

***In vitro* biocontrol analysis of *Alternaria alternata* (Fr.) Keissler under different environmental conditions**

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Abstract

The species *Trichoderma harzianum* was analyzed as possible biocontrol agent of *Alternaria alternata* under different environmental conditions (water activity and temperature). The strains were analyzed macroscopically to obtain the Index of Dominance. The analysis was completed using two microscopic techniques. *T. harzianum* showed dominance on contact over *A. alternata* at all testing temperatures and water activities tested except at 0.95 a_w and 15 °C, at which *T. harzianum* inhibited *A. alternata* at a distance. Biocontrol was governed by different mechanisms such as competition for space and nutrients, mycoparasitism, and possible antibiosis. Temperature and water activity significantly influenced fungal growth rate.

Key words: *Alternaria alternata*, Index of Dominance, rice, *Trichoderma harzianum*, water activity

Introduction

The composition and aspect of pre- and post-harvest rice grains may be affected by a group of microorganisms causing grain deformation and/or pigmentation. This grain alteration is commonly known as “pecky rice” [1].

The main agents responsible for this phenomenon are fungal species, among which is *Alternaria alternata* (Fr.) Keissler. This filamentous fungus generates small dark brown spots or specks that partially cover the surface of the lemma, palea and sterile lemmas, or even affect the inside of the grain. The resulting effects are a reduction in the seedlings size, loss of germination power, weight loss, brittle grains that fracture during grinding due to the resulting gypsum-like texture, and particularly the possible generation of biologically undesirable active compounds such as the mycotoxins.

Although the presence of a fungal species depends on many different factors, e.g.,

production region, variety, soil properties, farming techniques, state of the plant, climate, harvesting conditions, harvesting technique, drying system, conservation procedure in the storehouse. *Alternaria* is frequently found in the Valencian rice fields and in 1999 was the third more important fungal species affecting rice in this area [2]. Similarly, a study performed over rice samples from a different storage period revealed the presence of this fungal species in percentages of about 58%, 72% and 82% in rice varieties as common as Senia, Bahía and Bomba, respectively [3].

The fight against rice pests and diseases in the Valencian region is conditioned by the particular environmental characteristics of the land, as it belongs to the Natural Park of la Albufera. In this sense, it is important to study alternative pesticides such as biological control agents that minimize environmental impact.

The filamentous fungus *Trichoderma harzianum* is a mycoparasite that has been successfully tested as a biocontrol agent over a wide variety of

airborne and soilborne plant pathogens [4]. Among them are *Fusarium oxysporum* f.sp. *lycopersici*, *Botrytis cinerea*, *Pseuoperonospora cubensis*, *Sclerotinia sclerotiorum*, *Sphaerotheca fusca*, *Lasiodiplodia theobromae*, *Rhizoctonia solani* [5–9]. We possess at our laboratory a strain of *T. harzianum* currently under patenting process that has controlled other fungal species.

The aim of this work was the analysis of the possible antagonistic effect of *T. harzianum* over *A. alternata* under different conditions of temperature and water activity. The experiment was undertaken macroscopically and microscopically using both light microscopy (LM) and cryo-scanning electron microscopy (cryo-SEM).

Materials and methods

Fungal isolates

Alternaria alternata BVB 2102 was isolated at the laboratory of Agroforest Ecosystems of the School of Rural Environments and Enology from samples of rice grain collected from different rice fields and cooperatives of the main rice producing areas in Valencia.

Trichoderma harzianum BVB 1010 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.

All fungal strains were kept in Potato Dextrose Agar (PDA).

Culture medium

For the *in vitro* analysis rice extract agar (REA) was used as base medium [10]. To set the water activity (a_w), different amounts of glycerol and distilled water were added to the medium following the procedure described by Sempere and Santamarina [10]. The medium was aseptically distributed in Petri plates 90 and 150 mm in diameter.

Measurement of mycelial growth

Each Petri plate was inoculated with two disks (8 mm diameter) of each fungus species 45 mm apart, obtained from the growing margins of pure

colonies of *T. harzianum* and *A. alternata* grown in PDA at 25°C for 5 days; plates were incubated for 8 weeks at 15 and 25°C.

To maintain the water activity during the testing period, Petri plates of the same value were placed in polyethylene boxes containing solutions with the corresponding water activity value –0.995, 0.98, 0.95 and 0.90: 3, 11, 23.5, and 42 g glycerol/100 mL of distilled water, respectively. Water activity was checked using an Aqualab (Decago, Inc., Pullman, WA, USA).

Petri plates were examined daily, and the diameter of the growing colonies was measured in two directions at right angles to each other. Measurements were carried out for 5 days. In all cases the linear regression of increase in radius (in mm) against time (in days) was used to obtain the growth rates ($\text{mm} \cdot \text{day}^{-1}$) for each set of treatment conditions. The computer software used was Microsoft Excel 2003. The experiment was repeated four times.

Type of fungus interaction and determination of Index of Dominance (I_D)

Fungal growth rate was observed, and the plates were kept for eight weeks to analyze the macroscopic evolution of the fungal species. Finally, fungal interaction mode was identified, and each fungus was given a numerical value: 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). The Index of Dominance was developed to measure the ability of a species to dominate under a particular set of environmental conditions [11].

Assessment of antibiosis

The antibiosis zone was measured as a growth inhibition halo around *A. alternata* (mm). Treatments were compared with the diameter of each *A. alternata* control.

Microscopic analysis of fungal interaction

Microscopic analysis was performed over REA and full rice grain. The rice grain variety used in

the experiment was Bomba, typical of the designation of origin Rice of Valencia.

For the analysis in the synthetic medium, both fungal species were inoculated in REA squares 5 mm apart, mounted on a glass slide in a glass rod inside a Petri Plate of 90 mm under conditions of total asepsis. A coverslip was placed on the film. To maintain the levels of water activity, filter-paper disks impregnated with different water activity levels (0.995, 0.98 and 0.95 a_w ; 2.5, 11 and 23.5 g glycerol/100 mL distilled water) were aseptically placed on the Petri plates. After 5 days, the slide was then observed by light microscope.

For the analysis of fungal interaction in rice grains, first the samples were sterilized with a solution of sodium hypochlorite. For setting the water activities, the rice grains were deposited 48 h in the solutions described above. Water activity was measured and set with an Aqualab (Decagon, Inc., Pullman, WA, USA). The rice grains were deposited on a glass slide as described above. The fungal species were inoculated every 3 mm and were left to grow for 15 days. The experimental program was developed under conditions of total asepsis. The sample was mounted on a stub and was frozen in liquid nitrogen. It was subsequently sublimed at $-90\text{ }^{\circ}\text{C}$ for 15 min, covered with gold for 30 s, and visualized by scanning microscope at 10 kV.

Statistical analysis

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

Growth study

Maximum growth rate in both fungal species occurred at a temperature of $25\text{ }^{\circ}\text{C}$ and a water activity of 0.995, with values of 17 mm day^{-1} for *T. harzianum*, and of 6 mm day^{-1} for *A. alternata*. These results are similar to those observed for individual growth (data not shown) or to those

reported by Santamarina et al. in their ecophysiological study of *T. harzianum* in PDA at different temperatures and water activities [12].

At $25\text{ }^{\circ}\text{C}$ *T. harzianum* exhibited higher growth rates than *A. alternata* for all water activities tested, except at 0.90 a_w . These differences were greater with increasing water activities. At $15\text{ }^{\circ}\text{C}$ both fungal species showed a similar behaviour, except at 0.95 a_w at which *A. alternata* also exhibited a higher growth rate (Figure 1). *T. harzianum* and *A. alternata* did not show any growth at 0.85 a_w during the 8 testing weeks.

The statistical analysis of variance (ANOVA) revealed that the individual parameters, a_w and T, and their interaction, ($a_w \times T$), have a significant effect on the fungal growth rate of both species when growing together ($P < 0.01$) (Table 1).

According to Lacey and Magan [13], water activity and temperature greatly affect fungal growth. Temperature affects the growth of the mycelium and the formation and germination of

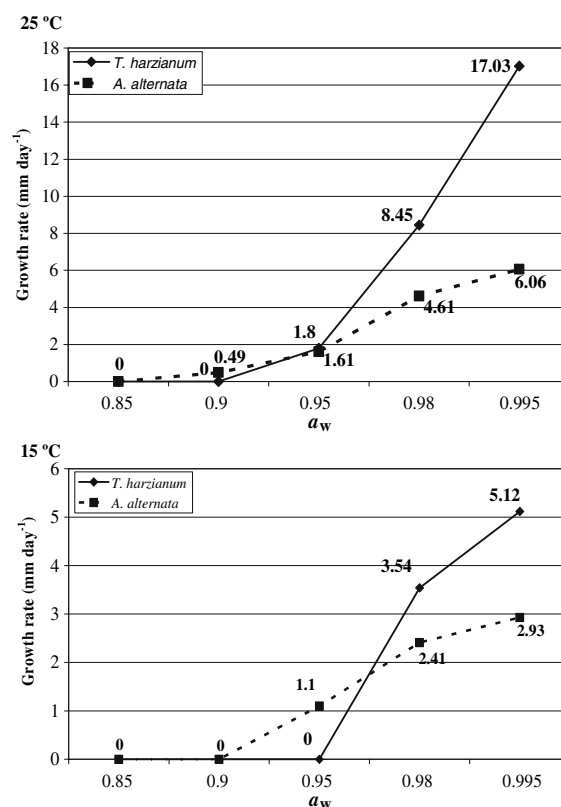


Figure 1. Temperature-dependent influence of water activity on the growth rate of *T. harzianum* and *A. alternata* grown jointly in Rice Extract Agar.

Table 1. Analysis of variance of the growth rate of *T. harzianum* and *A. alternata*; significance of water activity (a_w), temperature (T) and their interaction ($a_w \times T$)

<i>T. harzianum</i>				
FACTOR	DF	MS	F-ratio	P-value
a_w	4	6372.76	74.66	0.0000**
T	1	5434.03	63.66	0.0000**
$a_w \times T$	4	1822.11	21.35	0.0000**
<i>A. alternata</i>				
a_w	4	1403.2	85.35	0.0000**
T	1	631.901	38.44	0.0000**
$a_w \times T$	4	131.595	8.00	0.0000**

DF, Degrees of freedom; MS, Mean squares
**Indicates that the factor elicited a significant effect ($P < 0.01$)

the conidia. Water availability determines whether a fungal spore can germinate, the speed of germination and the metabolic and gas exchange activity of the fungus [13].
On the other hand, although initially fungal interaction did not affect growth rate, when the colonies got in contact, *A. alternata* growth rate decreased in particular at the fungal interaction front line. These results coincide with those

reported by Mónaco et al. when confronting *A. alternata* against different strains of *Trichoderma harzianum* in PDA [14]. This culture medium showed a reduction of 41–61% in the mycelial growth of *A. alternata*. On the other hand, Rocco and Perez reported an increase in the growth rate of *T. harzianum* when both fungal species were confronted in different culture media, probably induced by the presence of *A. alternata* [15].

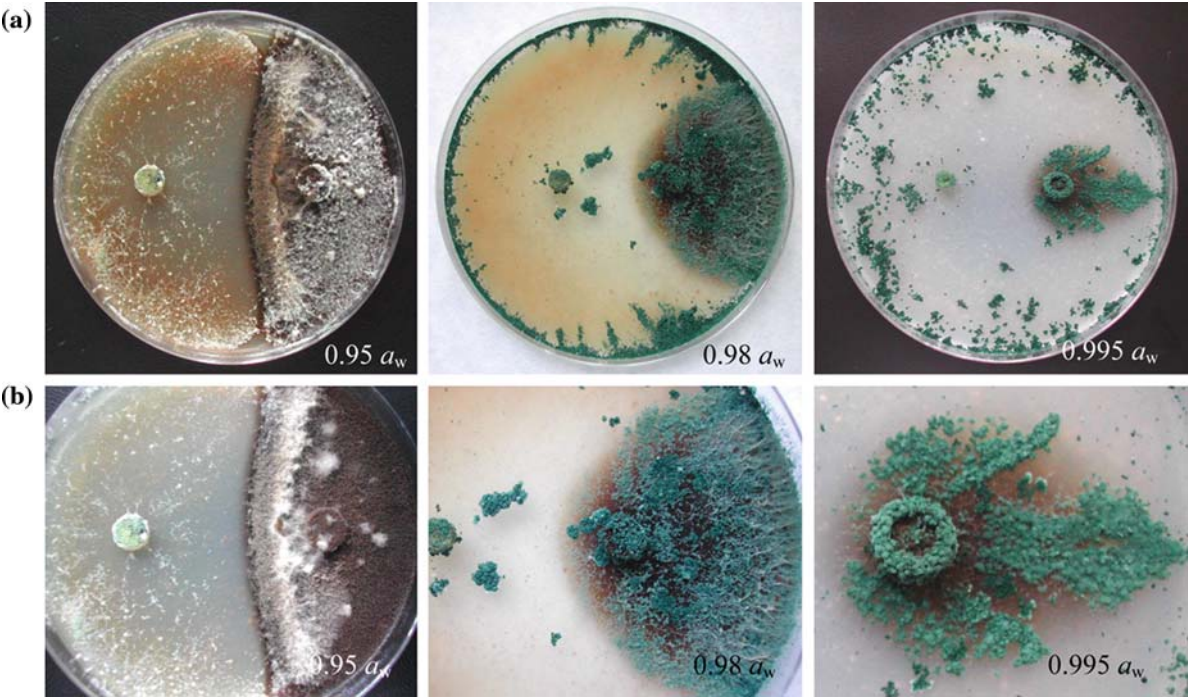


Figure 2. Interaction between *T. harzianum* and *A. alternata* after 8 weeks at 25°C and at different water activities Row (a) *T. harzianum* overgrowing *A. alternata* on Rice Extract Agar. Row b: Detail of fungus interaction. Left: *T. harzianum*. Right: *A. alternata*.

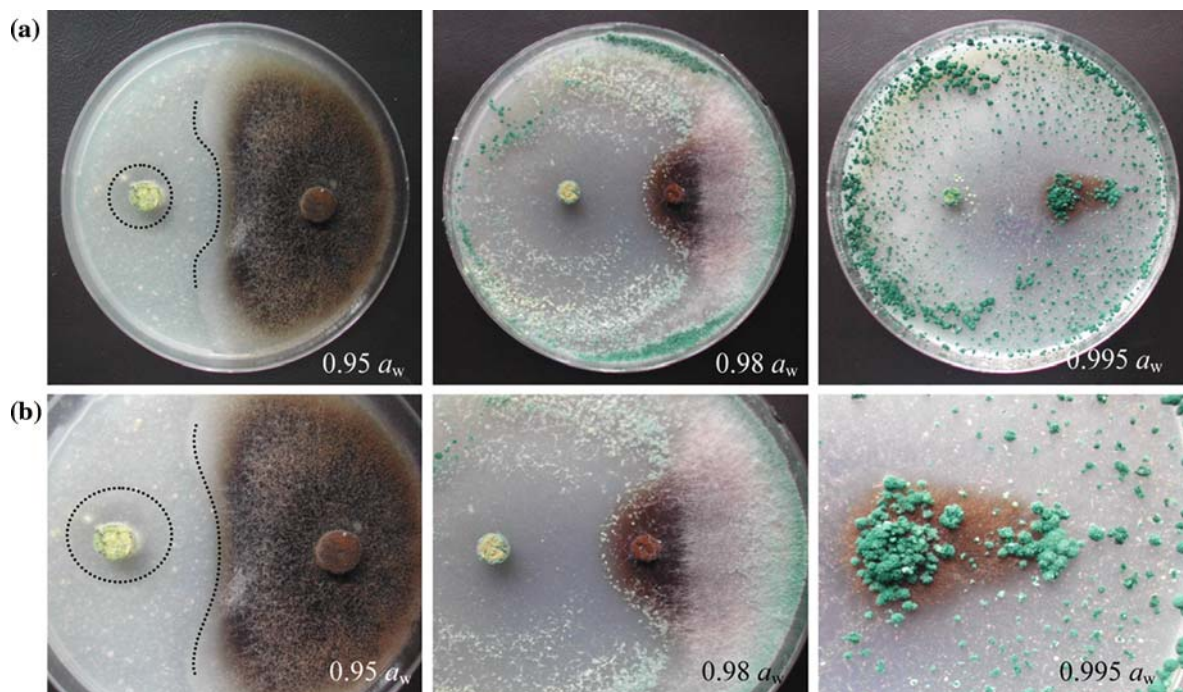


Figure 3. Row (a) Interaction between *T. harzianum* and *A. alternata* after 8 weeks at 15°C and at different water activities. Row (b) Detail of fungus interaction. Left: *T. harzianum*. Right: *A. alternata*. (0.98 and 0.995 a_w) Dominance on contact of *T. harzianum* against *A. alternata*. *T. harzianum* overgrowing *A. alternata* on Rice Extract Agar. (0.95 a_w) *T. harzianum* inhibiting *A. alternata* growth at a distance. ---Line of separation between both colonies.

Macroscopic study of fungal interaction

The same type of fungal interaction was obtained for all water activities and temperatures analyzed, except at 0.95 a_w and 15°C.

In the first case, *T. harzianum* inhibited *A. alternata* on contact. The two fungi were growing until both colonies got in contact; later, *T. harzianum* grew through *A. alternata* (Figure 2 and 3). These results coincide with those reported by Santamarina and Roselló when they confronted the same antagonist species over *Verticillium dahliae* and *Rhizoctonia solani* [8].

For the calculation of the Index of Dominance, *T. harzianum* was assigned a value of 4 and *A. alternata* a value of 0.

According to method proposed by Magan to Lacey [11], at 0.95 and 15 °C, mutual antagonism of the species at a distance was observed, with a separation between both fungal species of more than 2 mm (Figure 3). Each fungal strain was assigned a value of 3. The authors have found that antagonism is not mutual, but is produced by *T. harzianum* over *A. alternata*. Thus when comparing the growth rates of *A. alternata* grown individually with the results obtained from the

Table 2. Index of Dominance (I_D). I_D refers to sum of scores at 25 °C and 15 °C for *T. harzianum* competing with *A. alternata* 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>A. alternata</i>	0	0	0	X	0
15°C	<i>T. harzianum</i>	4	4	3	X	11
	<i>A. alternata</i>	0	0	3	X	3

Dominance on contact (4 for the dominant species, 0 for the inhibited species). Mutual antagonism at a distance (3 for both species). (X) Analysis of interaction was discarded

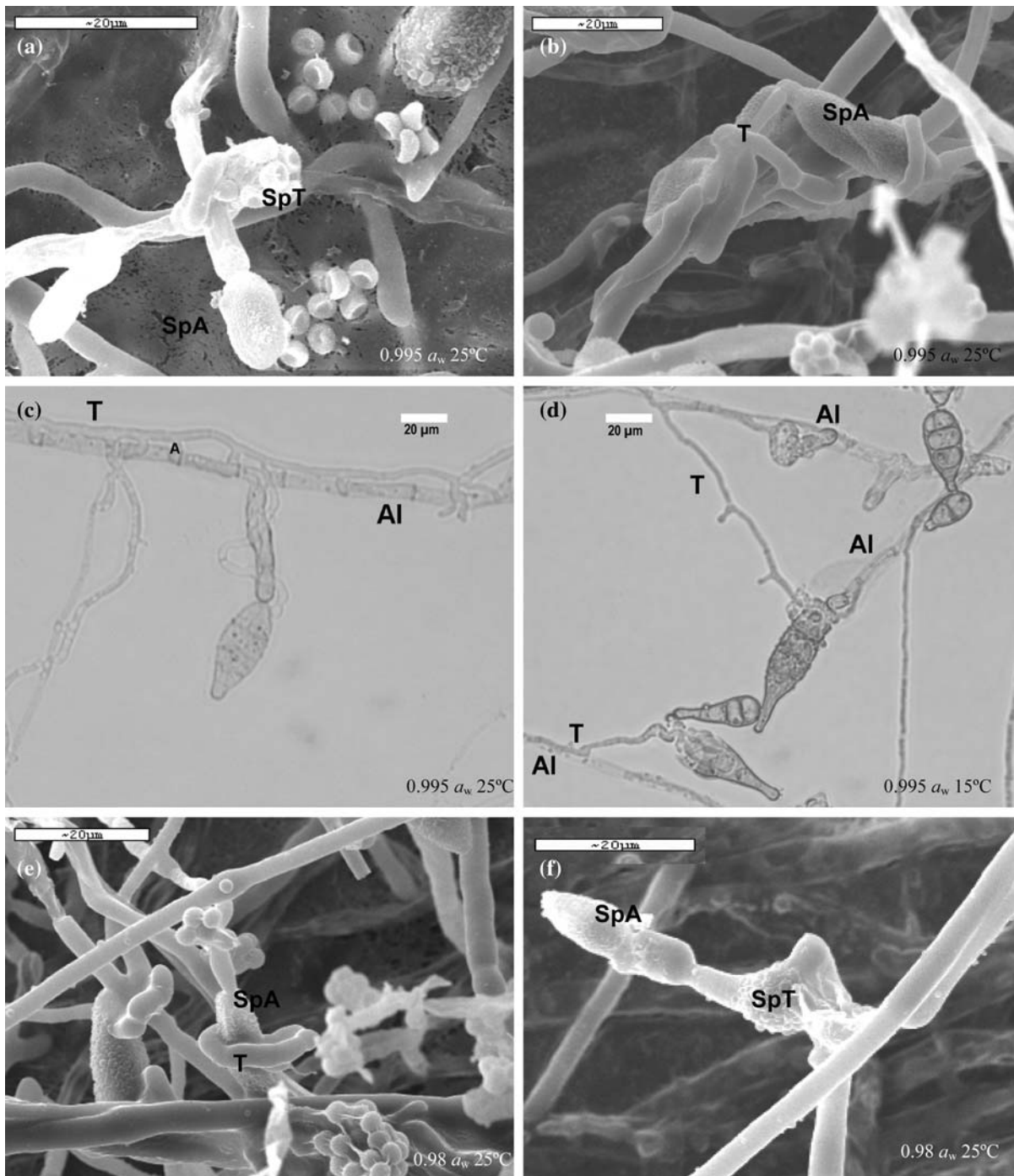


Figure 4. Micrographs of cryo-SEM (a, b, e, f) and light microscopy (c, d) of fungal interaction between *T. harzianum* and *A. alternata* at different temperatures and water activities. (a) *T. harzianum* is in the process of parasitizing a hyphae of *A. alternata* (b) *T. harzianum* hyphae coiling around and penetrating *A. alternata* spore. (c) Shows the appressoria-like structures. (e) Hooks of *T. harzianum* attached to spore of *A. alternata*. (d, f) Dissolution of the cell walls of *A. alternata* and *T. harzianum* hyphae penetrating spore and hyphae of this species. T: hyphae of *T. harzianum*. AI: hyphae of *A. alternata*. A: appressoria. SpA: spore of *A. alternata*. SpT: Spores of *T. harzianum*. a: $\times 2000$. b, e, f: $\times 1500$. c, d: $\times 400$.

strain grown dually, the later decreased. Nevertheless the growth of *T. harzianum* was very similar in both situations (data not shown).

The inhibition of fungal growth at a distance, as observed in this study, is commonly ascribed to antibiotic production by the antagonist [16, 17]. Most *Trichoderma* strains produce volatile and nonvolatile antibiotics and toxic metabolites that impede colonization by antagonized microorganisms; harzianic acid, alamethicins, tricholin, petai-bols, viridin, etc., have been described [18, 19, 20].

Analysis of fungal growth was discarded in this study for water activities of 0.90 since the development of the strains was minimal after eight testing weeks.

Finally, the numerical values used in the Index of Dominance revealed that *T. harzianum* was the dominant species over *A. alternata* at 25°C and 15°C (Table 2).

Light microscopy and cryo-SEM observations

The microscopic analysis showed that in interaction dominance on contact, when *T. harzianum* hyphae contacted *A. alternata* hyphae, they grew parallel to or coiled around the other and formed appressoria (Figure 4a, b, c and e). The coiling reaction is typical of mycoparasitic interactions [21].

Subsequently *T. harzianum* passed through the cell wall of *A. alternata*, using the contents of the host hyphae as nutrient source and grew inside it (Figure 4c and d).

Penetration inside *A. alternata* is promoted by the release of chitinase enzymes by the antagonist species that weaken cell wall tissue. The release of these enzymes is indicated by means of perforated hyphae fungal cell walls [22].

These molecules catalyse the breakdown of chitin, a primary component of fungal cell walls [23]. Subsequently, as suggested in Lorito et al. the release of antibiotics permeate the perforated hyphae and prevent resynthesis of the host cell wall [24].

As a result of *T. harzianum* attack, some *A. alternata* hyphae exhibited morphological changes such as deformation, increase of cellular vacuolisation, cell wall disintegration and dissolution of cytoplasm (Figure 4d and 4f).

At 0.95 a_w and 15°C a separation front line was observed between both colonies, confirming the results obtained in the macroscopic analysis. This

behaviour was possibly due to the secretion of metabolites which inhibited fungal growth of *A. alternata*.

The analysis performed in this work reveals that the mechanisms by which *T. harzianum* antagonizes *A. alternata* vary depending on the conditions of temperature and water activity. Similarly, different action modes can synergistically act on the same biocontrol agent.

From the macroscopic point of view, *T. harzianum* fights for space and nutrients, antagonizes with *A. alternata* due to its high growth rates, reduces the growing space of *A. alternata*, and grows over it once there is no more free space left.

This preliminary study suggests that *Trichoderma harzianum* antagonizes *A. alternata* through different mechanisms such as competition for space and nutrients, mycoparasitism and possible antibiosis.

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References

1. Pincirolì M, Sisterna MN, Bezus R, Vidal AA. Manchado del grano de arroz: efecto de la fertilización nitrogenada. Revista de la Facultad de Agronomía. La Plata 2003; 105: 88–96.
2. Piñeiro F., García J. La sanidad en el arroz valenciano. Enfermedades. Vida Rural 2000; 108: 26–30.
3. Santamarina P, Serna R, Asensi C, Roselló J. Evolución de la micoflora del arroz durante el periodo de almacenamiento. Phytoma 2002; 142: 113–117.
4. Chet I. *Trichoderma*: Application, Mode of Action and Potential as a Biocontrol Agent of Soilborne Plant Pathogenic Fungi. In: Chet I, ed. Innovative Approaches to Plant Disease Control, Wiley, New York, 1987: 137–160.
5. Chet I, Inbar J. Biological control of fungal pathogens. Applied Biochem Biotechnol 1994; 48: 37–43.
6. Golam M, Ilag LL. Potential for Biocontrol of *Lasiodiplodia theobromae*(Pat.) Griff. & Maubl. in Banana Fruits by *Trichoderma* species. Biol Control 1999; 15: 235–240.

7. Elad Y. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Prot* 2000; 19: 709–714.
8. Santamarina MP, Roselló J. Influence of temperature and water activity on the antagonism of *Trichoderma harzianum* to *Verticillium* and *Rhizoctonia*. *Crop Prot* 2006; 25: 1130–1134.
9. Moussa TTA. Studies on biological control of sugarbeet pathogen *Rhizoctonia solani* Kühn. *OnLine J Biol Sci* 2002; 2: 800–804.
10. Sempere F, Santamarina MP. Ecofisiología de *Drechslera oryzae* Subram. & Jain en condiciones *in vitro*. *Phytoma* 2006; 178: 49–50.
11. Magan N, Lacey J. The effect of water activity, temperature and substrate on interactions between field and storage fungi. *Trans Br Mycol Soc* 1984; 82: 83–93.
12. Santamarina MP, Roselló J, Serna R. Estudio de la ecofisiología del agente de biocontrol *Trichoderma harzianum* Rifai. *Phytoma* 2002; 142: 110–112.
13. Lacey J, Magan N. Fungi in cereal grains: their occurrence and water and temperature relationships. In: Chelkowski J, ed. *Cereal Grain: Mycotoxins, Fungi and Quality in Drying and Storage*, Elsevier, Amsterdam; 1991: 77–118.
14. Mónaco C, Cisterna M, Perelló A, Dal Bello G. Preliminary studies on biological control of the blackpoint complex of wheat in Argentina. *World J Microbiol Biotechnol* 2004; 20: 285–290.
15. Roco A, Pérez LM. *In vitro* biocontrol activity of *Trichoderma harzianum* on *Alternaria alternata* in the presence of growth regulators. *EJB Electronic J Biotechnol* 2001; 4: 68–73.
16. Fokkema NJ. The role of saprophytic fungi in antagonism against *Drechslera sorokiniana* (*Helminthosporium sativum*) on agar plates and on rye leaves with pollen. *Physiol Plant Pathol* 1973; 3: 195–205.
17. Blakeman JP, Fokkema NJ. Potential for biological control of plant diseases on the phylloplane. *Ann Rev Phytopathol* 1982; 20: 167–192.
18. Vey A, Hoagland RE, Butt TM. Toxic metabolites of fungal biocontrol agents. In: Butt TM, Jackson C, Magan N, eds. *Fungi as Biocontrol Agents: Progress, Problems and Potential*, CAB International, Bristol; 2001: 311–346.
19. Calistru C, McLean M, Berjak P. *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. A study of the production of extracellular metabolites by *Trichoderma* species. *Mycopathologia* 1997; 137: 115–124.
20. Calistru C, McLean M, Berjak P. *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. 1. Macroscopical and microscopical observations of fungal interactions. *Mycopathologia* 1997; 139: 115–121.
21. Harman GE, Howell CHR, Viterbo A, Chet I, Lorito M. *Trichoderma* species—opportunistic, avirulent plant symbionts. *Nature Reviews* 2004; Microbiology 2.
22. Rousseau A, Benhamou N, Chet I, Piché Y. Mycoparasitism of the extramatrical phase of *Glomus intraradices* by *Trichoderma harzianum*. *Phytopathology* 1996; 86: 434–443.
23. Zeilinger S, Galhaup C, Payer K, Woo SL, Mach RL, Fekete C, Lorito M, Kubicek CP. Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fungal Genet Biol* 1999; 26: 131–140.
24. Lorito M, Farkas V, Rebuffat S, Bodo B, Kubicek CP. Cell wall synthesis is a major target of mycoparasite antagonism by *Trichoderma harzianum*. *J Bacteriol* 1996; 178: 6382–6385.

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