Colegio de Postgraduados

Frequency of fungi associated with strawberry dry wilt and *in vitro* antagonistic impact of *Trichoderma harzianum* and *Bacillus subtilis*

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ABSTRACT

Objective: To determine the frequency of fungi associated with strawberry dry wilt and to evaluate the antagonistic impact of *Trichoderma harzianum* and *Bacillus subtilis*.

Design/Methodology/Approach: Three sampling sessions were conducted in strawberry plantations in the Zamora Valley to isolate and identify fungi associated with strawberry dry wilt and to determine their frequency. *In vitro* antagonism tests were performed between *Trichoderma harzianum*, *Bacillus subtilis*, and fungi isolated from strawberry plants showing wilt symptoms. Additionally, the percentages of mycelial growth inhibition were determined.

Results: Six fungi were isolated from diseased plants showing wilt symptoms. The most frequent fungi were: *Neopestalotiopsis* sp. (54.7%), *Fusarium oxysporum* (50.6%), and *Rhizoctonia solani* (40.5%). *Trichoderma harzianum* inhibited >90% of the radial growth of *Rhizoctonia solani* mycelia, of *Cylindrocarpon* sp., >80% of *Fusarium solani*, and *F. oxysporum* mycelia, and 77.7% of *Neopestalotiopsis* sp. mycelia. *Bacillus subtilis* recorded the highest antagonism against *Rhizoctonia solani* (57%).

Study Limitations/Implications: This research faced no limitations.

Findings/Conclusions: *In vitro* tests determined that *Trichoderma harzianum* can inhibit the mycelial growth of fungi associated with strawberry dry wilt. *Bacillus subtilis* had a lower capacity to inhibit the mycelial growth of the confronted fungi than *Trichoderma harzianum*; however, it was the most effective bacterium against *Rhizoctonia solani*.

Keywords: strawberry, wilt, phytopathogenic fungi, biological control.

Citation: Ceja-Torres, L. F., Mateo-Hernández, M. A., Ortega-Cendejas, X. M., & Gutiérrez-Hernández, G. F. (2024). Frequency of fungi associated with strawberry dry wilt and *in vitro* antagonistic impact of *Trichoderma harzianum* and *Bacillus subtilis*. *Agro Productividad*. https://doi.org/10.32854/agrop.v17i11.3138

Academic Editor: Jorge Cadena Iñiguez
Associate Editor: Dra. Lucero del Mar Ruiz Posadas
Guest Editor: Daniel Alejandro Cadena Zamudio

Received: May 23, 2024.
Accepted: September 17, 2024.
Published on-line: December XX, 2024.

Agro Productividad, 17(11) supplement. November. 2024. pp: 215-223.

INTRODUCTION

Mexico is the fourth largest strawberry producer worldwide, with an annual production of 578,142 tons and a *per capita* consumption of 1.9 kg. Michoacán is the most important producing state in the country, exceeding 354,000 tons, generating $8,113 million pesos, and accounting for 58.1% of the domestic production value [1]. However, strawberry

production is impacted by diseases such as wilt —caused by a consortium of fungi, with a variable incidence of the causal agents.

One of the most sustainable alternatives for the control of the fungal consortium responsible for strawberry wilt is the use of antagonistic fungi and bacteria. This biological control makes these microorganisms a valuable tool for agroecosystems, eliminating the need for chemical inputs [4]. *In vitro* research has shown an adequate antagonistic level of the T-H4 strain of *Trichoderma harzianum* against fungi associated with strawberry cultivation [5]. For their part, Pérez-Rodríguez *et al.* [6] reported that *Trichoderma harzianum* and *T. viridae* hindered the mycelial growth of fungi associated with strawberry crops. *Bacillus* strains have been proved to have a high potential as antagonists of *Fusarium oxysporum* [7]; additionally, *Bacillus subtilis* TS06 had an *in vitro* impact on the *Fusarium oxysporum* and *Verticillium dahliae* fungi that cause strawberry wilt [8] and inhibit the mycelial growth of *Fusarium equiseti* and *Fusarium solani* [9]. Consequently, the objective of this research was to determine the frequency of fungi associated with strawberry dry wilt and to evaluate the *in vitro* antagonistic impact of *Trichoderma harzianum* and *Bacillus subtilis* isolated from the rhizosphere of strawberry plants.

MATERIALS AND METHODS

Isolation of fungi associated with strawberry dry wilt and their frequency

The roots and crowns from wilted plants collected during three samplings from February to April 2024 in established plantations in the Zamora Valley, Michoacán, were washed to remove soil. Subsequently, they were sectioned into small pieces: 1 to 2 cm long roots and 1 cm thick crowns. The pieces were then subjected to a surface sterilization, immersing them in 3% sodium hypochlorite for 2 minutes and rinsed three times with sterile distilled water. The root and crown pieces were dried on sterile paper towels for 5 minutes and immediately placed onto a potato dextrose agar (PDA) medium. The inoculated dishes were incubated at 28 °C until colony growth was observed. Once the colonies had developed and after they had been identified, the isolation frequency of each fungus was determined.

Purification and identification of fungi

The mycelial growth of the various isolated fungi was transferred and purified using the hyphal tip technique on 2% water agar. Cultural identification was based on the color, appearance, and growth pattern of the colonies. Morphological identification was conducted using a compound microscope at 10x and 40x magnification, referencing the keys proposed by Nelson *et al.* [10], Sneh *et al.* [11], and Crous [12].

In vitro inhibition tests

Trichoderma harzianum was inoculated at one end of the Petri dish (a 5 mm diameter disc with antagonist growth) and the pathogenic fungus was inoculated at the opposite end. In the case of *Bacillus subtilis*, the bacteria were streaked at one end of the dish using the single-streak technique, while the pathogenic fungus was inoculated at the opposite end. As a control, a slice with the pathogen's mycelium was placed in the center of each Petri dish containing only PDA. All confrontation tests were performed in triplicate, while

the control fungi were tested in duplicate. The Petri dishes were incubated at 28 °C and the radial mycelial growth of the fungi (both confrontation and control) was measured in millimeters every 24 hours. The bioassay concluded once the mycelia of the control fungi had filled the Petri dish (8-12 days).

Determination of the inhibition percentage

The inhibition percentage of the mycelial growth of the pathogenic fungi was determined using the formula proposed by Patil *et al.* [13].

$$inhibition\,\% = \frac{D1 - D2}{D1} \times 100$$

Where $D1$ is the average radial growth of the fungus without antagonist and $D2$ the average radial growth of the fungus with antagonist.

Statistical analysis

The "percentage of mycelial growth inhibition" variable was subjected to an analysis of variance and Tukey's test ($p \leq 0.05\%$) using the Statistical Analysis System (SAS) software package. Prior to the analysis of variance, the percentage values were transformed using the arcsine transformation.

RESULTS AND DISCUSSION

Identification of fungi associated with strawberry dry wilt

Six fungi were isolated from strawberry plants with wilt disease. *Fusarium oxysporum* colonies are pink and may turn violet over time. *F. oxysporum* produced both microconidia and macroconidia. The latter were slightly curved and had 3 to 5 septa; intercalary chlamydospores were also observed. *Fusarium solani* is a fast grower that produces white mycelium, as well as microconidia and macroconidia; its chlamydospores usually appear as single entities.

Rhizoctonia solani has white, cottony colonies. As part of its specific characteristics, it forms hyphae at right angles and its septum is close to the point of origin of hyphal branching. Older colonies develop constricted mycelium. *Cylindrocarpon* sp. exhibited dark brown colonies with slow growth. Its conidiophores are short, while its macroconidia are generally cylindrical and develop septate. *Neopestalotiopsis* sp. developed a white, cottony colony with abundant acervuli on the surface. Under a compound microscope, light brown to dark brown macroconidia with septate hyphae were observed. *Alternaria* sp. displayed dark green mycelium; it produced light brown chains of oval conidia, with transverse and longitudinal septate.

Frequency of isolation of fungi associated with strawberry dry wilt

The most frequently isolated fungi associated with strawberry dry wilt were *Neoestalotiopsis* sp. (54.7%, second sampling), *Fusarium oxysporum* (50.6%, first sampling),

and *Rhizoctonia solani* (40.5%, third sampling). Less frequent fungi included *Fusarium solani*, *Cylindrocarpon* sp., and *Alternaria* sp. (Table 1). This variability in the frequency of isolated fungi may have been caused by the varied humidity and temperature conditions of the different times during which the samples were collected. Additionally, differences in fungal inoculum levels across plots and the susceptibility of various strawberry varieties grown in the Zamora Valley should be considered.

In vitro antasgonism tests with *Trichoderma harzianum*

Overall, the percentages of fungal growth inhibition increase over time. The inhibition of *Fusarium oxysporum* is high during the first 3 days of confrontation (54.5%); afterwards, progress is gradual, increasing by ≈10% every third day, until it reaches 83.7%. A similar percentage (85.8%) was recorded for *Fusarium solani* (Figure 1).

There were statistically significant differences ($p \leq 0.05$): the highest percentage of inhibition by *Trichoderma harzianum* against fungi associated with strawberry wilt was observed with *Rhizoctonia solani* (94%), with an outstanding increase (50%) in inhibition from the third to the sixth day. Other fungi that were satisfactoriy inhibited by this antagonist were *Cylindrocarpon* sp. (89.1%) and *Neopestalotiopsis* sp. (77.7%), with significant statistical differences between them. However, *Neopestalotiopsis* sp. was inhibited in only 6 days (Figure 1).

Trichoderma harzianum exhibited a strong antagonism against its competitors, demonstrating complete in vitro dominance, but with significant differences among the various fungi (Figure 2). These results are consistent with the findings of Guédez *et al.* [14] about pathogenic fungi that impact strawberries in the postharvest period. Additionally, the T-H4 strain of *Trichoderma* recorded an effective *in vitro* antagonism against strawberry fungi such as *Colletotrichum* sp., *Pestalotiopsis* sp., *Alternaria* sp., *Rhizoctonia* sp., and *Curvularia* sp. [5]. The biocontrol impact of *Trichoderma harzianum* and *T. viride* has also been demonstrated through a significant reduction of the mycelial growth of *Alternaria* sp., *Fusarium* sp., and *Rhizoctonia solani*, isolated from strawberry cultivation [6].

Table 1. Percentage of fungal isolations associated with strawberry dry wilt in three sampling conducted in the Zamora Valley, Michoacán.

Fungi	Frequency of isolations		
	Sampling (February)*	Sampling (March)	Sampling (April)
F. oxysporum	50.6%	20.0%	22.5%
F. solani	22.8%		
Rhizoctonia solani		10.5%	40.5%
Alternaria sp.	8.9%	7.4%	
Cylindrocarpon sp.	17.7%	7.4%	
Neopestalotiopsis sp.		54.7%	37.0%

*79 isolations in February, 95 in March, and 89 in April (2024).

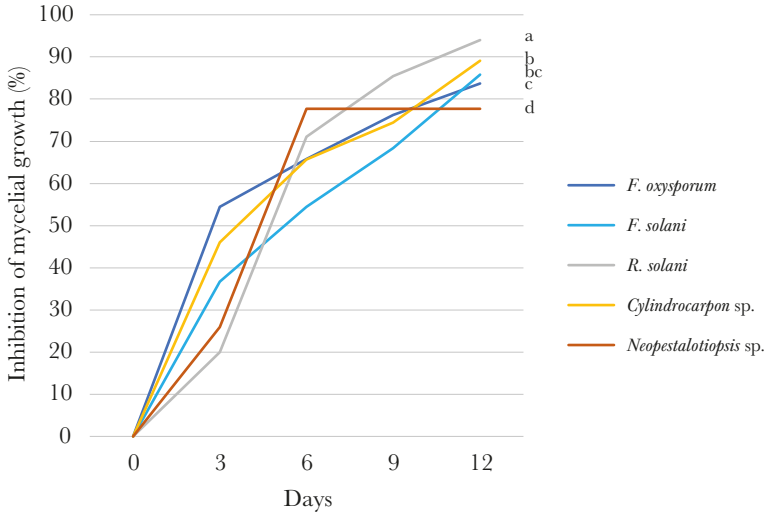

Figure 1. Percentage of mycelial growth inhibition caused by *Trichoderma harzianum* on fungi associated with strawberry dry wilt.

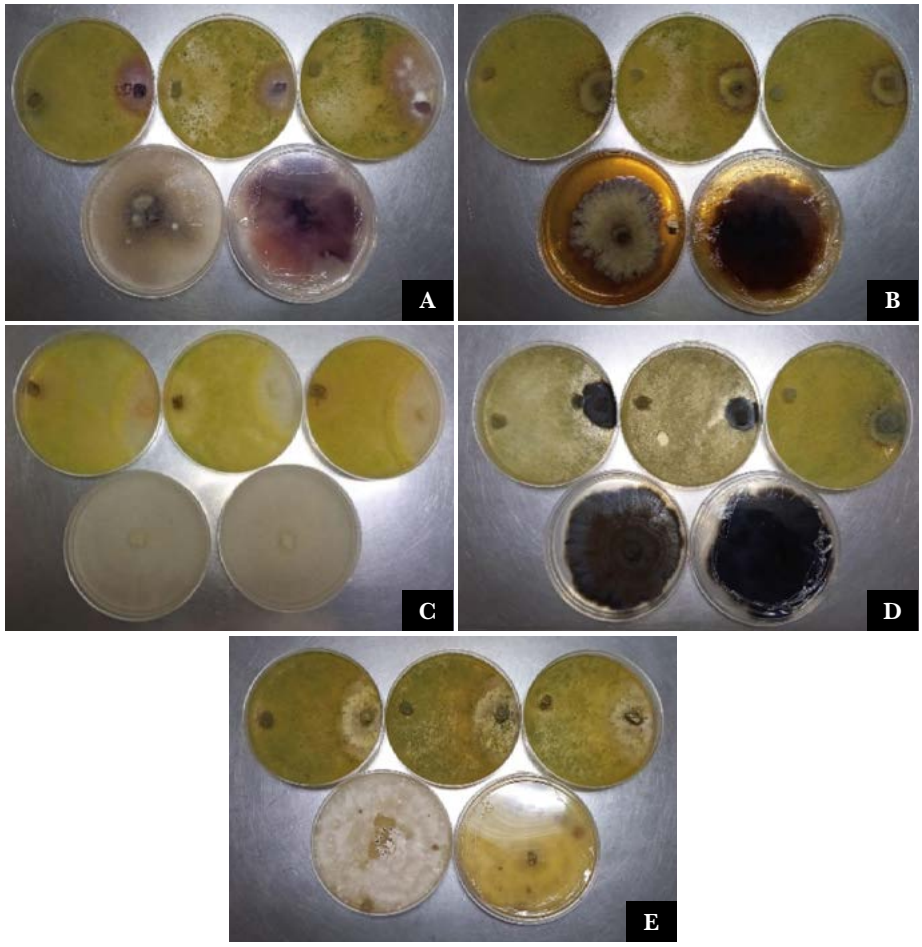

Figure 2. *Trichoderma harzianum* confrontation performed in triplicate *vs. Fusarium oxysporum* (A), *Fusarium solani* (B), *Rhizoctonia solani* (C), *Cylindrocarpon* sp. (D), and *Neopestalotiopsis* sp. (E). Growth of control fungi performed in duplicate.

Trichoderma exhibits several remarkable mechanisms that explain its microbial control of phytopathogens, including antibiosis, cell lysis, mycoparasitism, competition for space and nutrients, and environmental persistence [15-17]. Likewise, during the mycoparasitism process, *Trichoderma* species adheres to the hyphae of phytopathogenic fungi, frequently wrapping itself around them, and penetrating them; eventually, the degredation of their cells causes their complete weakening [18,19].

In vitro antagonism tests with *Bacillus subtilis*

Bacillus subtilis successfully inhibited the mycelial growth of some fungi associated with strawberry dry wilt. However, the inhibition percentages for three of the fungi were below 45%, while *Neopestalotiopsis* sp. recorded 53.2%, and the highest percentage was observed against *Rhizoctonia solani* (57%), with significant differences between them (Figures 3 and 4). These results are considerably lower than those reported by Jamali *et al.* [20], who achieved an 84% inhibition of *Rhizoctonia solani* in dual cultures with the *Bacillus subtilis* strain RH5 and concluded that the antagonistic activity of this bacterium is caused by the production of hydrolytic enzymes (chitinases, proteases, cellulases) and the synthesis of antimicrobial substances (bacillisin, surfactin, fengycin). In this regard, antibiosis is the most common mode of action of genus *Bacillus* [21].

Of the five fungi that the bacterium was tested against, only *Cylindrocarpon* sp. remained uninhibited until the second day. According to Michel-Aceves *et al.* [22], a shorter contact time (days) between the pathogen and antagonist causes greater aggressiveness in the antagonist and lower resistance in the phytopathogen. In this case, the bacterium only inhibited 45% of the mycelial growth of *Cylindrocarpon* sp.

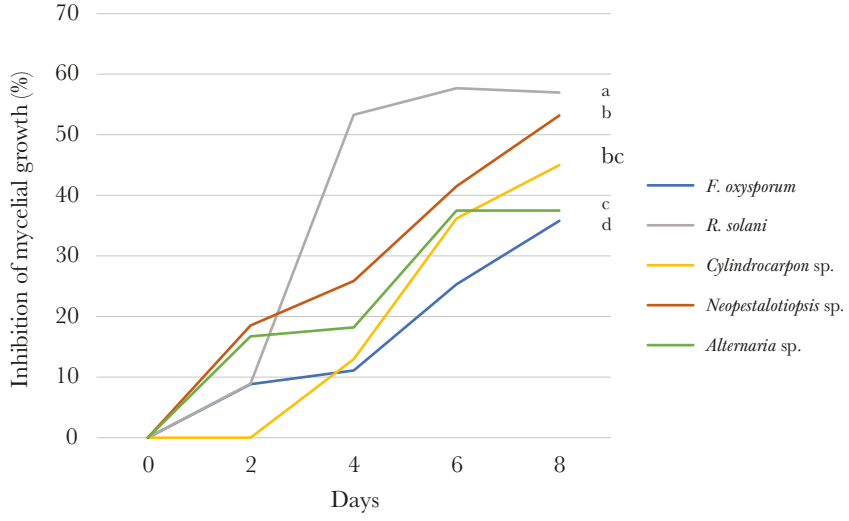

Figure 3. Inhibition percentages of the mycelial growth caused by *Bacillus subtilis* on fungi associated with strawberry dry wilt.

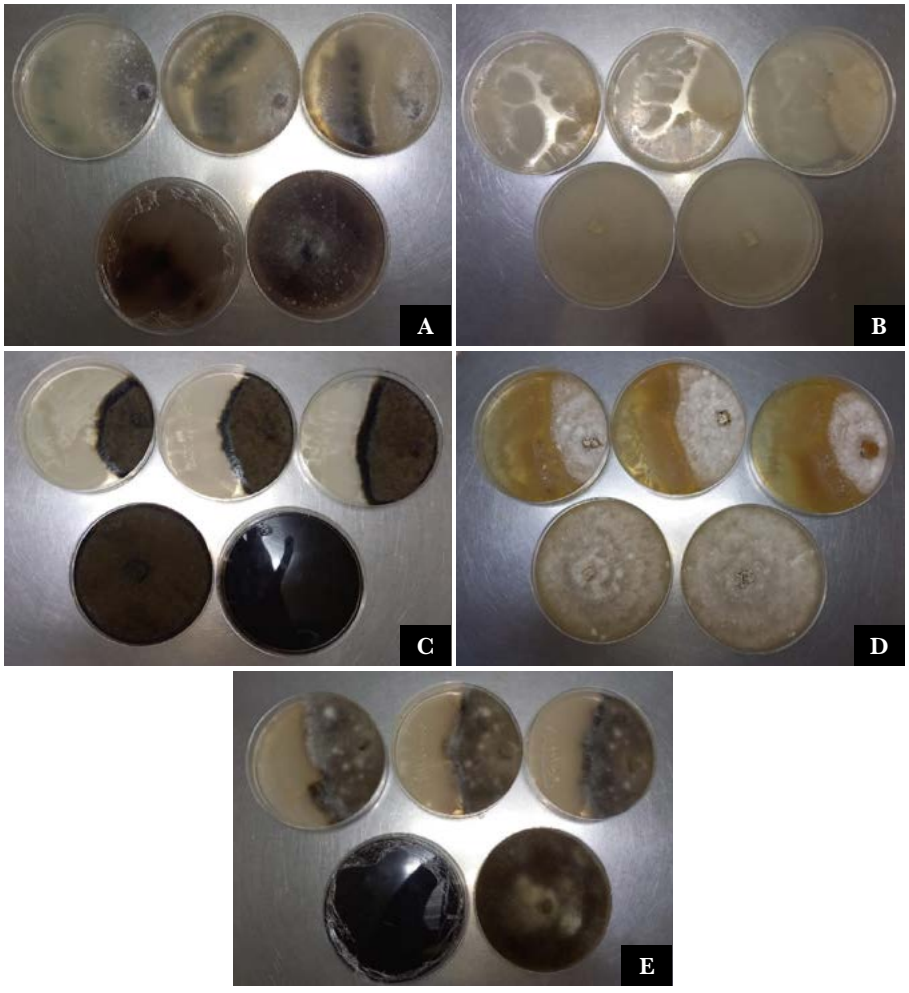

Figure 4. *Bacillus subtilis* confrontation performed in triplicate *vs Fusarium oxysporum* (A), *Rhizoctonia solani* (B), *Cylindrocarpon* sp. (C), *Neopestalotiopsis* sp. (D), and *Alternaria* sp. (E). Growth of control fungi performed in duplicate.

CONCLUSIONS

The most common fungi associated with strawberry dry wilt were *Neopestalotiopsis* sp. (54.7%), *Fusarium oxysporum* (50.6%), and *Rhizoctonia solani* (40.5%). *Trichoderma harzianum* can inhibit the mycelial growth of fungi associated with strawberry dry wilt. It mainly inhibited *Rhizoctonia solani* (over 90%), *Cylindrocarpon* sp., *Fusarium solani*, and *Fusarium oxysporum* (over 80%), and *Neopestalotiopsis* sp. (77.7%). *Bacillus subtilis* showed a lower capacity as an antagonist to inhibit the aforementioned fungi than *Trichoderma harzianum*. In this case, the fungus that was most effectively inhibited by the bacterium was *Rhizoctonia solani*.

ACKNOWLEDGEMENTS

The authors would like to thank the Instituto Politécnico Nacional for the funding provided for the development of project SIP 20240643 which was the basis for this article.

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