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Phytopathological Note

Trichoderma afroharzianum and homeopathic substances on the *in vitro* growth of fungi associated with fungal diseases

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ABSTRACT

Background/Objective. *Trichoderma spp.* is a sustainable alternative, as well as some homeopathic substances that are emerging as potential antifungal agents; making them promising solutions for combating and controlling diseases in crops of economic importance. The objective of the study was to evaluate the antagonism of *Trichoderma afroharzianum* and homeopathic substances against four fungi responsible for diseases of horticultural importance.

Materials and Methods. The antagonism of *T. afroharzianum* against *Phytophthora capsici*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Fusarium oxysporum* was evaluated using the dual culture technique. The percentage of inhibition was estimated every 24 hours by measuring mycelial growth and type of antagonism. Homeopathic substances derived from essential oil of *Tagetes remotiflora*, *Tagetes arenicola*, and *Tagetes lucida* were evaluated using the poisoned agar technique on all organisms. A completely randomized design with four replicates was used. The data were analyzed using Tukey's mean comparison test ($p \leq 0.05$).

Results. *T. afroharzianum* inhibited over 50% of the fungi and stopped their growth in all cases. Only in the interaction between *P. capsici* and *T. afroharzianum* did the antagonist grow over *P. capsici* and deform the hyphae. Homeopathic oil of *T. remotiflora* in 6CH dinamization reduced the growth of *R. solani* (21.95 *vs* 25.38 mm in the control), *F. oxysporum* (18.52 *vs* 21.35 mm in the control), and *T. afroharzianum* (20.87 *vs* 22.86 mm in the control), while 200CH dinamization of *T. arenicola* stimulated the growth of *T. afroharzianum* (25.15 *vs* 22.86 mm in the control), *P. capsici* (23.85 *vs* 16.18 mm in the control) and *B. cinerea* (25.98 *vs* 18.48 mm in the control).

Conclusion. *T. afroharzianum* showed a greater ability to counteract the growth of *P. capsici* and *B. cinerea.* Homeopathic substances may have applications as inhibitors and stimulators of mycelial growth; further research is needed to better guide their applications.

Keywords: Inhibition, Growth, Antagonist, Fungal disease, Agrohomeopathy

INTRODUCTION

Several crops of major economic importance face phytosanitary problems caused by *P. capsici*, *R. solani*, *B. cinerea*, and *F. oxysporum* (Andrade-Hoyos *et al.*, 2019; Cheung *et al.*, 2020). These microorganisms infect a wide range of hosts worldwide and can damage all parts of the plant, including the crown, roots, and fruits (Parada-Rojas *et al.*, 2021). Their ability to remain dormant under unfavorable conditions makes them difficult to control (Senapati *et al.*, 2022). Therefore, it is important to protect plants from the seedling stage through transplantation. At these stages, the use of fungicides has become essential for managing fungal diseases (González *et al.*, 2021). However, excessive use poses risks to the environment and soil by reducing microbiological and biochemical activity. Moreover, many organisms have developed resistance, rendering fungicides ineffective (Baćmaga *et al.*, 2019; Samal *et al.*, 2023). A sustainable alternative is the use of biocontrol agents such as *Trichoderma* spp., which display mycoparasitic activity, compete for space and nutrients, and have shown antagonism against at least 29 fungal species associated with plant diseases (Geng *et al.*, 2022; Guzmán-Guzmán *et al.*, 2023).

T. afroharzianum has been shown to inhibit *R. solani*, *F. oxysporum*, *P. capsici*, and *B. cinerea* (Mokhtari *et al.*, 2017; Mokhtari, 2018; Zhao *et al.*, 2021). However, it has also been reported as a cereal pathogen (Pfordt *et al.*, 2023). Therefore, its use is focused on other horticultural or ornamental species. In addition, the behavior of these species varies depending on the geographic region from which the isolates originate (Miranda, 2022), highlighting the importance of their evaluation.

On the other hand, some homeopathic substances have been proposed as potential antifungal inputs. To date, few studies have evaluated the effectiveness of homeopathic essential oils. Studies such as Oliveira *et al.* (2017) have shown that the homeopathic essential oils of *Eucalyptus citriodora* and *Cymbopogon citratus* reduce the germination of *Alternaria solani* and *Corynespora cassiicola*. Homeopathic substances are safe and low-cost, making them a sustainable option; however, their effects on other fungi and on biocontrol agents such as *T. afroharzianum* must be assessed, since some species of the genus *Tagetes* have fungistatic effects (Serrato *et al.*, 2014). It is proposed to evaluate homeopathics derived from the essential oils of *Tagetes remotiflora*, *T. arenicola*, and *T. lucida*, which will help determine the compatibility of their combined application and the potential use of these substances in future evaluations with other microorganisms. The objective of this study was to evaluate the antagonism of *T. afroharzianum* and homeopathic substances against four fungi associated with diseases of horticultural importance.

Molecularly identified strains were used (Ruíz-González *et al.*, 2024) with the following GenBank accessions: *T. afroharzianum* (PP843541.1), *F. oxysporum* (PP729638.1), *R. solani* (PP713022.1), *P. capsici* (PP922286.1), and *B. cinerea* (PP401673.1). These strains were obtained from the Laboratory of Genetic Resistance at the Universidad Autónoma Chapingo (Texcoco, Mexico). The *T. afroharzianum* strain was isolated from soil samples collected in La Cabaña, Acaxochitlán, Hidalgo (N 20° 08’ 43.63’’ E 98° 09’ 37.32’’). The fungi were reactivated on potato dextrose agar (PDA), and antagonism was evaluated using the dual culture technique on PDA. Discs of 3.5 mm diameter containing inoculum from both the antagonist and the pathogens were obtained with a cork borer. All inocula were five days old and were placed 2 cm from opposite edges of the Petri dishes. Each treatment was replicated four times. Controls consisted of Petri dishes with individual cultures of

each fungus. Incubation in darkness was carried out at 22 °C for *B. cinerea* and 25 °C for *F. oxysporum*, *R. solani*, and *P. capsici*. Mycelial growth was measured every 24 h in the direction of colony expansion until the control plates without antagonist completely covered the Petri dish. The inhibition percentage was calculated using the formula described by Ezziyyani *et al.* (2004).

Antagonism was determined using the following scale: 1. The antagonist grows over the fungus and covers 100% of the Petri dish; 2. The antagonist covers 75% of the Petri dish, inhibits the fungus, and can grow and sporulate on it; 3. Neither organism is dominant, each covers 50% of the surface (antagonist and fungus); 4. The fungus covers 75% of the Petri dish, inhibits the antagonist, and can grow and sporulate on it; and 5. The fungus grows over the antagonist and covers 100% of the Petri dish (Bell *et al.*, 1982).

The homeopathic essential oils were prepared by Dr. Felipe de Jesús Ruíz Espinoza (Professor-Researcher) at the Centro Regional Universitario del Anáhuac, Universidad Autónoma Chapingo. The centesimal scale (CH: centesimal Hahnemannian) was used. In an amber vial, 99 drops of ethyl alcohol (4.95 mL) and one drop of oil (0.05 mL) were placed. Succussion was then performed as described by Maldonado *et al.* (2015) to obtain the 1CH dilution. From the 1CH dilution, one drop (0.05 mL) was taken and mixed with 99 drops of alcohol, followed by succussion, resulting in the 2CH dilution. This process was repeated to obtain the 6CH and 200CH dynamizations evaluated in this study (Maldonado *et al.*, 2015). The homeopathics were prepared individually for each species: *T. remotiflora* (6CH-R and 200CH-R), *T. arenicola* (6CH-A and 200CH-A), and *T. lucida* (6CH-L and 200CH-L) at 6CH and 200CH dynamizations. In 250 mL Erlenmeyer flasks, PDA medium (100 mL) was prepared and combined with 10 drops (0.5 mL) of the corresponding homeopathic substance (each substance was prepared separately). This resulted in six different homeopathic treatments (6CH-R, 6CH-A, 6CH-L, 200CH-R, 200CH-A, 200CH-L) and one control without homeopathics. Each treatment was evaluated individually for each fungus. The fungal inoculum was placed in the center of the Petri dishes and incubated as described above. At this stage, the homeopathic substances were evaluated as a possible antifungal input; therefore, they were not tested in dual culture. Growth (mm day^{-1}) of the microorganisms was measured every 24 h. The experiments were conducted under a completely randomized design with four replications, and the data were subjected to analysis of variance and Tukey's test ($\alpha = 0.05$) in SAS OnDemand for Academics.

T. afroharzianum showed inhibition greater than 67% against *B. cinerea* and *P. capsici*, which was statistically different from the inhibition observed for *F. oxysporum* (63%). In *R. solani*, inhibition reached 64%, with no statistical differences (Figure 1). In this study, inhibition of *B. cinerea* was 13% higher (Figure 1) compared with the results reported by Zhao *et al.* (2021) using the same fungi. This difference may be because *T. afroharzianum* helps degrade oxalic acid, a pathogenic factor of *B. cinerea* (Wu *et al.*, 2022). Reported inhibition by *T. afroharzianum* against *R. solani*, *F. oxysporum*, and *P. capsici* has been 98, 95, and 84.7%, respectively (Mokhtari *et al.*, 2017; Mokhtari, 2018), showing that *T. afroharzianum* can respond differently to the same fungi. From this perspective, *in vivo* evaluation is the next step to confirm the antagonism of *T. afroharzianum*, since species of the same genus are known to promote plant growth, improve nutrient use efficiency, and enhance resistance (Yao *et al.*, 2023).

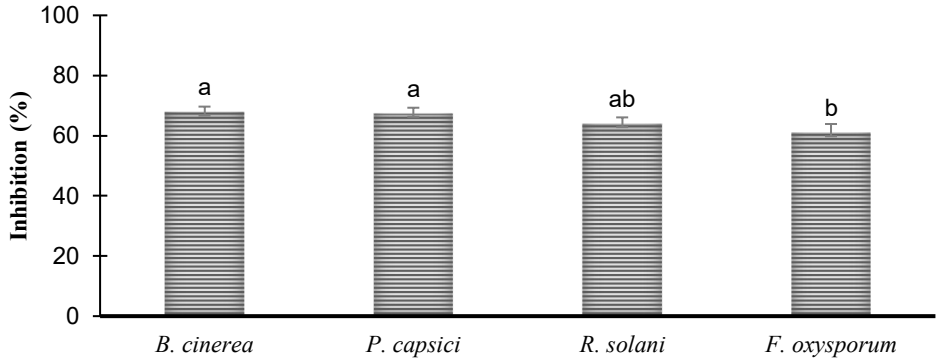

Figure 1. Mycelial growth inhibition of *B. cinerea* (5 days), *P. capsici* (11 days), *R. solani* (8 days), and *F. oxysporu*m (9 days) due to the effect of *T. afroharzianum* under *in vitro* conditions. Mean ± standard deviation (n=4) with different letters are statistically different (Tukey, $p \leq 0.05$).

The mycelial growth of *T. afroharzianum* was twice as high when grown alone (TO) (Figure 2). From the second day onward, statistical differences were observed compared with its growth in dual culture with the other fungi (Figure 2). This may be because some fungi produce bioactive metabolites such as pigments, antioxidants, polysaccharides, and enzymes that can interfere with the growth of other organisms (Shankar and Sharma, 2022).

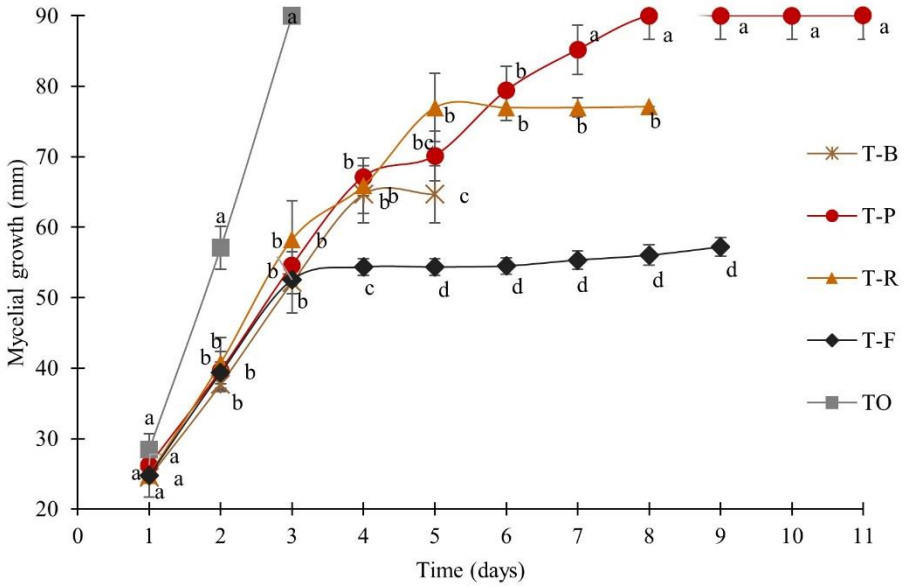

Figure 2. *In vitro* mycelial growth (mm) of *T. afroharzianum* in dual culture against *B. cinerea* (B), *R. solani* (R), *P. capsici* (P), and *F. oxysporum* (F). TO: control, T: *T. afroharzianum*, Mean ± standard deviation (n=4) with different letters are statistically different (Tukey, $p \leq 0.05$).

Hyphal contact between *B. cinerea*, *P. capsici*, and *F. oxysporum* was observed on the fourth day, and in *R. solani* on the third day (Figure 3). In all cases, *T. afroharzianum* (the antagonist) inhibited the growth of the fungi. The interaction between *P. capsici* and *T. afroharzianum* (Figures 2 and 3) showed type one antagonism, where the antagonist grew over *P. capsici*, deformed its hyphae, and completely covered the Petri dish (Figure 3). In *R. solani* and *B. cinerea*, type two antagonism was observed, with the antagonist covering 75% of the Petri dish, inhibiting the growth of both fungi, and sporulating over them (Figure 3). In *F. oxysporum*, type three antagonism was observed; although *T.*

afroharzianum exhibited greater growth (Figure 2), it was not able to grow over *F. oxysporum* (Figure 3). The mycoparasitism observed (Figure 3) may be explained by the secretion of phytotoxic compounds and cell wall–degrading enzymes by *Trichoderma* spp. When these enzymes degrade fungal cell walls, oligomers are released, which in turn induce the production of additional phytotoxic compounds and degrading enzymes (Mukherjee *et al.*, 2012). Cheng *et al.* (2012) reported that the mycoparasitic process of *Trichoderma* spp. involves two steps: an initial stage of hyphal coiling and a later stage of hyphal coiling, each occurring at different rates.

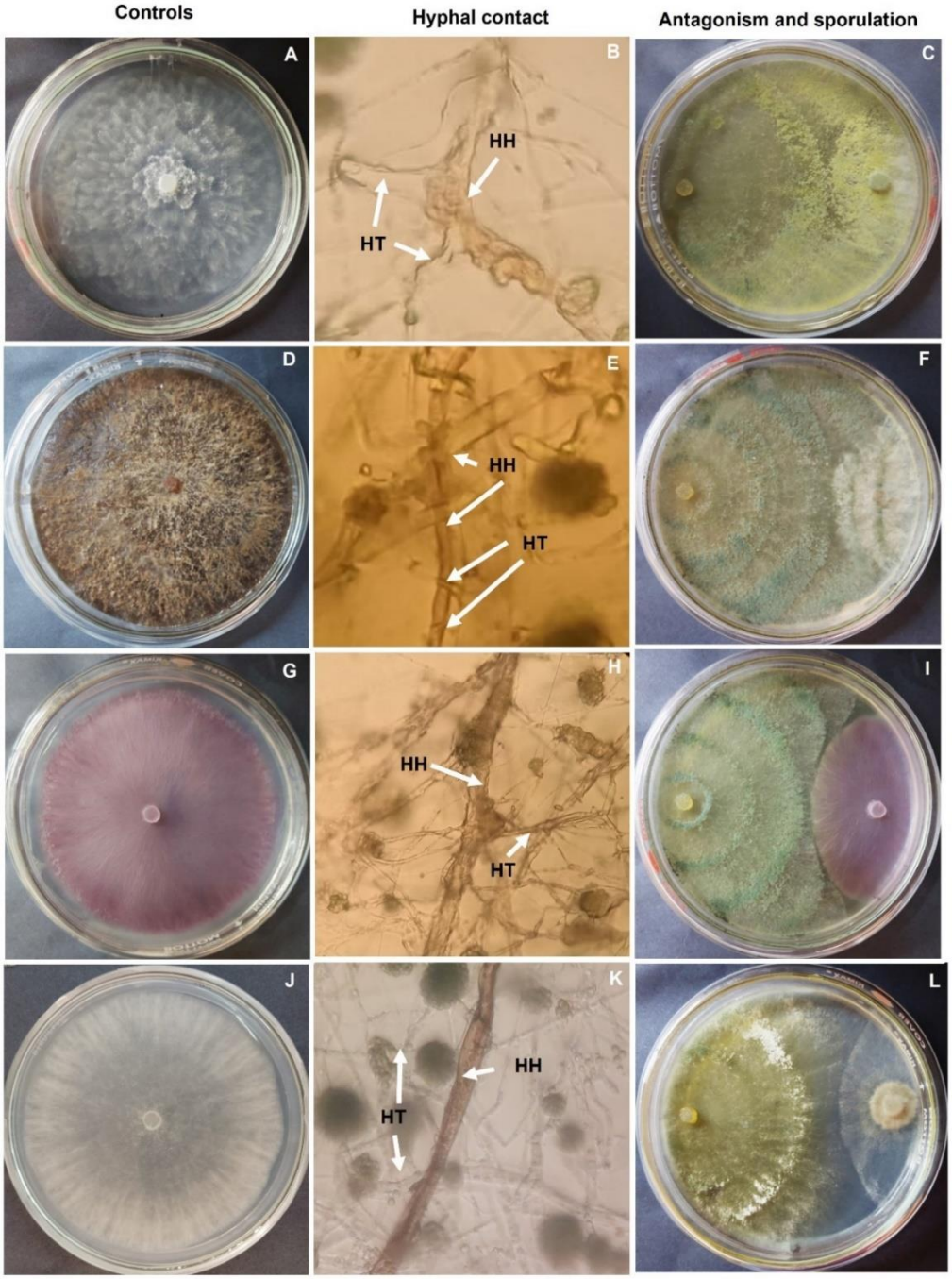

Figure 3. Individual mycelial growth (mm) of controls*: P. capsici* (A), *R. solani* (D), *F. oxysporum* (G) and *B. cinerea* (J) and antagonism in dual culture at the moment of hyphal contact (B, E, H, K) and sporulation after the controls covered the Petri dish (C, F, I, L). HH: fungal hyphae, HT: *T. afroharzianum* hyphae.

On the other hand, the homeopathic substance 6CH of *T. remotiflora* (6CH-R) reduced the growth of *F. oxysporum*, *R. solani*, and *T. afroharzianum*, whereas the 200CH of *T. arenicola* (200CH-A) stimulated the growth of *B. cinerea*, *P. capsici*, and *T. afroharzianum* (Figure 4). *P. capsici* showed the greatest growth with all the homeopathic treatments. In addition, *T. afroharzianum* exhibited increased growth with 6CH-A and 200CH-A. Oliveira *et al.* (2017) tested homeopathics at 6, 12, 30, 60, 100, and 200CH derived from the essential oils of *E. citriodora* and *C. citratus*, and all dynamizations reduced the germination of *A. solani* and *C. cassiicola*. In the present study, the 6CH dynamization also inhibited *F. oxysporum*, *R. solani*, and *T. afroharzianum* (Figure 4). Regarding stimulating effects of essential oils, Ćosić *et al.* (2010) and Simas *et al.* (2017) observed stimulation of *Fusarium subglutinans* and *Penicillium digitatum* with *Rosmarinus officinalis* essential oil and with (−) limonene and γ-terpinene, respectively. Likewise, homeopathics at 12, 24, 30, and 60CH prepared from *Corymbia citriodora* and *Eucalyptus* oil stimulated defense mechanisms in *Phaseolus vulgaris* var. Carioca (Oliveira *et al.*, 2014). A similar effect was observed here with *T. arenicola* (200CH-A), which stimulated the growth of *B. cinerea*, *P. capsici*, and *T. afroharzianum*.

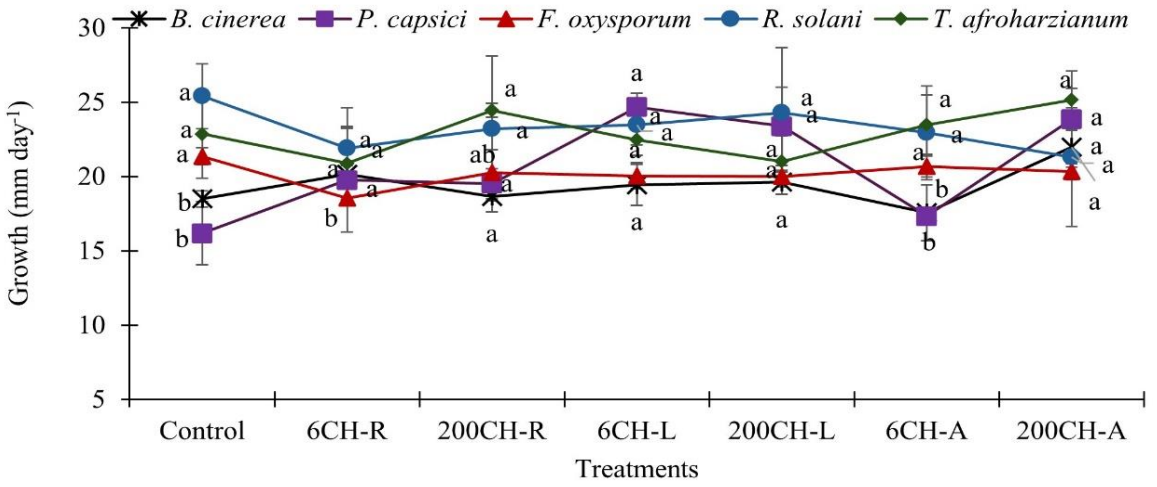

Figure 4. Fungal growth dynamics due to the effect of homeopathic substances on *B. cinerea*, *P. capsici*, *F. oxysporum*, *R. solani*, and *T. afroharzianum*. CH: centesimal hahnemannian, R: *T. remotiflora*, L: *T. lucida*, A: *T. arenicola*. Mean ± standard deviation (n=4) with different letters are statistically different (Tukey, $p \leq 0.05$).

The marked inhibition and antagonism of *T. afroharzianum* against *P. capsici*, *R. solani*, and *B. cinerea*, along with its ability to grow and sporulate over them, confirm its effectiveness as an important biological control agent and a strong candidate for future *in vivo* evaluations. On the other hand, although the homeopathic substances did not show a consistent effect, the 200CH-A preparation of *T. arenicola* stimulated the growth of *T. afroharzianum* (25.15 vs. 22.86 mm in the control), providing an important basis for further studies. While some homeopathic substances (6CH-R) inhibited *F. oxysporum*, *R. solani*, and *T. afroharzianum*, caution should be exercised in their use, since others (200CH-A) also promoted the mycelial growth of *P. capsici* and *B. cinerea*.

Conflict of interest

All authors declare no conflict of interest.

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