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Article

In Vitro Antagonistic Activity of *Trichoderma harzianum* against *Fusarium sudanense* Causing Seedling Blight and Seed Rot on Wheat

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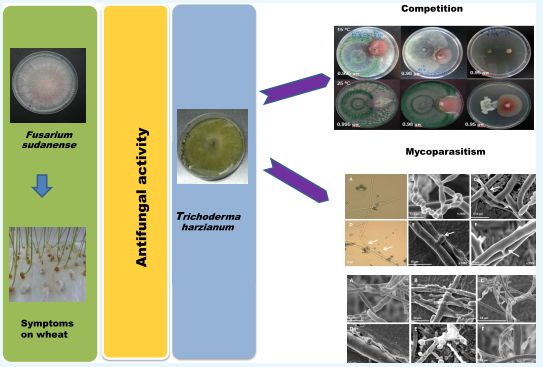
ABSTRACT: *Fusarium sudanense* is a novel fungus recently isolated from asymptomatic samples of wheat grains in Argentina. The fungus caused symptoms of seedling blight and seed rot on wheat after artificial inoculations. It is known that the production of mycotoxins by pathogens belonging to the *Fusarium* genus is harmful to human and animal health. Moreover, the warm and humid conditions that are favorable for growth and mycotoxin production of these species put the Argentinian wheat production area at a high risk of mycotoxin contamination with this novel pathogen. The aim of this work was to evaluate the antagonistic effect of *Trichoderma harzianum* against *F. sudanense* under *in vitro* tests at different environmental conditions. Fungi were screened in dual culture at different water activities (α_w) (0.995, 0.98, 0.95, and 0.90) and temperatures (25 and 15 °C). The growth rate of the fungi, interaction types, and dominance index were evaluated. Also, the interaction between *T. harzianum* and *F. sudanense* was examined by light and cryo-scanning microscopy. *T. harzianum* suppressed the growth of *F. sudanense* at 0.995, 0.98, and 0.95 α_w at 25 °C and 0.995 and 0.98 α_w at 15 °C. Macroscopic study revealed different interaction types between *F. sudanense* and *T. harzianum* on dual culture. Dominance on contact where the colonies of *T. harzianum* overgrew the pathogen was the most common interaction type determined. The competitive capacity of *T. harzianum* was diminished by decreasing the temperature and α_w. At 0.95 α_w and 15 °C, both fungi grew slowly, and interaction type "A" was assigned. Microscopic analysis from the interaction zone of dual cultures revealed an attachment of *T. harzianum* to the *F. sudanense* hyphae, penetration with or without formation of appressorium-like structures, coiling, plasmolysis, and a veil formation. According to our results, *T. harzianum* demonstrated capability to antagonize *F. sudanense* and could be a promising biocontrol agent.



INTRODUCTION

Current agriculture faces several global problems linked with its intensification and loss of biodiversity among other factors, which led to a forced search to look for more sustainable long-term alternatives. Accordingly, it is imperative to investigate more efficient management of available resources, which may result in benefits from environmental and economic points of view. One of these alternatives is to use fungi and their metabolites in the biological control of plant diseases; some of which produce secondary metabolites used to defend plants that are a very important source of chemical diversity.[1,2] There is still much to explore about metabolism, ecology, and the role of bioactive fungi in the expression of diseases in important crops like cereals. This implies a very broad and interesting field to search for secondary metabolites with various activities applicable as a potential source of bio-input as part of the Integrated Disease Management Strategies in Plant Health.[3,4] This potential provides us with a new tool in the current search for sustainable alternatives for agricultural pest management using new environmentally friendly bioproducts. That is, unlike synthetic chemical fungicides, their use does not represent negative effects, such as water and soil contamination or toxicity on pollinating insects, animals, or humans.

Fusarium sudanense S.A. Ahmed, Al-Hatmi & de Hoog is a novel species in the "*Fusarium fujikuroi* species complex" (FFSC)[5] that was isolated from asymptomatic samples of wheat grains in Argentina causing seedling blight and seed rot on wheat.[6] Thus, according to recent references, this suggests that the fungus could be a pathogen in a latent phase.[7] It is also known that latent pathogens may produce disease symptoms in

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certain plant growth stages or under certain environmental or nutritional conditions.

According to Shi et al.,[8] *Fusarium* spp. infects cereal crops in different regions, which causes major yield losses or reduces crop quality worldwide. In wheat diseases, fungi are one of the major limiting factors for the wheat yield and quality.[9] In particular, an asymptomatic period has been registered in the hosts of some species in the *Fusarium* genus. However, these fungi can break latency and switch to a pathogenic lifestyle, which induces symptoms in their hosts.[10] Additionally, the production of mycotoxins by pathogens belonging to the *Fusarium* genus with severe risks to human and animal health, has been reported by different authors.[11,12] Interestingly, mycotoxins produced by fungi belonging to the genus *Fusarium* continue to be discovered on widely known species and/or on novel species.[13]

F. sudanense on wheat grains in Argentina is a new phytosanitary risk that can cause significant losses during the implantation of the crop. The Argentine Pampas is one of the most highly productive areas of food in the world and currently contributes 2% of wheat global production. Moreover, the country is among the main exporters of grain and products from this crop in the world market.[14] However, despite an increased understanding of mycotoxigenic *Fusarium* spp. and mycotoxins in Argentina, knowledge on practices and measures to control both the fungus and its toxins is very limited.[15] For this reason, we focused on new innovative eco-friendly strategies for its control to minimize yield losses and prevent or reduce possible mycotoxin production. Accordingly, biological control by employing beneficial fungi helps reduce environmental contamination and resistance caused by the application of synthetic chemical products. In recent years, many research studies conducted in Argentina have focused on developing alternatives to chemicals for sustainable wheat disease management, such as using biopesticides.[15] In agreement with this new paradigm for disease control, this work aims to evaluate the biological control of *F. sudanense* with a fungal strain belonging to the *Trichoderma* genus, which was extensively studied as an antagonist against different important phytopathogens.[16−21] Interestingly, previous studies conducted in Valencia (Spain) have recorded that *Trichoderma harzianum* (CECT 20736) successfully antagonizes several rice pathogens by *in vitro* assays.[22]

It is also known that both biotic and abiotic factors, such as temperature and moisture, affect fungal growth.[23] Previous ecophysiological studies in *F. sudanense* have demonstrated that fungus growth is affected by temperature and water availability in culture media.[4] However, the impact of such factors for *F. sudanense* in the presence of potential antagonists is currently unknown. Therefore, the impact of the main environmental requirements for fungal growth will provide a better understanding of its behavior in nature when it interacts with other fungi.

The objectives of the present work were to (i) evaluate the effectiveness of *T. harzianum* (CECT 20736) against *F. sudanense* under different water activities and temperature conditions, (ii) macro- and microscopically determine the interaction types between both species under different conditions, and (iii) analyze the mechanisms of action involved in antagonizing the pathogen using light microscopy and cryo-scanning electron microscopy.

RESULTS

Fungal Interaction in the Dual Culture. *F. sudanense* and *T. harzianum* growth was affected by water activity and temperature as shown in Table 1 for the single factors.

Table 1. Analysis of Variance on the Growth Rates of *F. sudanense* and *T. harzianum* at Four Water Activities (α_w), Two Temperatures, and Their Interactions

source of variation	DF[a]	MS[b]	*P* value[c]
repetition	3	0.015	
species	1	135.344	≤0.001
temperature	1	331.467	≤0.001
α_w[d]	3	269.349	≤0.001
species and temperature	1	10.232	≤0.001
species and α_w	3	67.040	≤0.001
temperature and α_w	3	60.400	≤0.001
species and temperature and α_w	3	10.835	≤0.001
species and temperature and α_w	3	0.072	0.358
residual	31	0.065	

[a]DF: degrees of freedom. [b]MS: mean squares. [c]Fisher test ($P \leq 0.05$). [d]α_w: water activity.

Considering the main factors, *T. harzianum* had a higher growth rate compared to *F. sudanense*. The average of the growth rates of both species increased at 25 °C compared to 15 °C (an average of 6.50 and 1.94 mm/day respectively, not shown) and increased with the increment in the water activity.

However, double interactions species × temperature; species × water activity; temperature × water activity and the triple interaction species × temperature × water activity were also significant (Table 1). The significant species × temperature × water activity interaction is represented in Figure 1. At 15 °C, *T. harzianum* had significantly higher growth rate at 0.98 and 0.995 α_w than *F. sudanense*, whereas at 0.95 and 0.90 α_w, no statistically significant differences were detected according to the LSD ($P \leq 0.05$) between both species (Figure 1A). At 25 °C, the highest growth rates also corresponded to *T. harzianum* at 0.995 and 0.98 α_w, whereas at 0.95 and 0.90 α_w, *F. sudanense* showed higher values than *T. harzianum* (Figure 1B).

Macroscopic Study of the Interaction. Figures 2 and 3 show the interactions between *F. sudanense* and *T. harzianum* on the dual culture on PDA adjusted at different α_w values and incubated at two temperatures after four weeks. Initially, both fungi were grown until they came into contact. Later, *T. harzianum* inhibited *F. sudanense* growth at 0.995, 0.98, and 0.95 α_w at 25 °C and at 0.995 and 0.98 at 15 °C. *T. harzianum* grew and surrounded the *F. sudanense* colony at 0.995 and 0.98 α_w at both temperatures (Figures 2 and 3). *T. harzianum* overgrew the *F. sudanense* colony at 0.995, 0.98, and 0.95 α_w at 25 °C (Figure 3). At 15 °C and 0.995 α_w, *T. harzianum* also overgrew and surrounded the *F. sudanense* colony, while at this temperature and 0.98 α_w, *T. harzianum* showed incipient overgrowth of the *F. sudanense* colony. Interestingly, *F. sudanense* produced an intense violet pigment in media under all the evaluated conditions (Figure 2).

Table 2 shows different interaction types and the I_D between *F. sudanense* and *T. harzianum* in dual cultures at different water activities and temperatures after four weeks of incubation. *T. harzianum* antagonized *F. sudanense* at 0.995, 0.98, and 0.95 α_w and 25 °C, whereas it dominated at 0.995 and 0.98 α_w and 15 °C. As shown in Table 2, dominance upon

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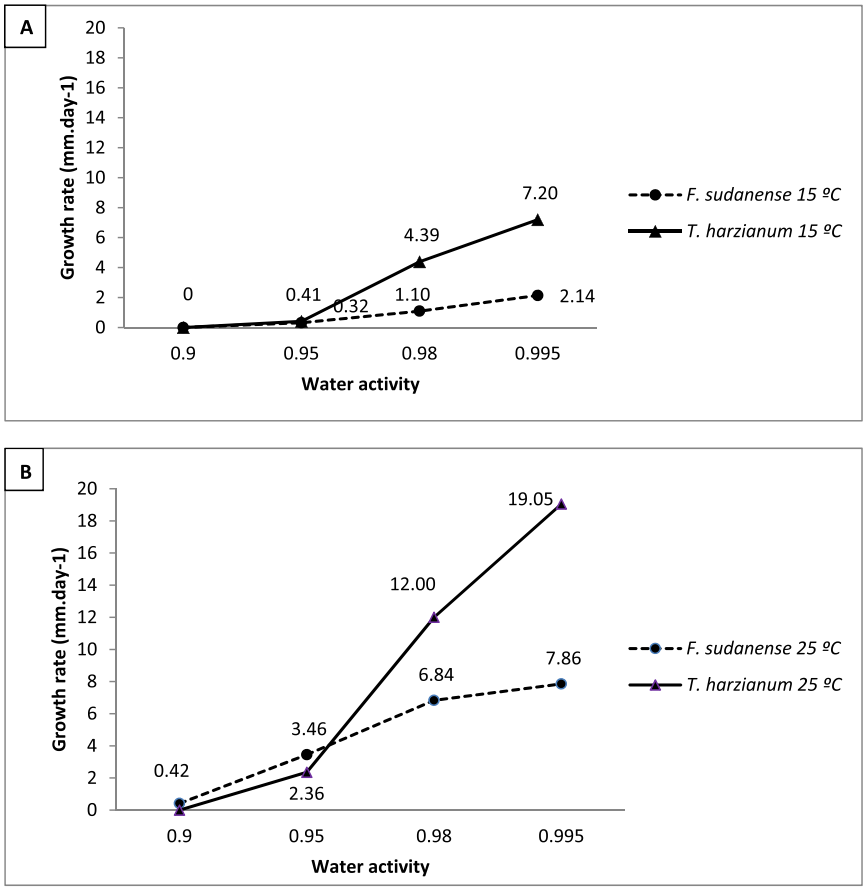

Figure 1. Influence of water activity (α_w) and temperature on the growth rates of *F. sudanense* in dual culture with *T. harzianum*. A: 15 °C, B: 25 °C.

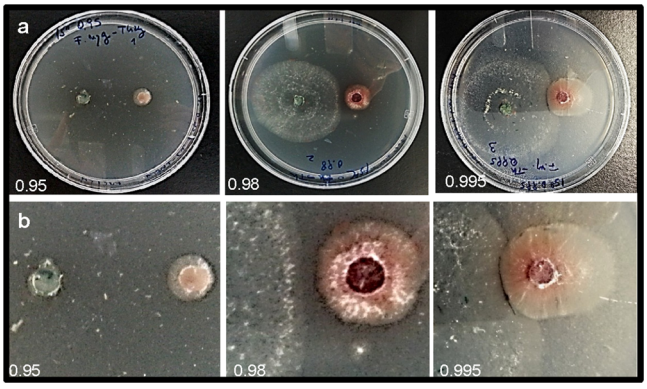

Figure 2. Dual cultures of *F. sudanense* and *T. harzianum* under 0.95, 0.98, and 0.995 α_w at 15 °C. (a) Original plates and (b) enlarged images. *T. harzianum* did not grow at 0.90 α_w.

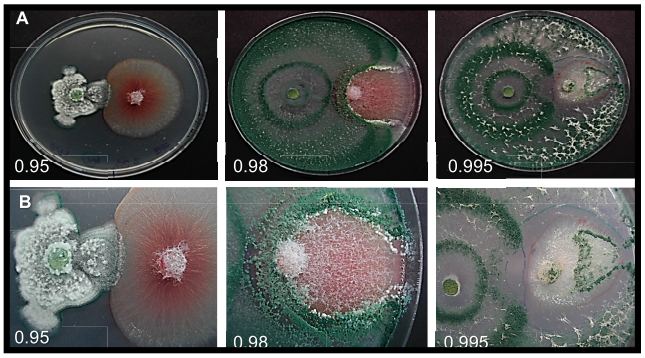

Figure 3. Dual cultures of *F. sudanense* and *T. harzianum* under 0.95, 0.98, and 0.995 α_w at 25 °C. (a) Original plates and (b) enlarged images. *T. harzianum* did not grow at 0.90 α_w.

contact was the main interaction observed on the dual cultures. The interaction "D" type (4/0) was assigned because *T. harzianum* inhibited *F. sudanense* colonies and, as mentioned above, this fungus overgrew the *F. sudanense* colonies after four weeks of incubation, except at 0.95 α_w and 15 °C. The competitive ability of *T. harzianum* diminished as the water activity and temperature decreased. Under these conditions, both fungi grew slowly, and interaction "A" was assigned.

The numerical values assigned to each fungus (I_D) showed that *T. harzianum* was the species that dominated *F. sudanense* except at the lowest α_w. The I_D indicated the antagonistic potential of *T. harzianum* against *F. sudanense*. This index clearly assessed this fungus's ability to reduce *F. sudanense* growth under different water activity and temperature conditions except at 0.95 α_w and 15 °C, and 0.90 α_w.

Microscopic Analysis of the Interaction. The microscopic observations from the interaction zone revealed initial contact between both fungi and the attachment of *T. harzianum* to the *F. sudanense* hyphae. Penetration with or without the formation of appressorium-like structures was also observed (Figure 4A–F). Tight attachment, conidia concentration, and hyphae woven or intertwined into a compact mass with strong hyphal compression were easily observed from the interaction zones section under light and cryo-scanning electron microscopy (Figure 4G–I). The presence of *F.*

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Table 2. Index of Dominance (I_D) and Types of Interaction between *F. sudanense* and *T. harzianum* in Dual Cultures at Different Conditions after Four Weeks of Incubation

		α_w[a]				
temperatures	species	0.995	0.98	0.95	0.90[c]	I_D[b]
25 °C	*F. sudanense*	0	0	0		0
	T. harzianum	4	4	4		12
type of interaction		D	D	D		
15 °C	*F. sudanense*	0	0	2		2
	T. harzianum	4	4	2		10
type of interaction		D	D	B		

[a]α_w: Water activities. [b]I_D: index of dominance according Magan and Lacey (1984).[36] Values were obtained by adding the values assigned to each species according to the interaction with the different water activities. Types of interaction and corresponding values: A - mutual intermingling (1), B - mutual antagonism on contact or with free space between fungus colonies <2 mm (2), C - mutual antagonism at a distance (3), D - dominance on contact (4 for the dominant species, 0 for the inhibited species), and E - dominance at a distance (5 for the dominant species, 0 for the inhibited species). [c]Interaction discarded because there was no growth.

sudanense hyphae with a wrinkled or shrunken appearance, compared to the hyphae grown alone, was observed (Figure 5B,D) (plasmolysis).

The *F. sudanense* hyphae that were coiled by the *T. harzianum* hyphae were observed (Figure 4A,C). Interestingly, a veil-like formation that wrapped false heads and microconidia of *F. sudanense* was observed (Figure 4E,F). Thus, *T. harzianum* antagonized *F. sudanense* with initial contact by coiling and hyphae plasmolysis. Clear evidence for direct mycoparasitism was herein recorded.

DISCUSSION

Growth of *F. sudanense* and *T. harzianum* was significantly affected by water activity and temperature in PDA medium. These results indicated that, when α_w and temperature values were high, both fungi rapidly developed, whereas growth was slower under nonoptimal conditions (α_w and temperature values were low), or no growth was detected after six days.

The optimal conditions for *F. sudanense* growth were similar to those previously described for others *Fusarium* spp., such as *F. oxysporum* f. sp. *lycopersici*, *F. sambucinum*, *F. verticilliodes*, and *F. proliferatum*.[24−26] In addition, different authors have demonstrated the influence of abiotic factors, such as water activity and temperature, on the ability of different *Fusarium* species to germinate, grow, and produce mycotoxins.[27,28]

Our results indicated that *T. harzianum* CECT 20736 suppressed *F. sudanense* growth at 0.995 and 0.98 α_w at 15 and 25 °C. In particular, the growth rates for *T. harzianum* CECT 20736 evaluated corroborate previous observations made by

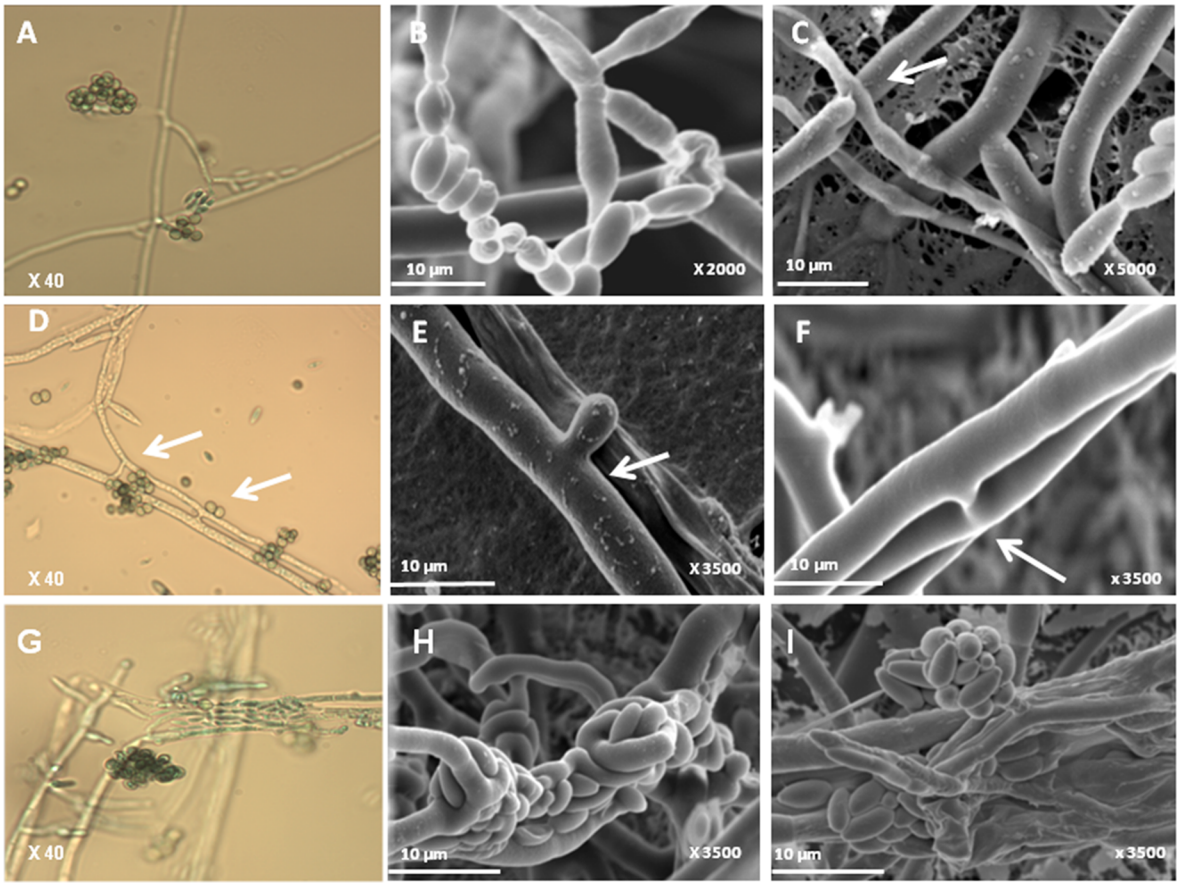

Figure 4. Micrographs of the interaction between *F. sudanense* and *T. harzianum*. (A, D, G) Light micrographs and (B, C, E, F, H, I) cryo-scanning electron micrographs showing the initial contact (A, B) and contact and penetration (C, D, E, F) with or without the formation of appressorium-like structures (E). conidia concentration of F. sudanense (G) and hyphae woven and intertwined into a compact mass with a strong hyphal compression (H, I). The arrow indicates the contact and penetration zones.

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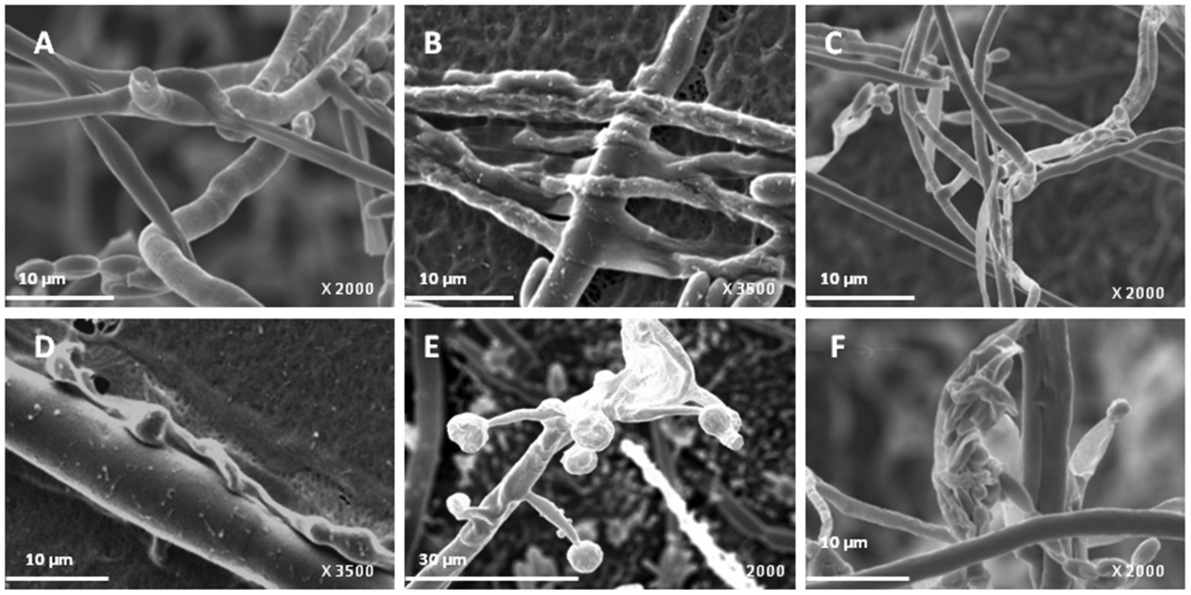

Figure 5. Cryo-scanning micrographs of different types of mycoparasitism used by *T. harzianum* against *F. sudanense*. (A, C) Coiling, (B, D) plasmolysis, and (E, F) veil formation wrapping the false heads and microconidia of *F. sudanense*.

Sempere and Santamarina[22,26] with the same strain and agree with those reported by Santamarina and Roselló[29] using *T. harzianum* LBVB 1010.

According to the results obtained in dual culture assay and our macroscopic study, both fungal species required a high moisture content to achieve their optimal growth. Moreover, the highest *T. harzianum* growth rates versus those of *F. sudanense* allowed us to suggest that this strain offers a very high potential as a biocontrol agent against *F. sudanense* by using competition for space or resources as its main mechanisms. Our microscopic studies also detected that *T. harzianum* used direct mechanisms (mycoparasitism) to control *F. sudanense*, such as penetration, plasmolysis, and coiling among others. These results suggest that the *T. harzianum* strain evaluated in this study could release cellular enzymes capable of penetrating and degrading cell walls and causing plasmolysis, such as chitinase, protease, and glucanase enzymes. Several authors have demonstrated that competition for space or resources and mycoparasitism are the main mechanisms used by different *Trichoderma* species to carry out the biocontrol of fungal phytopathogens.[5,17−19,30−34] The fungal ability to occupy certain ecological niches in nature depends on their capacity to compete against other microorganisms and environmental conditions to successfully develop among other factors.[35] The cited authors recognized a range of interspecific fungal interactions that are influenced by environmental conditions. We herein used the method proposed by Magan and Lacey[36] to determine the type of interaction and the I_D that would indicate the ability of a fungus to dominate under different *in vitro* conditions. Determining in advance how the species behave upon interacting and under different environmental conditions (water availability and temperature) is important since it allows us to know which species will dominate in a specific ecosystem.

Different *Fusarium* species are known to cause latent infections, which are considered one of the highest parasitism levels.[37] Verhoeff[38] stated that true latent infection must involve a parasitic relation that eventually induces symptoms. In agreement with what Sinclair states,[37] knowledge of fungal-causing latent infections contributes to developing more effective disease management measures. Interestingly, we isolated and identified *F. sudanense* from asymptomatic wheat grains of different samples, which causes seedling blight and seed rot.[4] Thus, we suggest that it could be a latent pathogen awaiting specific environmental conditions to switch to a pathogenic state. Consequently, its early detection in grains could avoid disease and loss of wheat crops. It is important to highlight that the environmental conditions for optimal *F. sudanense* growth obtained herein (high humidity and temperate temperatures) and in another study by the same authors[4] occur in the typical agro-ecological wheat region of Argentina until stem elongation, which is growth stage 30 according to Zadoks et al.[39] As *T. harzianum* also grew under these same conditions, we suggest that both fungi can interact and, consequently, *T. harzianum* can dominate *F. sudanense* in field scenarios. These findings are of much importance today because biological control is an environmentally friendly and safer tool to be considered among current integrated disease management strategies applied to wheat crops.

CONCLUSIONS

In conclusion, the results herein presented showed that the *T. harzianum* strain studied had successful biocontrol activity and ability to compete against *F. sudanense*. Competition for space and nutrients and direct mycoparasitism were the mechanisms used by *T. harzianum* for the biological control of *F. sudanense*. The initial contact between both fungi and the attachment of *T. harzianum* to the *F. sudanense* hyphae, its penetration with or without the formation of appressorium-like structures, coiling, mycelial plasmolysis, and a clearly demarcated veil formation were all recorded for the first time.

According to our results, by demonstrating the ability and aggressive competitive nature of *T. harzianum*, we could protect wheat seedlings against *F. sudanense* when applied to seed or soil treatments.

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MATERIALS AND METHODS

Fungal Strains. The *F. sudanense* strain was obtained from asymptomatic wheat grains of the cultivar Klein Yarará at the Laboratory of the CIDEFI, FCAyF, UNLP, Buenos Aires province (Argentina), and incorporated into the Colección Española de Cultivos Tipo (CECT) of Universidad de Valencia (UV, Spain), accession no. CECT 20938; GenBank accession nos. MF540540, MF581918, and MH411162.

The *T. harzianum* strain (CECT 20736) was provided by the CECT (UV), and it was previously obtained from corn grains from samples of Valencia, Spain. The fungus was identified by the Centraalbureau voor Schimmelcultures (CBS), Fungal Biodiversity Centre and included in the Banco de Datos de Biodiversidad de la Comunidad Valenciana (BDBCV-General: 659374) (https://www.gbif.org/occurrence/1501023929).

Both strains were maintained on potato dextrose agar (PDA) at the Laboratorio de Botánica, Escuela Técnica Superior de Ingeniería Agronómica y del Medio Natural (ETSIANM) of UPV, Spain, until used.

Interaction between *F. sudanense* and *T. harzianum*. To evaluate the antifungal activity of *T. harzianum* against *F. sudanense* at different water activities and temperatures, both fungi were kept on 2% PDA and incubated at 25 °C in the dark for seven and five days, respectively. In order to prepare culture media with different water availabilities, PDA (pH 5.5) was used as a basic medium, which was adjusted by adding glycerol and distilled water following the technique by Sempere and Santamarina.[40] The prepared water activities (α_w) were 0.90, 0.95, 0.98, and 0.995. The dual culture technique was used to screen the potential biocontrol of *T. harzianum* against *F. sudanense*. Mycelial disks (8 mm diameter) of each fungus obtained from the edge of the colonies developed on 2% PDA were placed on the opposite side (45 mm apart) in 90 mm Petri dishes containing PDA adjusted to different α_w values. The controls were performed by placing only disks of *F. sudanense* and *T. harzianum* on all the adjusted media. Plates with the same water activity were placed inside hermetic plastic boxes containing a glycerol water solution with an equilibrium relative humidity value identical to the α_w of the plates.[40] Then, they were incubated at 15 and 25 °C in darkness. Treatments were performed by combining the four water activities and incubating at both temperatures. Each treatment was replicated four times. Fungal *F. sudanense* and *T. harzianum* growth was examined at 24 h intervals for six days, and the diameter of the growing colony was measured in two directions at right angles to each other. A linear regression of the increase in radius (in mm) against time (in days) was used to obtain the growth rates (mm/day) for each set of treatments using the computer software Microsoft Excel. An analysis of variance (ANOVA) of the growth rates obtained was performed using GenStat Release 12.1 (2009) where the factors were species, water activities, and temperatures. Means were compared by an LSD test at $P \leq 0.05$.

Macroscopic Study of the Interaction. After obtaining the growth rates, Petri plates were maintained for 4 weeks at 15 and 25 °C to analyze the macroscopic evolution of the fungal species growing in the interaction. At this time, the index of dominance (I_D) of each fungus, which indicates the ability of fungi to compete or dominate under the prescribed environmental conditions, was determined. Previously, numerical values were given to both fungi to obtain the I_D. With these values, interaction types were determined following Magan and Lacey:[36] mutual intermingling (1), mutual antagonism on contact or with a free space among fungus colonies <2 mm (2), mutual antagonism at a distance (3), dominance upon contact (4 for the dominant species, 0 for the inhibited species), and dominance at a distance (5 for the dominant species, 0 for the inhibited species). The results were presented for each α_w and temperature.

Microscopy Study of the Interaction. In order to elucidate the mechanisms of antagonism used by *T. harzianum* against *F. sudanense* under *in vitro* conditions, a dual microculture technique was used. Both fungal species were inoculated 5 mm apart on PDA squares adjusted at 0.995 α_w (99% equilibrium relative humidity), mounted on a glass rod inside 90 mm Petri plates under sterile conditions. Coverslips were placed on the agar. The α_w level was maintained by placing filter paper disks impregnated with a solution at 0.995 α_w and then were aseptically placed on the Petri dishes. Five plates with dual microcultures were incubated at 25 °C for 3−5 days until observation. The controls were the plates with each fungus alone. A microscopic study was performed with the samples of individual fungi and the samples from the interaction zone from the dual microcultures. Examinations were made under a light microscope (Olympus PM-10AK3) at the Laboratorio de Botánica of the ETSIANM of the UPV, Spain.

The cryo-scanning electron analysis was undertaken using dual microculture but no coverslips were placed on the PDA adjusted at 0.995 α_w. Samples of the interaction zone *F. sudanense*−*T. harzianum* were mounted on a stub and frozen on liquid nitrogen. Then, samples were sublimed at −90 °C for 15 min, covered with gold for 30 s according the technique used by Sempere and Santamarina.[41] A scanning electron microscope JEOL JSM 5410 was used at the UPV Electronic Microscopic Service (Spain).

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Notes

The authors declare no competing financial interest.

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