% Tween-20. The concentration of each treatment was recorded with the aid of a Neubauer camera (Laboroptik; Bad-Homburg, Germany) in a 131-CLED compound microscope (National; Beijing, China) at 40×.

2.4. Compatibility of Trichoderma Isolates with Phi

To evaluate the compatibility of *Trichoderma* sp. with Phi, suspensions of isolates Taz-001, Taz-013 and Taz-016 isolates were prepared and inoculated on PDA medium under the same conditions and Phi concentrations as in the previous experiment. The compatibility of the *Trichoderma* isolates with Phi was evaluated by comparing the spore concentration obtained in the control against those obtained with each Phi concentration.

2.5. In Vitro Compatibility of Phi with *Trichoderma* sp. in the Control of *Fusarium* sp.

In vitro compatibility was studied using the dual culture technique. At the base of the standard Petri dish, a diameter line (90 mm) was drawn, and two points were marked equidistant from the center towards the edge (22.5 mm) of the Petri dish. Thus, the inoculation points for the pathogen and the antagonist were determined. For each confrontation, 5 μL of the *Trichoderma* sp. suspensions (Taz-001, Taz-013, or Taz-016) and *Fusarium* sp., adjusted with 0.03% Tween-20 to 1×10^6 mL^{-1} spores, were inoculated. The three *Trichoderma* sp. isolates combined with 1000, 4000, and 8000 μg mL^{-1} Phi were evaluated against *Fusarium* sp. in PDA medium adjusted to pH 5.7 using NaOH 1 N. The control treatment was the pathogen without the presence of Phi or *Trichoderma*. All

the treatments were incubated at 28 °C for 10 d, and the percentage of mycelial growth inhibition (MGI) was evaluated.

2.6. Compatibility of Phi and *Trichoderma* sp. in the Control of *Fusarium* sp. in Sugarcane Plants

To evaluate the compatibility of Phi with *Trichoderma* sp. in the control of *Fusarium* sp. in sugarcane plants (in planta assay), eighty 3 L plastic pots were established under greenhouse conditions. The pots were filled to two-thirds capacity with the sterile substrate mixture (121 °C, 15 lb/in^2, 15 min), consisting of ITSB agricultural soil and peat moss (Cosmopeat®) provided by Cosmocel (San Nicolás de los Garza, Nuevo León, Mexico) at a 2:3 ratio (*v*:*v*). In each container, a sugarcane cutting bud was planted, and the corresponding treatments were applied. The distribution across the experimental containers was randomized, resulting in fourteen treatments of the three factors (*Trichoderma* sp., Phosphite, and *Fusarium* sp.) listed in Table 1 for better reader understanding.

Table 1. Treatments evaluated to test the compatibility of *Trichoderma* sp. and phosphite (Phi) in the control of the *Fusarium* species complex causing pokkah boeng in sugarcane (*Saccharum* spp.).

Factors	Treatments													
	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**T12**	**T13**	**T14**
Trichoderma sp.	−	−	−	−	−	−	+	+	+	+	+	+	+	−
Phi (μg mL^{-1})	−	500	1000	2000	4000	8000	−	500	1000	2000	4000	8000	−	−
Fusarium sp.	+	+	+	+	+	+	+	+	+	+	+	+	−	−

Presence: +; absence: −. For this experiment, only the *Trichoderma* isolate Taz-016 was used, as it showed the most efficient control of *Fusarium* sp.

Trichoderma inoculations (T7-T13) were conducted by immersing the cuttings (each containing a healthy and well-developed bud, previously washed with 2% sodium hypochlorite) in a spore suspension adjusted with 0.03% Tween-20 to 1 × 10^8 cells mL^{-1}, for 30 min. Treatments that only included Phi application from phosphorous acid (Sigma-Aldrich®) adjusted to a pH of 5.7 with NaOH 1 N were immersed in the same way for 30 min at each concentration (T2-T6). In the combination of Phi with *Trichoderma* (T8–12), 50 mL of each of the Phi concentrations was added directly to the cuttings previously treated with *Trichoderma*. The *Fusarium* control and absolute control treatments (T1 and T14) consisted of immersing the cuttings in 0.03% Tween-20 for the same period.

Once the sugarcane seedlings reached 30 cm in height (~30 d), treatments with *Fusarium* (+F) were inoculated at the base of the plant through the drenching of 50 mL *Fusarium* sp. suspension, adjusted to 1 × 10^8 spores mL^{-1} with 0.03% Tween-20, while the rest of the treatments without *Fusarium* (−F) received 50 mL 0.03% Tween-20 with no *Fusarium* sp. spores.

During the evaluation, Phi was applied on three occasions (at days 8, 15 and 22 after planting, like preventive treatment). *Trichoderma* was applied twice by drenching (100 mL), using the same inoculation concentration at days 15 and 30 after planting. Two fertilizations were applied using soluble monoammonium phosphate up to field application strength (N-P-K (12-61-0)) at a concentration of 3 g L^{-1}. These were applied at days 0 and 30 after planting. The seedlings were watered with 250 mL tap water every morning.

After 60 days, the compatibility of the agents (Phi and *Trichoderma* sp.) were evaluated in the control of *Fusarium* sp. For this analysis, each plant was separated into culm, leaf, and root. The presence or absence of red rings at the base of the culm, caused by the infection of the *Fusarium* species complex, was evaluated. Plant height, culm length, and leaf dry weight were quantified in each experimental unit for each treatment tested.

At the end of the evaluation, samples were taken from the roots of the seedlings inoculated with the *Trichoderma* isolate (Taz-016) and *Fusarium* sp. for their re-isolation. Using these samples, Koch's postulates were confirmed, and the presence of the *Fusarium* species complex and/or *Trichoderma* sp. as biocontrol agent(s) in the rhizosphere was verified.

2.7. Experimental Design

For this study, an experiment with a completely randomized block design was established to analyze the variables conidiogenesis and inhibition percentage in the in vitro tests, as well as for the analyses of the morpho-physiological variables of the sugarcane seedlings in in planta tests. Six replicates per treatment were run, and all tests were conducted twice. Two statistical models were used: (1) generalized mixed linear model (GMLM) with a negative binomial response (λij, φ) for the responses evaluated as conidiogenesis in *Fusarium* sp. and *Trichoderma* sp., and (2) mixed linear model (MLM) for the MGI (%) of *Fusarium* sp., to study the effect of Phi, and the compatibility of Phi with *Trichoderma* sp. in vitro and in planta.

2.8. Statistical Analysis

The statistical analysis of the evaluated variables was done using the glimmix procedure (PROC GLIMMIX) with Statistical Analysis System 9.4 (SAS) for Windows. Means were compared using Fisher's LSD test with a significance of 5%.

3. Results

3.1. Effect of Phi on In Vitro Fusarium sp. *Growth*

In this study, the evaluated concentrations (from 500 to 8000 µg mL^{-1} Phi) presented a fungistatic effect on the mycelial growth of the pathogen, with an inhibition range from 26.0 to 81.0% (Figure 1).

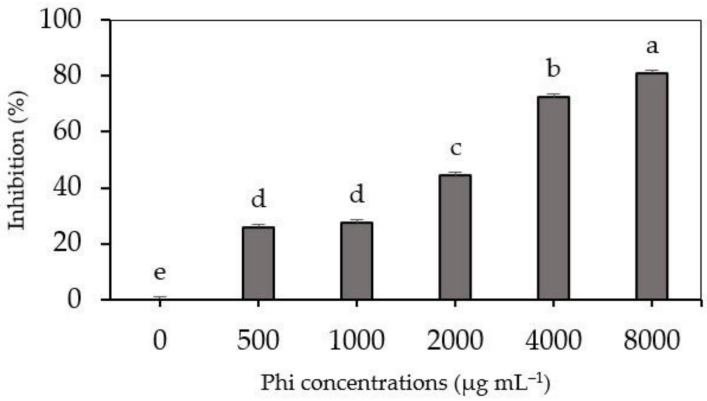

Figure 1. Mycelial growth inhibition (MGI) of *Fusarium* sp. in PDA culture medium, with the addition of Phi (0, 500, 1000, 2000, 4000 and 8000 µg mL^{-1} Phi). Means ± SD with different letters are statistically different (Fisher's test; α = 0.05). Phi–phosphite.

A fung-istatic effect on MGI (%) was observed when Phi was added to the medium. A fungicidal effect from 500 µg mL^{-1} was observed on the conidiogenesis of *Fusarium* sp., as it decreased by 99.7% compared to the control (Table 2).

Table 2. Conidiogenesis of *Fusarium* sp. (spores mL^{-1}) with Phi addition to the PDA culture medium.

Phi Concentration (µg mL^{-1})	Conidiogenesis (Spores mL^{-1})
0	$1.3 \times 10^9 \pm 0.3 \times 10^9$ a
500	$3.0 \times 10^6 \pm 0.7 \times 10^6$ b
1000	$3.2 \times 10^6 \pm 0.7 \times 10^6$ b
2000	$1.1 \times 10^6 \pm 0.3 \times 10^6$ c
4000	$1.0 \times 10^6 \pm 0.2 \times 10^6$ c
8000	$9.0 \times 10^5 \pm 0.2 \times 10^5$ c

Means ± SD with different letters are statistically different (Fisher's test; α = 0.05).

3.2. Compatibility of Trichoderma Isolates with Phi

In the compatibility experiment between *Trichoderma* and Phi, there was a significant ($p \leq 0.001$) decrease in the conidiogenesis of the fungus. There were significant differences in all three isolates evaluated when increasing the Phi concentration in the medium (Figure 2). Nevertheless, mycelial growth continued until the whole dish was colonized (7 d after inoculation).

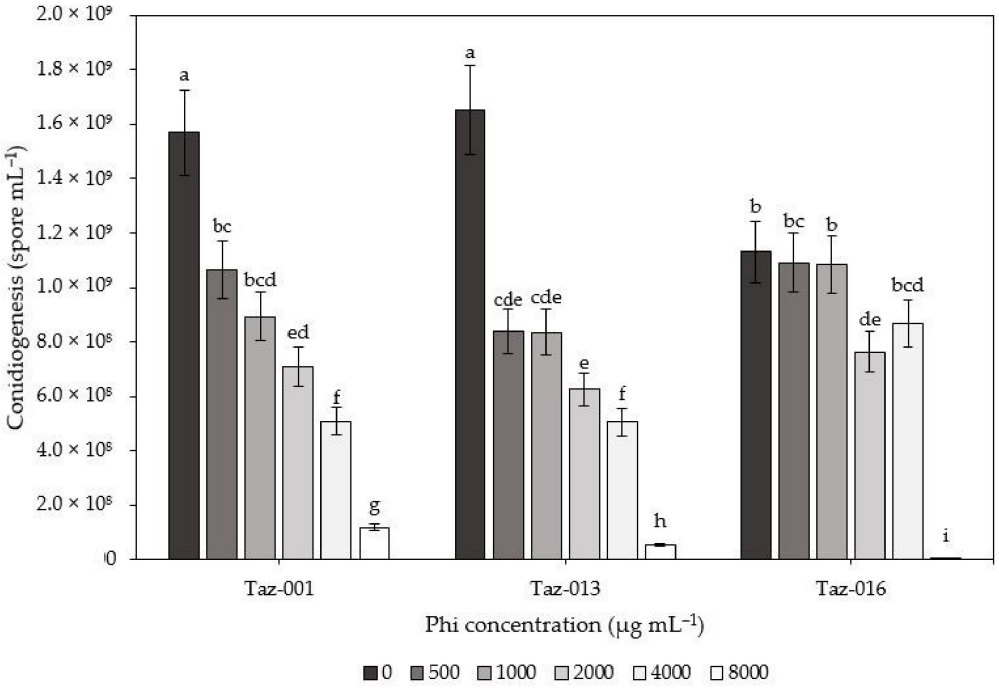

Figure 2. Conidiogenesis of *Trichoderma* sp. isolations (Taz-001, Taz-013 and Taz-016) determined as the number of spores per mL established in PDA medium supplemented with different concentrations of phosphite (Phi). Means with different letters indicate significant differences among treatments (Fisher's test; $\alpha = 0.05$).

The conidiogenesis of the Taz-016 isolate showed no statistical differences between concentrations of 500 and 1000 µg mL^{-1} Phi concerning the control, as it only decreased by 3.5 and 4.1%, respectively. On the other hand, Taz-001 showed significant differences at the same concentrations, as its conidiogenesis decreased by 32.1 and 57.0%, respectively. Likewise, Taz-013 decreased by 49.2 and 50.7%, respectively (Figure 2). The Taz-001 strain recorded higher conidiogenesis in the treatment with 8000 µg mL^{-1} than the other isolates; 52.6% more than Taz-013 and 97.9% more than Taz-016.

3.3. In Vitro Compatibility of Phi with Trichoderma sp. *in the Control of Fusarium* sp.

In the compatibility between Phi and *Trichoderma* sp. for the control of *Fusarium* sp. experiment, total colonization of the Petri dish was observed in the control treatments at 10 d after inoculation. In addition, a higher percentage of pathogen inhibition was recorded when Phi was applied combined with *Trichoderma* suspensions (Figure 3), which was taken as an indicator of compatibility.

Significant statistical differences were observed in the percentage of mycelial growth inhibition when increasing the Phi concentration in the medium ($p \leq 0.0001$). The highest Phi concentration (8000 µg mL^{-1}) inhibited *Fusarium* sp. by 77. 8%, while 4000 and 1000 µg mL^{-1} Phi caused 56.0 and 30.3% inhibition, respectively (Figure 4).

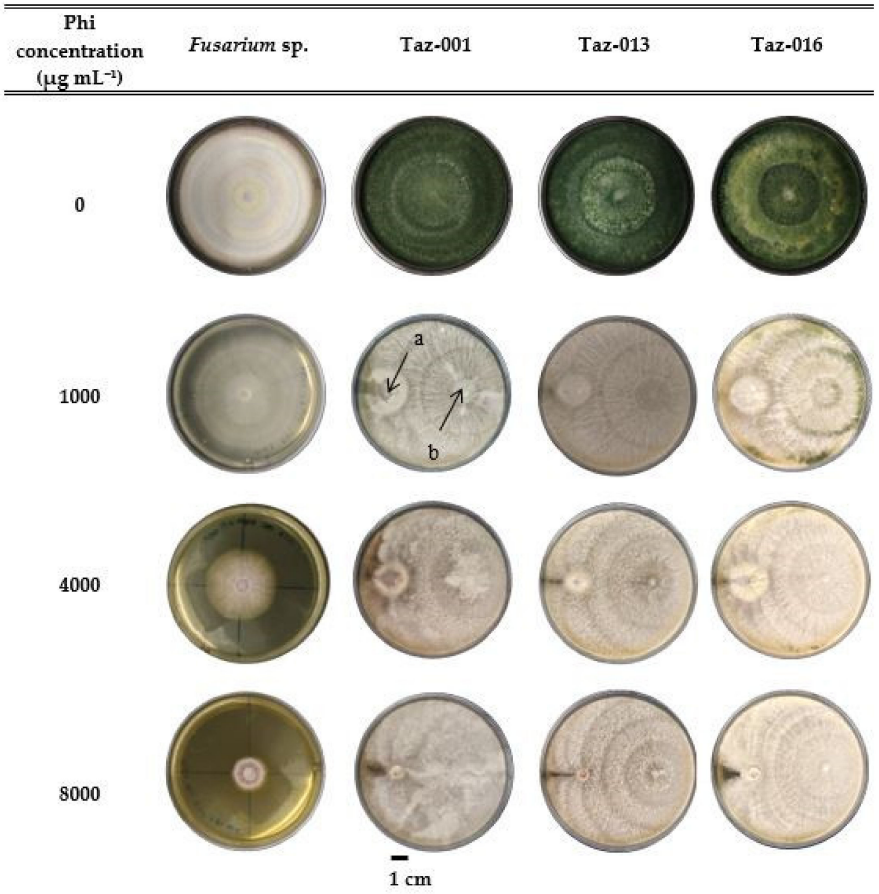

Figure 3. Growth of *Fusarium* sp. and *Trichoderma* sp. isolates after 10 d incubation in Petri dishes with three different Phi concentrations and a control without Phi. (a) Pathogen inoculation (*Fusarium* sp.); (b) bioregulator inoculation (*Trichoderma* sp.).

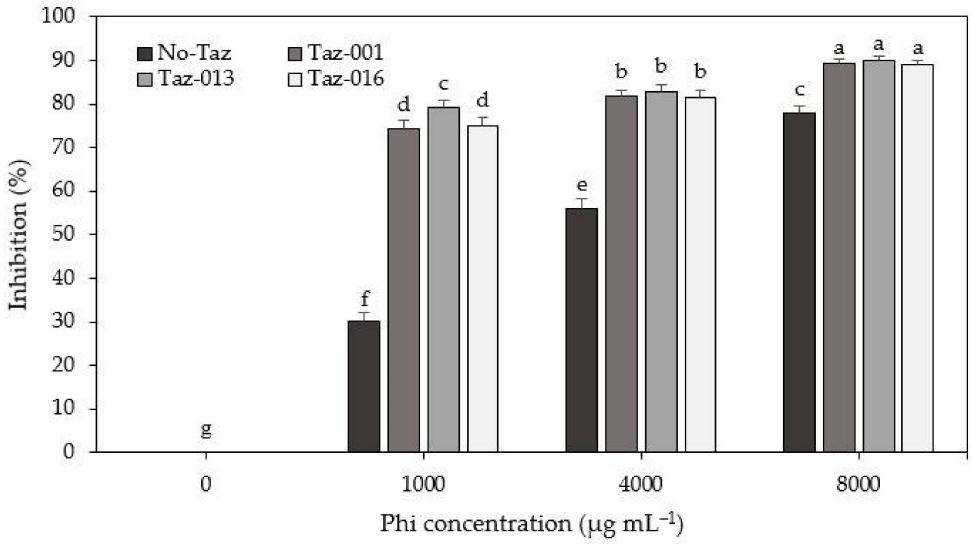

Figure 4. Mycelial growth inhibition (MGI) of *Fusarium* sp. with *Trichoderma* sp. (Taz-001, Taz-013, and Taz-016) and adding Phi to the culture medium. Means ± SD with different letters are statistically different (Fisher's test; $\alpha = 0.05$).

The highest inhibition percentages were observed with combining *Trichoderma* and Phi. When *Trichoderma* + 1000 μg mL^{-1} Phi were combined, at least 74.3% inhibition

was achieved. This means it increased by 44.0% more than in the Phi treatment alone, at the same concentration. When *Trichoderma* + 4000 µg mL^{-1} Phi was combined, the inhibition percentage increased by 25.2% concerning the treatment with Phi alone. Finally, when *Trichoderma* + 8000 µg mL^{-1} Phi was combined, the pathogen inhibition percentage increased by 11.2% compared to the treatment with Phi alone, at the same concentration (8000 µg mL^{-1}) (Figure 4).

3.4. Compatibility of Phi and Trichoderma sp., *in the Control of Fusarium* sp. *in Sugarcane Plants*

In this experiment, the treatments that included only Phi application (T2-T6), only *Trichoderma* sp. (T7 and T13), and combining the two fungicidal agents (T8–12), showed no symptoms of the presence of *Fusarium* sp., nor did treatment T14 (absolute control). However, treatment T1 showed necrotic lesions at the root and a red ring at the base of the culms (Figure 5A,B).

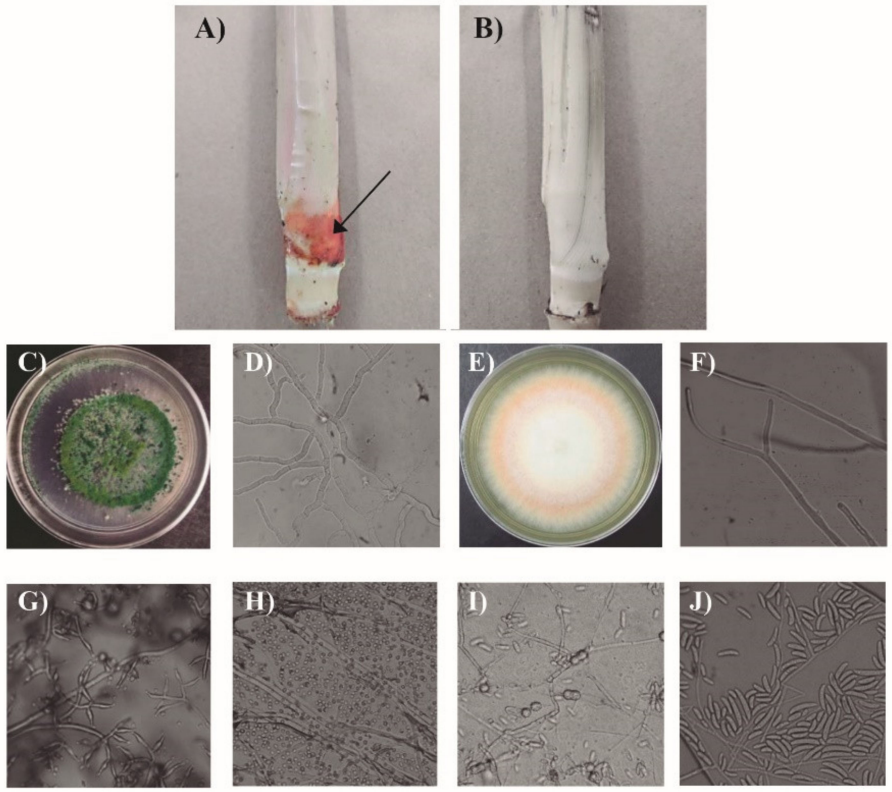

Figure 5. Base of the sugarcane culm exposed to the tested treatments and isolates of *Trichoderma* sp. and a *Fusarium* species complex from the roots of sugarcane plants to test the Koch's postulates. (**A**) Presence of a red ring at the base of the culm in plants inoculated with *Fusarium* sp. on the root without fungicidal agents; (**B**) absence of red ring at the base of the culm in plants inoculated with *Fusarium* sp. on the root with any of the combinations of fungicidal agents (*Trichoderma* + Phi); (**C**) *Trichoderma* sp. colony in PDA; (**D**) *Trichoderma* sp. septate mycelium.; (**E**) *Fusarium* sp. colony in PDA; (**F**) *Fusarium* mycelium; (**G**) phialides in *Trichoderma* isolate; (**H**) *Trichoderma* spores; (**I**) *Fusarium* resistance structures; (**J**) *Fusarium* spores.

In each of the treatments inoculated with *Trichoderma* sp. and *Fusarium* sp. the two isolates were obtained (Figure 5C–J), corroborating Koch's postulates.

In evaluating plant height, significant differences between treatments were observed ($p \leq 0.0001$). Treatment T11 (*Trichoderma* sp. Taz 016 + 4000 µg mL^{-1} Phi in the presence of *Fusarium*) improved plant height by 10.4% compared to the control. Moreover, the presence of *Fusarium* sp. without any fungicidal agent (T1) decreased plant height by 10.4%. Likewise, treatments T4 (2000 µg mL^{-1} Phi in the presence of *Fusarium*) and

T5 (4000 µg mL^{-1} Phi in the presence of *Fusarium*) decreased plant height by 11.6 and 11.9%, respectively (Table 3). For culm length, treatment T11 had the best response, with 30.8% increased plant height over the control ($p \leq 0.0001$). In comparison, the presence of *Fusarium* sp. without using control agents (T1) generated a decrease of 13.2% in the mean value of this variable compared to the control (Table 3).

Table 3. Effect of *Trichoderma* sp. (Tri) and phosphite (Phi; µg mL^{-1}) in the control of *Fusarium* sp. (Fus) on plant height, culm length, and leaf dry weight of sugarcane (*Saccharum* spp.).

Treatment	Plant Height (cm)	Culm Length (cm)	Leaf Dry Weight (g)
Fus (T1)	132.6 ± 4.7 ef	27.6 ± 2.0 d	10.6 ± 2.3 c
500 Phi vs. Fus (T2)	144.2 ± 4.3 cd	37.2 ± 1.9 ab	15.0 ± 2.1 c
1000 Phi vs. Fus (T3)	138.6 ± 4.7 edef	28.6 ± 2.0 cd	10.5 ± 2.3 c
2000 Phi vs. Fus (T4)	130.8 ± 4.7 f	28.7 ± 2.0 cd	10.5 ± 2.3 c
4000 Phi vs. Fus (T5)	130.3 ± 4.3 f	27.8 ± 1.9 d	10.3 ± 2.1 c
8000 Phi vs. Fus (T6)	136.5 ± 4.3 ef	30.0 ± 1.9 cd	9.8 ± 2.1 c
Tri vs. Fus (T7)	139.2 ± 4.7 cdef	32.4 ± 2.0 bcd	14.8 ± 2.3 bc
Tri + 500 Phi vs. Fus (T8)	162.8 ± 4.3 a	40.7 ± 1.9 a	20.4 ± 2.1 ba
Tri + 1000 Phi vs. Fus (T9)	149.3 ± 4.3 bc	32.5 ± 1.9 bcd	21.4 ± 2.1 a
Tri + 2000 Phi vs. Fus (T10)	147.3 ± 4.3 cd	33.3 ± 1.9 bc	23.9 ± 2.1 a
Tri + 4000 Phi vs. Fus (T11)	163.4 ± 4.7 a	41.6 ± 2.0 a	26.1 ± 2.2 a
Tri + 8000 Phi vs. Fus (T12)	141.6 ± 4.7 cdef	31.4 ± 2.0 cd	22.5 ± 2.2 a
Tri (T13)	160.6 ± 4.7 ab	32.6 ± 2.0 bcd	15.1 ± 2.2 bc
Control (T14)	148.0 ± 4.7 bcd	31.8 ± 2.0 bcd	14.0 ± 2.2 c

Means ± SD with different letters are statistically different (Fisher's test; $\alpha = 0.05$).

4. Discussion

4.1. Phi Significantly Decreases the Growth of the Fusarium Species Complex In Vitro

The action mechanism of Phi on fungi and oomycetes is complex and is not yet known in detail [18]. However, Phi has been shown to have direct effects on mycelial growth in soil pathogens like *Alternaria alternata* (90.0%), *Penicillium expansum* (50.0%), and *Phytophthora infestans* (26.0%), among others [25,32]. In this work, the concentrations of Phi exhibited a fungistatic effect on the mycelial growth (Figure 1), and the inhibitory effect of the different Phi concentrations on the vegetative development of *Fusarium* sp. was investigated. Interestingly, our results are in full agreement with those found elsewhere [33]. Phosphite proved to be effective in inhibiting the mycelial growth of *Fusarium solani* under all their experimental conditions (1–6 g L^{-1}) since none of the treatments showed mycelial growth. Phosphite-containing compounds inhibit the growth of plant pathogens through a direct fungistatic effect, which depends on the Phi concentration that accumulates in the fungus. In turn, growth is influenced by the phosphate concentration and the effectiveness of the phosphite oxidation system [33]. Potassium phosphite also partially inhibits developing *Alternaria alternata*, and concentrations of 229 and 531 µg mL^{-1} Phi cause 50.0 and 90.0% inhibition in conidial germination, respectively [32]. Furthermore, when applying 22.5–50.0 mM Phi, the sporulation of *Hyaloperonospora arabidopsidis* was decreased up to 97.0% [18]; hence, the amount of spores provides a valid estimate of the susceptibility of pathogens to Phi.

4.2. Compatibility between Trichoderma and Phi

A combined integrated disease control strategy that includes biological control agents and inorganic compounds requires a compatibility study of both components, especially sporulation capacity. In *Trichoderma* sp. and Phi, conidiogenesis provides a valid compatibility estimation, as these reproductive structures give this genus the capability to exert antagonism on plant pathogens [34]. In the present study, we observed differences in the compatibility percentages between *Trichoderma* sp. isolates and the different concentrations of Phi ($p \leq 0.001$), demonstrating that *Trichoderma* has a natural capacity to tolerate agents with fungicidal activity (i.e., "natural resistance" or "inherent resistance") [35]. The Taz-016 isolate showed greater compatibility (96.5 and 95.9%) with concentrations of 500 and 1000 µg mL^{-1} Phi, while Taz-001 (67.9 and 43.0%) and Taz-013 (50.8 and 49.4%) were less compatible with the same concentrations of Phi tested. The capacity of *Trichoderma*

to resist relatively high concentrations of toxic compounds (both synthetic and natural) depends on efficient cellular detoxification mechanisms carried out by a complex system of membrane pumps, which includes ABC transporters (ATP-binding cassette) that can provide a protection mechanism against toxic compounds and xenobiotic agents [36]. ABC transporters could explain the natural tolerance of *Trichoderma* to fungicidal agents and its capacity to successfully survive in environments with remnant Phi molecules.

The mycoparasitic capacity of *Trichoderma* varies among species and fungal isolates due to their origin and host where they develop [37]. The results observed in the differences in antagonism among the isolates evaluated herein may be related to the different agronomic management and sugarcane varieties from which they were obtained. The Taz-016 isolation was obtained from plants treated with intensive management using organosynthetic products. Due to this constant selection pressure, it could have developed the resistance inherent to the medium.

Despite the generalized decrease in conidiogenesis in the three *Trichoderma* isolates evaluated with Phi, mycelial growth continued until the whole Petri dish was colonized. This proves the capability of *Trichoderma* to survive in unfavorable environments [27]. Fungicides like orthocide, propiconazole, mancozeb, and chlorothalonil at concentrations between 0.03 and 0.2% do not inhibit the growth of *T. harzianum* (Th09) and *T. viride* (Tv11) by over 10.0% [38]. Compatibilities of 34.9% in 300 ppm and 97.9% in 50 ppm of fungicides copper oxychloride and 2-(trichloromethylsulfanyl)-3a,4,7,7a-tetrahydroisoindole-1,3-dione (16.6 to 25.0%) and *Trichoderma* have also been reported [39]. Potassium phosphonate and fosetyl-aluminum are 100.0% compatible with *T. viride* with only 0.5% mycelial growth inhibition [29], which suggests that Taz-016 is compatible with Phi doses $\leq$1000 μg mL^{-1}.

Several *Trichoderma* species can tolerate fungicidal agents since they adapt to diverse soil or substrate conditions, where they can produce lithic enzymes, antibiotics, and/or secondary metabolites and colonize them quickly [34]. Although some agricultural supplies can make it difficult for *Trichoderma* to act [40], they do not impede its development. This is because this genus shows ecological plasticity, developed by its wide geographical distribution, which depends more on the isolate than the species itself [41,42].

4.3. Phi Is Compatible with Trichoderma sp. *in In Vitro Control of the Fusarium Species Complex Causing Pokkah Boeng in Sugarcane*

The obtained results in the compatibility between Phi and *Trichoderma* sp. for the control of *Fusarium* sp. provide evidence that combining Phi and *Trichoderma* isolates increases the efficacy to control *Fusarium* sp., compared with the sole application of Phi. This can be attributed to the antifungal activity of the Phi molecules and the indirect mode of action of Phi through the stimulation of the natural defense mechanisms of plants. [18,19,34,43] and to the antagonistic capacity of the *Trichoderma* genus against *Fusarium* sp. [44,45]. By adding 0.075% and 0.1% (*v*/*v*) chitosan to a suspension of *T. harzianum*, the vegetative development of the opportunistic phytopathogenic fungus *Sphaeropsis sapinea* was significantly decreased ($\geq$40.0%) [46]. Furthermore, combining *T. viride* strain-CIAH240 with fungicides like triadimefon, thiophanate methyl, mancozeb, and alcidine at 50 mg g^{-1} improved the percentage of disease control efficiency to more than 70.0% during postharvest of Indian plum (*Ziziphus mauritiana*) [47]. The results of our work show that the *Trichoderma* isolates (Taz-001, Taz-013, and Taz-016) and the different Phi concentrations (1000, 4000, and 8000 μg mL^{-1}) are compatible since in all treatments, the *Trichoderma* isolates could colonize the entire Petri dish 10 d after inoculation. When Phi and *Trichoderma* are combined, they act synergistically on the inhibition of the vegetative development of *Fusarium* sp. In this relationship, Phi acts as a fungistatic or fungicidal agent, and the *Trichoderma* isolates act as antagonists by competing for space and nutrients, as well as mycoparasitism.

Under our experimental conditions, *Trichoderma* isolates grew considerably faster than *Fusarium* ones. This ability gives the antagonist an important advantage against the pathogen in competition for space and nutrients [48,49]. This mode of action is a mechanism of antagonism employed by *Trichoderma* sp. [50]. In our study, we considered the initial phase of the interaction between *Trichoderma* and *Fusarium*, which is characterized

by a noncontact competition of mycelia. The diffusible metabolites of both organisms decide the fate of the interaction [51]. Another key biological control mechanism for most *Trichoderma* strains is mycoparasitism mediated by producing chitinases and other enzymes that degrade the cell wall [37,52–54]. Mycoparasitism is a complex process displayed by *Trichoderma* strains [55], which involves recognizing the pathogen, producing endochitinases, and the physical binding of *Trichoderma* to the pathogen. Subsequently, *Trichoderma* strains secrete enzymes that hydrolyze the main structural compounds of fungal cell walls, chitin and β-glucan [48,56–58].

Although the concentration of 8000 μg mL^{-1} of Phi was the most effective to inhibit the in vitro development of *Fusarium* sp., in this work, it was observed that 8000 μg mL^{-1} of Phi inhibited the development and sporulation of native microorganisms as *Trichoderma* sp. In addition, it has been reported that Phi at high concentrations can cause phytotoxicity [16,22]. In the integrated disease and pest management approach, efficient control of pathogens is sought, with minimal impact on the environment. Accordingly, combining 1000 μg mL^{-1} Phi and *Trichoderma* sp. was chosen, where the compatibility of both factors was recorded. Compared to applying 1000 μg mL^{-1} Phi, combining both *Trichoderma* and 1000 μg mL^{-1} Phi resulted in a 44.0% increase in inhibition of *Fusarium*.

4.4. The Combination of Phi and Trichoderma sp. *Is Efficient in the Control of the Fusarium Species Complex Causing Pokkah Boeng in Sugarcane in the Planta Assay*

The treatments that included only Phi and *Trichoderma* sp. application and combining the two fungicidal agents showed no symptoms of the presence of *Fusarium* sp. (Figure 5A,B). This can be attributed to the fact that combinations of *Trichoderma* with fungicides significantly induce defense enzymes, such as peroxidases that catalyze lignification and suberization reactions in cell walls and stimulate the synthesis of phenolic compounds in plants while limiting fungal activity [40]. Treatment T11 improved plant height compared to the control, and the presence of *Fusarium* sp. (T1, T4 and T5) decreased plant height (Table 3). The use of *T. harzianum* isolates (root immersion) combined with a fungicidal agent like tebuconazole 250 EC (direct to the soil) has been reported to increase plant height in gerbera (*Gerbera jamesonii*) var. Donavan yellow, where management efficiency is maximized if both agents are compatible [59]. For culm length, treatment T11 (*Trichoderma* sp. Taz-016 + 4000 μg mL^{-1} Phi in the presence of *Fusarium*) increased plant height compared to the control (Table 3). Interestingly, combinations of *Trichoderma* spp. with comothiophanate-methyl increase vegetative growth in the common bean (*Phaseolus vulgaris*) [40]. *Trichoderma* sp. Taz-016 + 4000 μg mL^{-1} Phi in the presence of *Fusarium* increased dry leaf weight (Table 3). In tomato (*Solanum lycopersicum*), the combined use of *T. harzianum*, neem (*Azadirachta indica*) spray, and foliar spray of benzimidazole applied to control *Fusarium* increased fresh and dry weight of plants compared to the control [60]. This response is attributed to a greater pressure exerted on the pathogen by combining biocontrollers with fungicidal agents. Furthermore, a host that is resistant combined with biological control agents (i.e., *Purpureocillium lilacinum* and *Trichoderma harzianum*) and organic amendments (i.e., neem) can be used in managing *Fusarium* in diverse agricultural systems [61].

5. Conclusions

In the present study, we demonstrated that Phi has a direct action on mycelial growth and conidiogenesis of *Fusarium* sp. isolated from the rhizosphere of sugarcane. We also demonstrated the capacity of *Trichoderma* sp. to survive in environments in the presence of Phi. The *Trichoderma* isolates (Taz-001, Taz-013, and Taz-016) are potential biological control agents against *Fusarium* sp. Furthermore, it was evident that *Trichoderma* sp. and Phi act synergistically, inhibiting vegetative development and conidiogenesis of *Fusarium* sp. in vitro and the infection caused by the pathogen in sugarcane plants. This control strategy represents an effective alternative to the conventional management of pokkah boeng in the field, under a sustainability scheme that can control the disease while stimulating plant growth.

Author Contributions: Conceptualization, F.C.G.-M. and G.H.-R.; methodology, R.S.-P., G.H.-R., and F.C.G.-M.; software, J.S.-R. and J.V.H.-C.; validation, G.H.-R. and J.S.-R.; formal analysis, R.S.-P., G.H.-R. and J.S.-R.; investigation, R.S.-P. and G.H.-R.; resources, F.C.G.-M. and G.H.-R.; writing—original draft preparation, R.S.-P. and G.H.-R.; writing—review and editing, F.C.G.-M. and G.H.-R.; supervision, F.C.G.-M. and G.H.-R.; project administration, F.C.G.-M. and G.H.-R. All authors have read and agreed to the published version of the manuscript.

Funding: Mexico's National Science and Technology Council (CONACYT-Mexico) funded this research.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The Laboratory of Plant Nutrition of the College of Postgraduates in Agricultural Sciences Campus Montecillo provided space and infrastructure for some experiments carried out during this study. The Sugar Mill La Margarita S. A de C. V. (Vicente, Oaxaca, Mexico) provided the sugarcane samples for the study.

Conflicts of Interest: The authors declare no conflict of interest.

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