

Antagonistic interactions between fungal rice pathogen *Fusarium verticillioides* (Sacc.) Nirenberg and *Trichoderma harzianum* Rifai

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Received 18 February 2009 / Accepted 28 April 2009

Abstract - *Trichoderma harzianum* has been found to be a competitor and mycoparasite of *Fusarium verticillioides* which causes foot rot disease on rice. The experiment was undertaken macroscopically and microscopically. In total 6 treatments were performed combining three water activities (0.95, 0.98, 0.995) and two temperatures (15 and 25 °C). At all conditions tested, except at 0.95 a_w and 15 °C. *Trichoderma harzianum* acted as biocontrol agent through two synergistic mechanisms - competition and mycoparasitism - over *F. verticillioides*. At 0.95 a_w and 15 °C mutual antagonism by contact was observed. The effect of abiotic factors water activity and temperature on fungal growth was determined.

Key words: *Fusarium verticillioides*, Index of Dominance, mycoparasitism, rice, *Trichoderma harzianum*, water activity.

INTRODUCTION

Foot rot caused by *Fusarium verticillioides* (Sacc.) Nirenberg (teleomorph, *Gibberella fujikuroi*) is one of the oldest and most widespread diseases of rice, occurring in all the major rice growing areas with grain yield losses estimated between 4-20% (Reddy and Sathyanarayana, 2002).

The disease can be observed in the seedbed and the field. Infected seedlings are either taller than normal seedlings or stunted. In mature crops, infected plants may have a few tall, lanky tillers with pale green flag leaves. If the crop survives panicles are empty (Mew and Gonzales, 2002).

Fusarium verticillioides mainly colonize cereal grains before harvest but it may occur in stored grains when the humidity is high. This strain is most likely observed on the entire rice seed (about 57%) (Mew and Gonzales, 2002). Infected grains are discoloured and sometimes pink and white coloured due to the presence of mycelial mass.

This filamentous fungus is an important colonist of rice too, because of its ability to produce several mycotoxins. Has been reported the occurrence of fumonisin B1 and B2 in rice grains (Hinojo *et al.*, 2006).

Exist few data concerning biological control of *F. verticillioides*. Hinton and Bacon (1995) revealed that one isolate of *Enterobacter cloacae* associated, as an endophyte, with corn roots, stems and leaves, is antagonistic toward *F. verticillioides* and other toxic fungi associated with corn. Bevivino *et al.* (1998,

2000) assessed *Burkholderia cepacia* antagonism against this filamentous fungus on maize. Bacon *et al.* (2001) suggested that *Bacillus subtilis* has high potential for controlling this strain but also several fungal diseases of maize. Cavaglieri *et al.* (2004) found that seed maize bacterization with *Azotobacter armeniacus* at 10^6 and 10^7 inoculum levels totally inhibited the *F. verticillioides* counts. Later studies reported the ability of *Bacillus subtilis* to reduce rhizoplane and endorhizosphere *F. verticillioides* colonization at all inoculum and maize root levels tested, and suggested that the combination *Enterobacter cloacae* and *Microbacterium oleovorans* had the potential for the biological control of *F. verticillioides* as a maize seed inoculant (Cavaglieri *et al.*, 2005a, 2005b). *Arthrobacter globiformis* demonstrated important effects on *F. verticillioides* growth and fumonisin B1 suppression *in vitro* (Cavaglieri *et al.*, 2005c).

The experiment was undertaken to evaluate possible biological agent *Trichoderma harzianum* against the rice pathogen *Fusarium verticillioides* under different conditions of temperature, water activity and culture media. Initially a macroscopic study was realized and was completed with the cryo-scanning electron microscopy observations of the mycelial interactions.

MATERIALS AND METHODS

Microorganisms and culture medium. *Trichoderma harzianum* CECT 20736 was isolated from national corn grain samples currently forming part of the fungal collection of the Department of Agroforest Ecosystems in the School of the Rural Environment and Enology.

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Fusarium verticillioides was isolated at the laboratory of Agroforest Ecosystems of the School of Rural Environments and Entomology from samples of rice grain collected from different rice fields and cooperatives of the main rice producing areas in Valencia.

All fungal strains were kept in Potato Dextrose Agar (PDA). The synthetic medium used for the ecophysiological assay was Rice Extract Agar (REA) similar to rice composition with a pH of 5.5. To adjust the medium to the different water activities required, it was modified by adding different amounts of glycerol (Sempere and Santamarina, 2006a).

Determination of growth rates. The growth and antagonistic study of *T. harzianum* against *F. verticillioides* was determined by dual culture technique. Mycelial discs (8 mm diameter) were cut out from actively growing pure cultures of both strains on PDA at 25 °C for 5 days and placed at opposite sides, 45 mm apart, of 90 and 150 mm Petri plates containing REA.

In total 10 treatments were performed combining five water activities (0.85, 0.90, 0.95, 0.98 and 0.995) and two temperatures (15 and 25 °C).

Plates with the same water activities (a_w) were placed in water impermeable plastic containers together with two 100 ml beakers containing a glycerol water solution with an equilibrium relative humidity value identical to the a_w of the plates (Sempere and Santamarina, 2007). In this way, equilibration to the target a_w levels was achieved within 24 h, maintaining a constant relative humidity inside the Petri dishes and also controlling the a_w of the substrate.

The growth was measured after 5 days at intervals of 24 h according to the method described by Sempere *et al.* (2007). To calculate the growth rates ($\text{mm} \cdot \text{day}^{-1}$) a linear regression of the radius (mm) as opposed to the time (days) was carried out. The computer software used was Microsoft Excel 2003.

Each combination of microorganisms at different temperatures and water activities was replicated four times.

Antagonistic activity. Growth rates were obtained, and the plates were kept for 60 days to analyze the macroscopic evolution of the fungal species. The method proposed by Magan and Lacey (1984) was used to determine the type of fungus interaction and the Index of Dominance (I_D).

The interaction of each dual culture at different water activities and temperatures was examined, the type of interaction was determined, and numerical scores were assigned to obtain an Index of Dominance. Mutual intermingling (1); mutual antagonism on contact or with free space between fungus colonies < 2 mm (2); mutual antagonism at a distance (3); dominance on contact (4 for the dominant species, 0 for the inhibited species); dominance at a distance (5 for the dominant species, 0 for the inhibited species).

Analysis of fungal interaction was discarded in this study for water activities of 0.85 and 0.90 since the development of both strains after eight testing weeks was nil or minimal respectively.

Cryo-sem analysis of individual strains and fungal interactions. Microscopic analysis of fungal interaction was carried using the technique of *dual microculture*. Previously the strains were inoculated individually (Sempere and Santamarina, 2006b, 2007). Microscopic examination of *T. harzianum* and *F. verticillioides* grown alone was performed 7-15 days after inoculation and the dual culture between 15-30 days after inoculation.

The experiment was undertaken on rice extract agar and full rice grain. The Bomba rice grain variety was used, typical of the designation of origin Rice of Valencia.

Statistical treatment of data. The analysis of variance (ANOVA) with significance values of $P < 0.01$ was used to determine the influence of parameters water activity (a_w) and temperature (T), and of their interaction ($a_w \times T$) on dual fungal growth rates. STATGRAPHICS Plus 5.0 software (Stat Point, Inc., Herndon, Virginia, USA) was used in the study

RESULTS AND DISCUSSION

Ecophysiological study of *Trichoderma harzianum* and *Fusarium verticillioides*

Maximum growth rate of *F. verticillioides* grown dually occurred at a temperature of 25 °C and 0.995 a_w . The growth colony was of 5.16 mm day^{-1} (Fig. 1).

Sempere *et al.* (2004, 2007) reported the same maximum growth at 0.995 a_w and 25 °C for other rice *Fusarium* sp., *Fusarium culmorum* and *F. sambucinum*, grown individually and jointly others strains. Velluti *et al.* (2000) obtained the highest growth rate for *F. verticillioides* at 0.98 a_w and 25 °C on maize but this water activity was the maximum value experimented.

The minimum amount of water activity producing growth in REA occurred at 0.90 a_w . Although initially development was not registered at 0.95 a_w and 15 °C, and at 0.90 a_w and both temperatures, during the eight testing weeks it was observed. Previously values of 0.87 and 0.89-0.90 a_w have been described

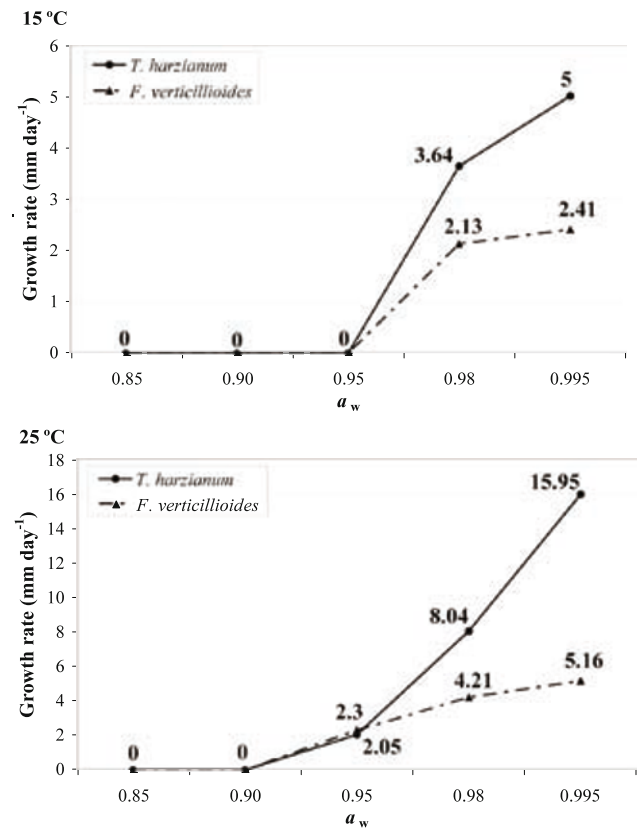


FIG. 1 - Temperature-dependent influence of water activity (a_w) on the growth rate of *Trichoderma harzianum* and *Fusarium verticillioides* grown jointly in Rice Extract Agar.

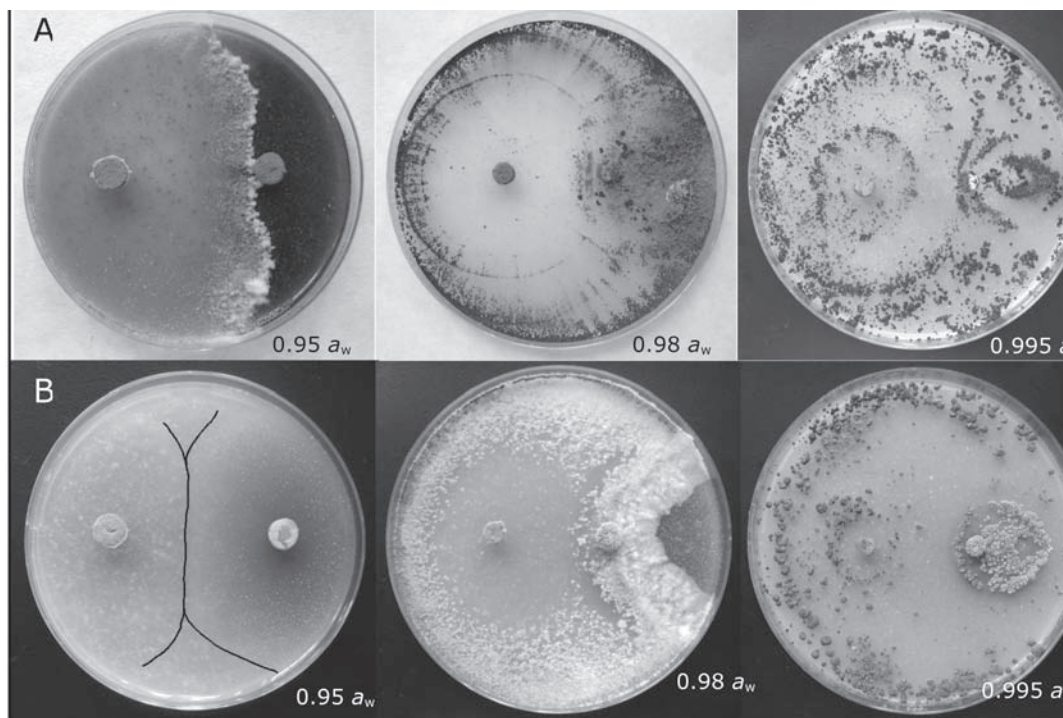


FIG. 2 - Dual cultures between *Trichoderma harzianum* (Left) and *Fusarium verticillioides* (Right) after 8 weeks at different temperatures and water activities. Row A: 25 °C. Row B: 15 °C. Dominance on contact of *T. harzianum* against *F. verticillioides* (0.98 and 0.995 a_w at both temperatures), *T. harzianum* overgrowing *F. verticillioides* on Rice Extract Agar (0.95 a_w 25 °C) and Mutual antagonism of *T. harzianum* and *F. verticillioides* (0.95 a_w 15 °C). — Line of separation between both colonies.

(Woods and Duniway, 1986; Marín *et al.*, 1995; Marín *et al.*, 1998; Pitt and Hocking, 1999).

Similar growth rate of *F. verticillioides* at 0.98, 0.995 a_w at 15 °C and 0.95 a_w at 25 °C was observed (Fig. 1).

The optimum radial growth of *T. harzianum* was at 0.995 a_w in both tested temperatures (Fig. 1). This species also grew to all the water activities, except at 0.85 a_w . Griffin (1963) reported minimal value of 0.91 a_w at 25 °C. This strain co-cultured with *Alternaria alternata* at the same environmental conditions and medium showed similar ecophysiological study (Sempere and Santamarina, 2007).

When comparing both fungi strains, *T. harzianum* presented higher growth rates than *F. verticillioides* at both temperatures, for high water activities studied, 0.995 and 0.98 (Fig. 1). For example, for 0.995 a_w 25 °C, the growth rate of *T. harzianum* was about 15.95 mm·day⁻¹, whereas the growth rate of *F. verticillioides* was 5.16 mm·day⁻¹. Both numerical values registered for species at 0.95 a_w and 25 °C were similar.

The growth rates of *T. harzianum* and *F. verticillioides* increased from 0.90 to 0.995 a_w at both temperatures on Rice Extract agar and grew faster at 25 °C than at 15 °C.

These results are similar to those observed for individual growth (data not shown). Initially fungal interaction did not affect growth rate, when the colonies got in contact, *F. verticillioides* growth rate decreased in particular at the fungal interaction front line (Fig. 2). This observation supported earlier reports that certain strains of *Trichoderma* inhibited *F. verticillioides* growth in other culture media (Calistru *et al.*, 1997a; Yates *et al.*, 1999). In this respect, Yates *et al.* (1999) found that the isolate of the *Trichoderma* sp. suppressed growth of this filamentous fungus colony with time, increasing from the 46% suppression observed on day 6 to a maximum of 91% by day 14.

All water and temperatures differed respect of mycelial growth inhibition caused by *Trichoderma harzianum*. Maximum inhibition of growth of *F. verticillioides* was observed at 0.995 a_w and 25°C. It was due to high growth rates of *T. harzianum* presented in these conditions (Fig. 2).

At 0.95 a_w and 15 °C colony's growth of both species decreased when the individual growth was compared.

Single factors (water activity, temperature) and one-way interaction had a significant effect on fungal growth of *T. harzianum* and *F. verticillioides* ($P < 0.01$) (Table 1). Previous studies have detailed the important influence of abiotic factors such as a_w , temperature on the ability of *Fusarium* species to germinate, grow and produce mycotoxins (Miedaner and Perkowski, 1996; Picco *et al.*, 1999; Hope and Magan, 2003; Llorens *et al.*, 2004).

TABLE 1 - Analysis of variance of the growth rate of *Trichoderma harzianum* and *Fusarium verticillioides*

Factor	DF	MS	F-ratio	P-value
a_w	4	5104.39	102.32	0.0000**
T	1	5975.29	119.77	0.0000**
$a_w \times T$	4	1537.86	30.83	0.0000**

a_w : significance of water activity, T: temperature, and $a_w \times T$: their interaction.

DF: Degrees of freedom, MS: Mean squares.

**Indicates that the factor elicited a significant effect ($P < 0.01$).

Dual cultures

Competition for foods and space was the mechanism macroscopically observed to be adopted by *Trichoderma harzianum* against *Fusarium verticillioides*. *Trichoderma harzianum* suppressed the growth of this strain through the overgrowth at all tested conditions, except at 0.95 a_w and 15 °C (Fig. 2).

This mechanism was also observed when Sempere and Santamarina (2007) confronted this strain against seedborne fungus *Alternaria alternata* at the same water activities and temperatures and when different *Trichoderma* species grew jointly *Rhizoctonia solani* (Shalini *et al.*, 2006).

According to method proposed by Magan and Lacey (1984), in this conditions *T. harzianum* inhibited *F. verticillioides* on contact. For the calculation of the Index of Dominance, *T. harzianum* was assigned a value of 4 and *F. verticillioides* a value of 0 (Table 2).

The first contact between *T. harzianum* and *F. verticillioides* at 0.995 a_w and 25 °C was observed after three days of inoculation. In the Fig. 2 it can be seen the colonies of *T. harzianum*, which aerial mycelium are greenish and whitish in colour and in some conditions formed concentric rings grew over the pink colonies of *F. verticillioides*.

At 0.95 a_w and 15 °C mutual antagonism occurred. Colony growth of *T. harzianum* and *F. verticillioides* was inhibited when both mycelia got in contact. The two fungi were growing until both colonies got in contact; later growth of *F. verticillioides* and *T. harzianum* stopped at the fungal interaction front line. Each fungal strain was assigned a value of 2.

Macroscopically, in all abiotic factors tested, no apparent changes of *F. verticillioides* colonies were observed. Colony morphology appeared similar to that of the fungus in singles cultures at the different temperatures and water activities. When Calistru *et al.* (1997a) co-cultured *F. verticillioides* and different aggressive *Trichoderma* strains (*T. harzianum* and *T. viride*) on PDA, the pink mycelium of *F. verticillioides* produced a white exudate and a yellow pigment, neither of which were observed when the strain grew alone.

The competitive capacity of *T. harzianum* was altered when changing the temperature and the water activity. Results revealed that *T. harzianum* was the dominant species over *F. verticillioides* at 25 and 15 °C (Table 2).

Several researchers have studied the potential of *Trichoderma* species as control organisms for phytopathogenic *Fusarium* strains. Prasad *et al.* (2002) showed that *Trichoderma harzianum* were an effective biocontrol agent of *Fusarium udum*. Thangavelu *et al.* (2004) obtained the same results for *F. oxysporum* f. sp. *cubense*. *Trichoderma asperellum* appeared to be a new alternative of biocontrol of *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (Cotxarrera *et al.*, 2002). Wang *et al.* (2005) demonstrated the effect of *Trichoderma* spp. on control of root rot disease of coneflower caused by *Fusarium*

solani. *Trichoderma harzianum* and *Trichoderma longibrachiatum* were effective in controlling *F. solani*, the cause of peanut brown rot root, in both naturally infested and artificially contaminated fields (Rojo *et al.*, 2007).

Study of mycelial interactions

Cryo scanning electron microscopic observations of *T. harzianum* (Fig. 3) grown alone or jointly *F. verticillioides* revealed that this species sporulated at 0.95, 0.98, 0.995 a_w and both temperatures. Individual colony growth of *T. harzianum* presented smooth and rough septate hyphae with conidia produced successively at the tip of the short inflated phialides (Fig. 3B and 3C). Immature conidiophores showed globose heads (Fig. 3A). Conidia's formation took place into globose heads at the tip of the phialides (Fig. 3A). The examination showed wall's remains between conidia's walls (Fig. 3C and 3D). Conidiophores pyramidally branched (Fig. 3B). Subglobose to short-ovoid conidia rough-walled (Fig. 3C and 3D).

For *F. verticillioides* the same *T. harzianum* environmental conditions of sporulation were obtained. Individually this filamentous fungus, with septate hyphae and simple or branched conidiophores formed microconidia or macroconidia occurred in false heads at the tip of the phialide. Too oval to clavate microconidia with 0-2 septate of *F. verticillioides* were produced in chains. Slender macroconidia with pedicellate basal cell. Conidia smooth-walled.

The dual cultures of *T. harzianum* and *F. verticillioides* at all tested conditions except at 0.95 a_w and 15 °C revealed myco-parasitism of *T. harzianum* over *F. verticillioides*. *Trichoderma harzianum* hypha established contact with the host by coiling around and later penetration into the false heads of macro and microconidia (Fig. 4A and 4B). Sometimes *T. harzianum* came in contact with hypha of *F. verticillioides* some grew parallel to, and penetrating it, coagulates its protoplasm, empties its contents and finally comes out producing sexual structures (Fig. 4C, 4E and 4F). It can be seen observed directly penetration of *T. harzianum* into false heads *F. verticillioides* (Fig. 4D).

Cheetham *et al.* (1997) reported that the mechanism in the interaction between the filamentous fungi *Fusarium culmorum* and *Trichoderma harzianum* was not solely competition for a single resource and suggested parasitism or production of antibiotics or secondary metabolites by antagonist. Observations of hyphal interaction of *T. harzianum*, *T. viride*, *T. virens* and the pathogen *Fusarium oxysporum* sp. *ciceris* indicated that antagonistic hyphae coiled around the hyphae of pathogen and killed them. In this study, occasionally *T. viride* hyphae formed hook or bunch like structures around the hyphae of the pathogen from where penetration took place. Hyphae of antagonist either coiled around the hyphae of the host before penetration or entered directly (Dubey *et al.*, 2007).

TABLE 2 - Index of Dominance (I_D). I_D refers to sum of scores at 25 and 15 °C for *Trichoderma harzianum* competing with *Fusarium verticillioides* based on the interaction scores for each species

Temperature	Fungus species	0.995 a_w	0.98 a_w	0.95 a_w	0.90 a_w	I_D
25 °C	<i>T. harzianum</i>	4	4	4	X	12
	<i>F. verticillioides</i>	0	0	0	X	0
15 °C	<i>T. harzianum</i>	4	4	2	X	10
	<i>F. verticillioides</i>	0	0	2	X	2

Dominance on contact (4 for the dominant species *T. harzianum*, 0 for the inhibited species *F. verticillioides*). Mutual antagonism on contact (2 for both species). X: Analysis of interaction was discarded.

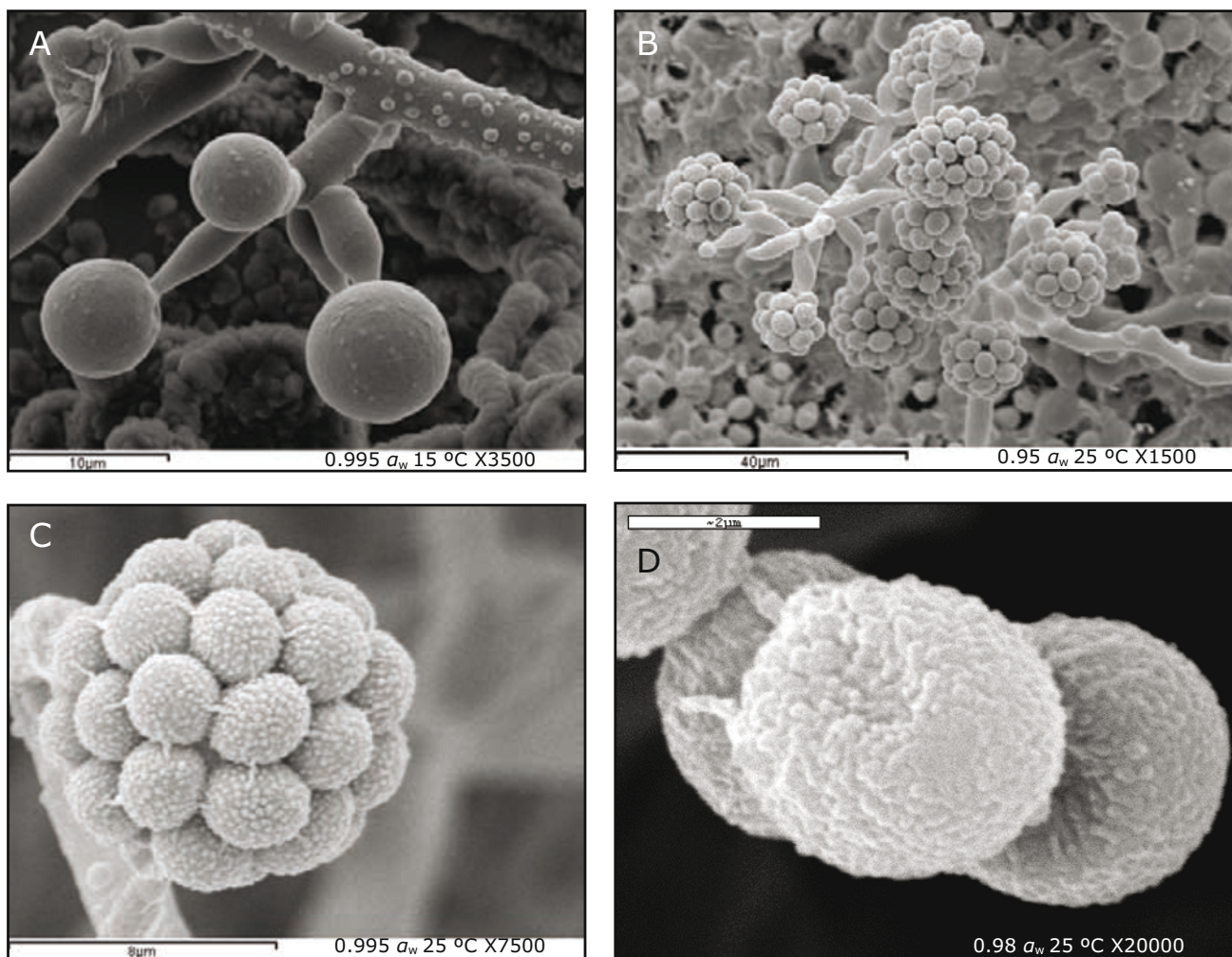


FIG. 3 - Cryo-scanning electron micrographs of morphological features of *Trichoderma harzianum* growth individually at different temperatures and water activities. A: immature conidiophores. Phialides with globose heads borne at the tip. B: conidiophores pyramidally branched with the conidia already formed. C: conidia formed into globose head at the tip of the phialides. D: detail of conidia walls.

Most fungi attacked by *T. harzianum* have cell walls that contain chitin as a structural back-bone and laminarin (β -1,3-glucan) as a filling material. Laminarin is a polymer of D-glucose in a β -1,3 configuration, arranged as helical coils, from which minor polymers of β -1,6-D glucose branch. Fungal cell walls contain more than 60% laminarin (Cohen-Kupiec *et al.*, 1999). The fact that *T. harzianum* penetrated the host mycelium and grew extensively within of *F. verticillioides* suggested cell wall-degrading enzymes, chitinases and β -1,3 glucanases, were produced. Moreover, it could be that other metabolites produced by *T. harzianum* might also have an important role.

Mycoparasitism was not observed between the two isolates at 0.95 a_w 15 °C. The authors suggested that this behaviour was due minimal growth of the species.

This study is the first detailed the formation of conidia of *Trichoderma harzianum* and mycoparasitism of *Fusarium verticillioides* at different environmental conditions. Early reports of *Trichoderma* species and *F. verticillioides* interactions revealed changes to the growth/ morphology of the pathogen in co-culture, no showed in none of the investigated samples did parasitism by *Trichoderma* species. No physical contact between fungi was observed and collapse and loss of turgor of *F. verticillioides* hyphae was produced. The research suggested that this

way was due extracellular metabolites and volatile compounds of antagonistic species (Calistru *et al.*, 1997a, 1997b).

This behaviour could be caused because the authors studied the interaction seven days after inoculation and this period the antagonists did not parasitize the host. Although it could be produced because the used strains in their study did not exert this mechanism.

Recently, has been reported the efficacy of *Trichoderma* sp. in reducing the disease severity index of maize root rot caused by *F. verticillioides* but no exists none investigation of the efficiency of this species on rice (Šrobárová and Eged, 2005).

Mycoparasites are fungi that can parasitize other fungi. This term is generally used to include parasites that coil around the host hyphae or overgrow other colonies on agar (Paul, 1999). Competition may play an important role in fungal interactions, and may occur as an independent phenomenon or in combination with other mechanisms (Whipps and McQuilken, 1993). In the present study *T. harzianum* exert an effect synergistic, competition and mycoparasitism, over *F. verticillioides* at all temperatures and water activities tested except at 0.95 a_w . The authors suggest that *T. harzianum* could be a good biocontrol agent of rice pathogen *Fusarium verticillioides*.

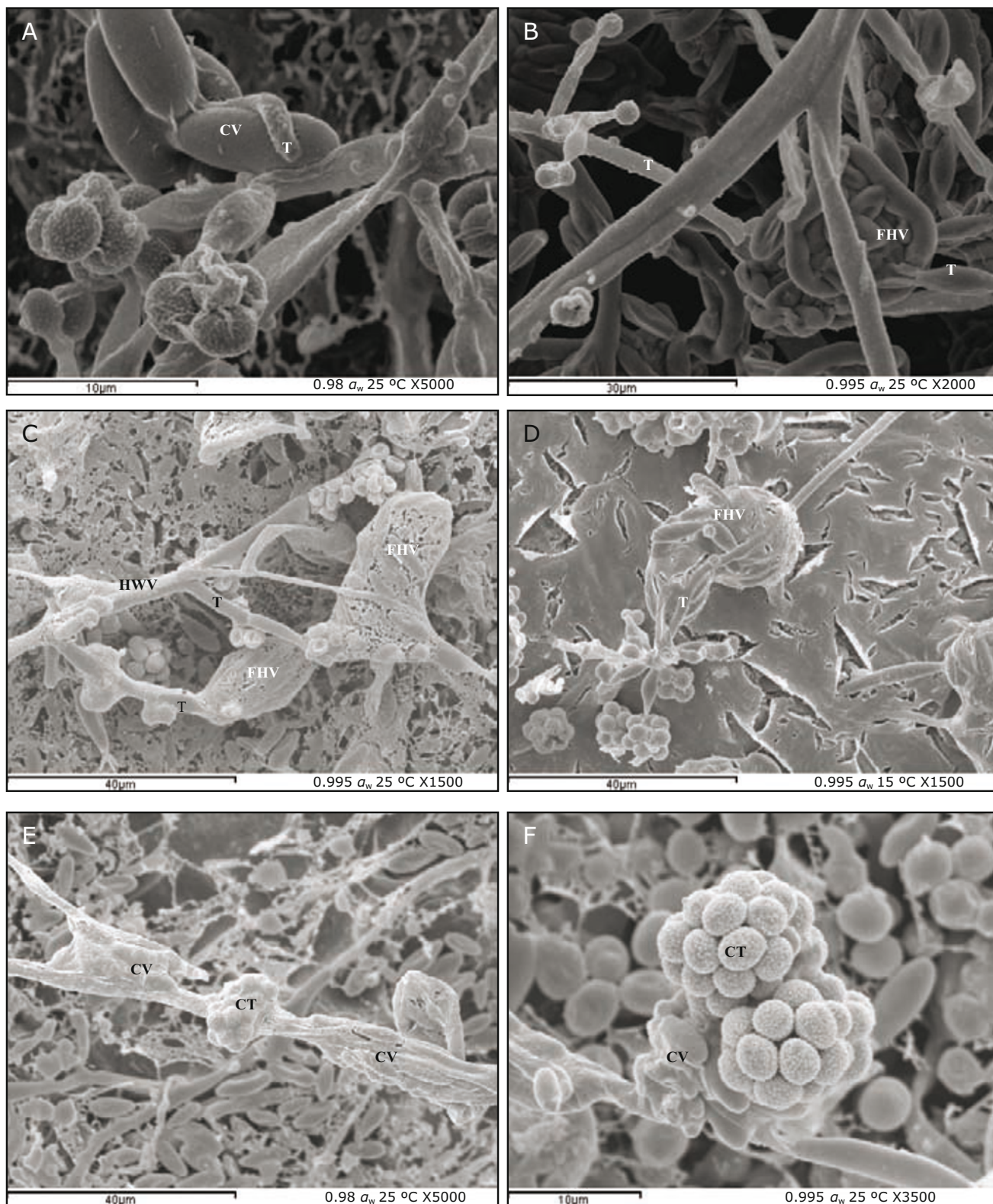


FIG. 4 - Cryo-scanning electron micrographs of dual culture of *Trichoderma harzianum* and *Fusarium verticillioides* at different temperatures and water activities. A: hypha of *T. harzianum* recognizing and coiling around *F. verticillioides* conidia. B: hyphae of *T. harzianum* coiling around and penetrating directly false head of *F. verticillioides*. C: hyphal wall degraded of *F. verticillioides* by the penetration and growth of *T. harzianum* inside it. D: directly penetration of *T. harzianum* inside false head in formation of *F. verticillioides*. E: conidia of *T. harzianum* coming out of hypha of *F. verticillioides*. F: conidia of *T. harzianum* coming out of *F. verticillioides* false head degraded. **CV**: conidia of *F. verticillioides*. **T**: hypha of *T. harzianum*. **FHV**: false head of *F. verticillioides*. **HWV**: hyphal wall of *F. verticillioides*. **CT**: conidia of *T. harzianum*.

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