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Biological control of one species belonging to the dominant mycobiota of rice of Valencia

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Abstract - The possible biological control of the seed-associated fungus, *Nigrospora oryzae* by *Trichoderma harzianum* under different environmental conditions was investigated. A study of the fungal growth in dual cultures revealed that *T. harzianum* inhibited by contact *N. oryzae* at all testing temperatures and water activities tested except at 0.95 a_w and 15 ºC, where *T. harzianum* inhibited pathogen growth before hyphal contact and exhibited an inhibition zone between the colonies of both fungi. Suppression of the sporulation, loss of turgor and cell collapse, wall's disintegration, coiling and penetration of *T. harzianum* around different structures of *N. oryzae* were observed by cryo-scanning electron microscopy. The effect of abiotic factors water activity and temperature on fungal growth was determined.

Key words: mycoparasitism, *Nigrospora oryzae*, Index of Dominance, rice, *Trichoderma harzianum*, water activity.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal grains grown worldwide. This crop is a primary food source for more than a third of the world's population and provides 27% of the dietary energy supply, and 20% of the dietary protein intake (Kush, 1997; Fresco, 2005).

Fungal diseases are one of the major factors limiting rice production (Kim *et al*., 2003). Biological control of fungal plant pathogens appears as an attractive and realistic approach, and numerous microorganisms have been identified as biocontrol agents (Massart and Jijakli, 2006). Studies made for suppressing these cereal pathogens are few. Within these studies *Pseudomonas fluorescens*, *Delftia tsuruhatensis, Bacillus mycoides* and *Streptomyces* spp. bacteria have been used as antagonistic against *Sarocladium oryzae*, *Rhizoctonia solani*, *Drechslera oryzae* and *Pyricularia oryzae* (Sakthivel and Gnanamanickam, 1986a, 1986b; Sakthivel *et al*., 1986; Chakrabarti and Chaudhari, 1992; Krishnamurthy and Gnanamanickam, 1998; Tian *et al*., 2004; Han *et al*., 2005; Nagarajkumar *et al*., 2004, 2005). *Candida* sp. and *Sporobolomyces roseus* yeasts have been employed too against these strains (Akai and Kuramoto, 1968; Rush *et al*., 1998).

Trichoderma species have been investigated as biological control agents (BCAs) for over 70 years, but it is only recently that strains have become commercially available (Hjeljord and Tronsmo, 1998; Hermosa *et al*., 2000). Others fungi have been studied with this purpose. In this way, *Penicillium oxalicum* and species de *Trichoderma* and *Gliocadium* have been successfully applied controlling rice pathogens *Rhizoctonia solani*, *Alternaria alternata*, *Nigrospora oryzae* and *Bipolaris oryzae* (Rosales and Mew, 1982; Roy and Sayre, 1984; Gokulapalan and Nair, 1986; Abdel-Fattah *et al*., 2007; Sempere and Santamarina, 2007a, 2007b).

In the Valencian autonomous region, typical rice production area, which owns the origin denomination Rice of Valencia, *Pyricularia*, *Alternaria*, *Phoma*, *Bipolaris*, *Nigrospora*, *Fusarium*, *Aspergillus*, and *Penicillium* genera have been isolated. Among them the seedborne fungus *Nigrospora oryzae* (Berk. & Broome) Petch (teleomorph: *Khushia oryzae* Huds.) has been found with high percentage (Piñeiro and García, 2000; Santamarina *et al*., 2002). This species affects different parts of the rice plant but stands out because of its contribution for pecky rice. Infected grains are discoloured and sometimes black and white coloured due to the presence of mycelial mass and contamination can occur preharvest, harvest and rice processing.

Understanding how biocontrol agents exert their protective effects is a prerequisite to their effective practical application (Massart and Jikali, 2006). The purpose of this paper was to investigate the interaction between *T. harzianum* and *N. oryzae* in co-culture at different environmental conditions and culture media using different techniques. In addition, an ecophysiological study of both strains was realised.

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MATERIALS AND METHODS

Fungal strains. *Nigrospora oryzae* DAE 7406 was isolated from rice grains collected from different fields and cooperatives of the main rice producing areas in Valencia. *Trichoderma harzianum* CECT 20736 was isolated from national corn grain samples.

The above mentioned strains were kept in the Department of Agroforest Ecosystems of the School of Rural Environments and Enology, Polytechnic University of Valencia, Spain.

Growth in paired cultures. The basic medium used was Rice Extract Agar (REA) with a pH of 5.5. The water activity (a_w) of this basal medium was 0.995, and this a_w was modified by the addition of different amounts of glycerol to obtain a_w levels of 0.98, 0.95, 0.90 and 0.85 (Sempere and Santamarina, 2006a). The experiments were carried out at 15 and 25 ºC in the dark.

The fungi disks of *T. harzianum* and *N. oryzae* (8 mm diameter) obtained from the growing margins of the fungus colonies grown in Potato Dextrose Agar (PDA) at 25 ºC for 5 days, were inoculated at opposite sides -45 mm apart-, of a 90 and 150 mm Petri plates containing REA.

Plates with the same water activities 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. 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.

Fungal growth was measured at right angles, five days at intervals of 24 h after inoculation. A linear regression of the data was performed in order to calculate the growth rate (mm·day^{-1}). The computer software used was Microsoft Excel 2003.

The experiment was repeated four times.

Macroscopic experiments. Macroscopic experiments were conducted in 60 days. Growth rates were obtained and Petri plates at 0.95, 0.98 and 0.995 water activities and both temperatures were examined macroscopically, the type of interactions was determined, and numerical scores were assigned to obtain an Index of Dominance according to method proposed by Magan and Lacey (1984). 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 Petri plates in this experiment for water activities of 0.90 and 0.85 was discarded.

Microscopic experiments. For cryo-scanning electron microscopy (cryo-SEM), two experiments were undertaken. Experiment 1: both strains were inoculated at the same time. Experiment 2: *N. oryzae* was inoculated three days later inoculation of *T. harzianum*. Microscopic analysis was performed on Rice Extract Agar and full rice grain. The rice grain variety used in the experiment was Bomba, typical of the designation of origin Rice of Valencia.

To study hyphal interactions in Rice Extract Agar, both 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. To maintain the levels of water activity, filter paper disks impregnated with different water activity levels (0.995, 0.98 and 0.95 obtained with 2.5, 11 and 23.5 g glycerol/100 ml distilled water) were aseptically placed on the Petri plates. For the analysis of fungal interaction in rice grains, first the samples were sterilised 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 (Sempere and Santamarina, 2006b, 2007a).

Microscopic examination of the dual microculture was performed between 5-30 days after inoculation.

Assessment of antibiosis. Growth of *N. oryzae* jointly *T. harzianum* was measured. Treatments were compared with the diameter of each REA plate control inoculated with *N. oryzae*.

Statistical analysis. The analysis of variance (ANOVA) with significance values of $P < 0.01$ was used to determine the influence of parameters water activity and temperature (T), and of their interaction (a_w x 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

Effects of water activity and temperature on mycelial growth

Figure 1 shows growth rates of *T. harzianum* and *N. oryzae* dually grown at 15, 25 ºC and at five different water activities.

Growth of the species studied was fastest at the highest a_w studied at all temperatures. The optimum a_w (0.995) for growth of both strains didn't vary with temperature. The maximum growth rates occurred at 25 ºC and 0.995 a_w for *T. harzianum* and *N. oryzae.* The growth rates of *T. harzianum* and *N. oryzae* were of 16 mm day^{-1} and 8.97 mm day^{-1} respectively.

At 25 ºC and five days, the minimum a_w for growth was 0.90 for *N. oryzae* and 0.95 for *T. harzianum*. At 15 ºC the

TABLE 1 - Analysis of variance of the growth rate of *Trichoderma harzianum* and *Nigrospora oryzae*; significance of water activity (a_w), temperature (T) and their interaction (a_w x T)

Factor	DF	MS	F-ratio	P-value
a_w	4	5826.55	97.40	0.0000**
T	1	4695.68	78.50	0.0000**
a_w x T	4	1196.92	20.01	0.0000**

DF: degrees of freedom, MS: mean squares. ** Indicates that the factor elicited a significant effect ($P < 0.01$).

minimum a_w was 0.95 for *N. oryzae* and 0.98 for *T. harzianum*. Growth of both species at 15 ºC and 25 ºC was minimal at 0.90 a_w in REA during the eight testing weeks. *Trichoderma harzianum* and *N. oryzae* did not show any growth at 0.85 a_w.

Similar results were obtained previously when *N. oryzae* and *T. harzianum* grown individually and jointly *Alternaria alternata* (Sempere and Santamarina, 2006b, 2007a).

Trichoderma harzianum presented higher growth rates than *N. oryzae* at both temperatures, for high water activities studied, 0.995 and 0.98. At 0.95 a_w the growth of *N. oryzae* was bigger (Fig. 1).

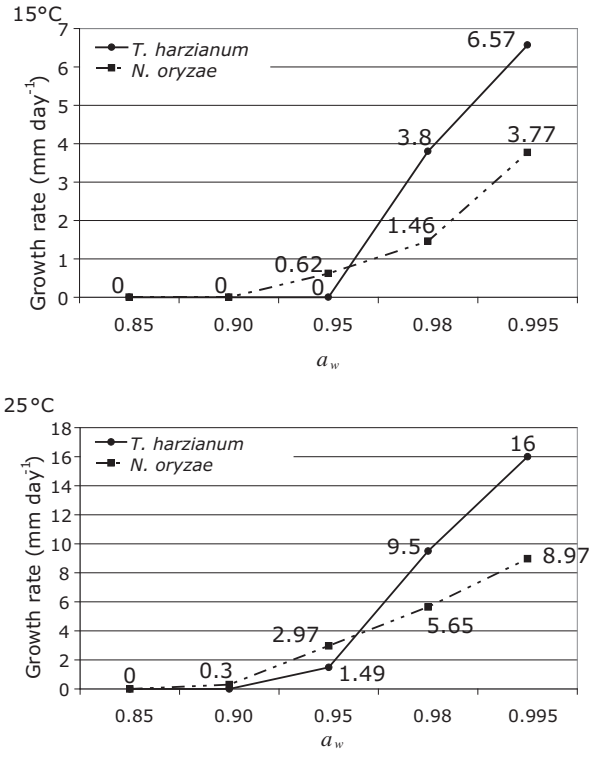

FIG. 1 - Ecophysiological study of *Trichoderma harzianum* and *Nigrospora oryzae* dual culture in Rice Extract Agar at different temperatures and water activities (a_w).

Expansion of mycelia of both colonies was the same in the direction of the other colony than it was when they were grown individually. When the colonies got in contact, *N. oryzae* growth rate decreased in particular at the fungal interaction front line. However at 0.95 a_w and 15 ºC *T. harzianum* inhibited pathogen growth before hyphal contact and exhibited an inhibition zone between the colonies of both fungi (Fig. 2). Thangavelu *et al*. (2004) found that *Trichoderma harzianum* was the most effective in inhibiting the mycelial growth of *Fusarium oxysporum* f. sp. *cubense in vitro*. Growth of *F. oxysporum* f. sp. *ciceris* was also suppressed by *T. harzianum* when these species were co-cultured on PDA (Hervás *et al*., 1998, Dubey *et al*., 2007).

Biotic and abiotic parameters determine the extent of fungal colonisation and, among them, water activity, temperature and fungal interactions are the most important (Torres *et al*., 2003). In this study, significant differences in water activity and temperature effects on growth rates were registered for *T. harzianum* and *N. oryzae* ($P < 0.001$) (Table 1).

Macroscopic observations of dual culture

Regarding the Index of Dominance (Table 2) and the Fig. 2, dominance on contact was the most common interaction of *T. harzianum* against *N. oryzae. Trichoderma harzianum* was assigned a value of 4 and *N. oryzae* a value of 0.

In Petri plates dual cultures, the first apparent contact between hyphae of the two fungi occurred at 0.995 a_w 25 ºC, 4 days after inoculation. As long as the water activity and temperature decreased the colonies got in contact later. In the subsequent days, *T. harzianum* mycelium continued growing and colonising the substratum already colonised by *N. oryzae*. The aerial mycelium and sporulation of *T. harzianum* were more abundant when this strain grew over *N. oryzae*.

In the Fig. 2 it can be seen the colonies of *T. harzianum*, which are greenish and whitish in colour grew over the colonies of *N. oryzae*. The colonies of *N. oryzae* grown alone were first brown with a wool-like aerial white mycelium irregularly distributed, more abundant at 25 than at 15 ºC. On aging, their colour changed to darker brown-black by the growing margin of the fungus colony and to brown in the middle (Sempere and Santamarina, 2006b). When this species faced to *T. harzianum* in some water activities these mycelial features were not observed even the *N. oryzae* colony was not detected.

In this way this assay observed that one of the mechanisms that presented *T. harzianum* as a biocontrol agent at high water activities, apart from temperature, was the competition for space and nutrients. This mechanism was described to play a role in the biocontrol of *Verticillium dahliae* and *Rhizoctonia solani* by this strain (Santamarina and Roselló, 2006).

In dual culture of *T. harzianum* and *N. oryzae* at 0.95 a_w and 15 ºC, an inhibition zone around the *Nigrospora* colony was observed, where macroscopically, no apparent contact of the hyphae of the two fungi was observed (Fig. 2). According to method proposed by Magan and Lacey (1984), mutual antagonism at a distance happens when a separation between both fungal species of more than 2 mm is observed. A value of 3 is assigned for each fungal strain. The authors have found that this antagonism is not mutual. *Trichoderma harzianum* colony antagonised *N. oryzae*. Thus when comparing the growth rates of *N. oryzae* grown individually with the results obtained from the strain grown jointly *T. harzianum*, second decreased. Nevertheless the

TABLE 2 - Index of Dominance (I_D) at different temperatures and water activities

Temp.	Fungus species	a_w 0.995	0.98	0.95	0.90	I_D
25 ºC	*T. harzianum*	4	4	4	X	12
	N. oryzae	0	0	0	X	0
15 ºC	*T. harzianum*	4	4	3	X	11
	N. oyzae	0	0	3	X	3

I_D refers to sum of scores at 25 and 15 ºC for *Trichoderma harzianum* competing with *Nigrospora oryzae* based on the interaction scores for each species. Dominance on contact (4 for the dominant species *T. harzianum*, 0 for the inhibited species *N. oryzae*). Mutual antagonism at a distance (3 for both species). (X) Analysis of interaction was discarded.

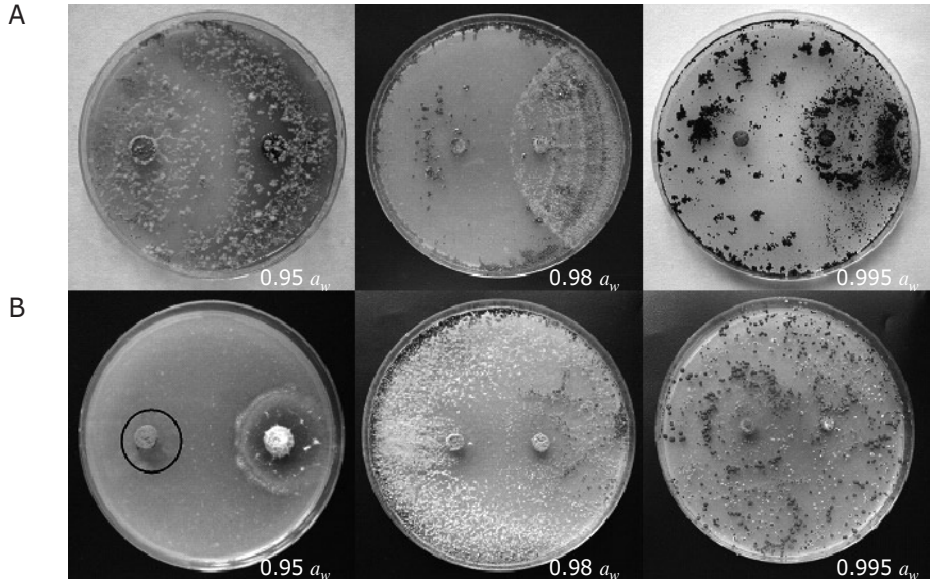

FIG. 2 - Petri plates showing the interaction between *Trichoderma harzianum* (left) and *Nigrospora oryzae* (right) after 8 weeks at different water activities and temperatures. Row A: 25 ºC. Culture of *N. oryzae* was inhibited by *T. harzianum*. Both species were growing until both colonies got in contact. Later *T. harzianum* grew trough *N. oryzae*. Row B: 15 ºC. The same type of interaction of Row A was registered (0.98 and 0.995 a_w), while *T. harzianum* inhibited *N. oryzae* growth at a distance (0.95 a_w), —— colony of *T. harzianum*.

growth of *T. harzianum* was very similar in both situations (data not shown).

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

Mycelial interactions between *Trichoderma harzianum* and *Nigrospora oryzae*

When *T. harzianum* and *N. oryzae* were inoculated at the same time, conidia of *N. oryzae* were not observed (data not shown). Macroscopic examination of the Petri plates showed discoloured aerial mycelium of *N. oryzae* (Fig. 2). These observations suggested that *T. harzianum* suppressed sporulation of its host.

Previously, this capacity was proposed as a potential strategy of biological control (Sutton and Peng, 1993; Köhl and Fokkema, 1998). In different studies, the antagonist *Clonostachys rosea* reduced and suppressed conidia of *Botrytis cinerea* (Sutton *et al*., 1997; Morandi *et al*., 2003). This behaviour was also observed when mycoparasitic *Pythium oligandrum* was confronted against *Fusarium culmorum* in dual culture (Davanlou *et al*., 1999). Studies of dual cultures of *T. harzianum* and other species revealed inhibition of germination but no suppression of conidia (Lorito *et al*., 1993a, 1993b; Zimand *et al*., 1996; Kapat *et al*., 1998; Golam Mortuza and Ilag, 1999).

Individual culture of *N. oryzae* sporulated at all conditions tested (0.995, 0.98 and 0.95 a_w at 15 and 25 ºC). For other strain belonging to the same species isolated from the rice grains of Valencia, only formed conidia at 0.995 and 0.98 a_w at 25 ºC (Sempere and Santamarina, 2006b). This filamentous fungus formed unicellular solitary conidia, globose or subglobose shaped with a smooth surface texture, irregularly grown from short or ill-defined conidiophores (Fig. 3A and 3B).

Microscopic observation of the inoculation of *N. oryzae* three days later inoculation of *T. harzianum* revealed the mechanism of mycoparasitism at all conditions tested except at 0.95 a_w and 15 ºC. One of the earliest events of the antagonistic process was the apparent affinity of *T. harzianum* for hypha and conidia of the *N. oryzae* (Fig. 3C and 3D). However, how *Trichoderma* species "recognise" their host and attack host cells is yet unknown. This process may involve hydrophobic interactions or interactions between complementary molecules present on the surface of both the host and the mycoparasite such as between lectins and carbohydrates (Whipps, 2001). Different researchers worked in this mycoparasite and others species for elucidated this process and signalling pathways that occur latter recognition of the host (Elad *et al*., 1983; Inbar and Chet, 1994; Neethling and Nevalainen, 1996; Omero *et al*., 1999).

Occasionally *T. harzianum* hyphae coiled around the hyphae and conidia of the pathogen from where penetration took place (Fig. 3E and 4D). Also, directly penetration of *T. harzianum* into hyphae and conidia of *N. oryzae* was observed (Fig. 3F, 4A, 4C, and 4D). Microscopic examinations showed loss of turgor and cell collapse of *N. oryzae* conidium (Fig. 3B) and disintegrated hyphal walls. Finally, *T. harzianum* utilised both their cell walls and cellular contents for nutrition and come out producing sexual structures (Fig. 3F, 4A, 4C, and 4D).

The enzymes chitinases and glucanases from *Trichoderma harzianum* have been demonstrated to play an important role in mycoparasitic action against different fungi and others organisms (Viterbo *et al*., 2002; Ezziyyani *et al*., 2004; Limón *et al*., 2004; Binod *et al*., 2007). However, fungal proteases may be significantly involved in antagonistic activity, not only in the breakdown of the host cell walls but also by inactivation of enzymes of pathogens

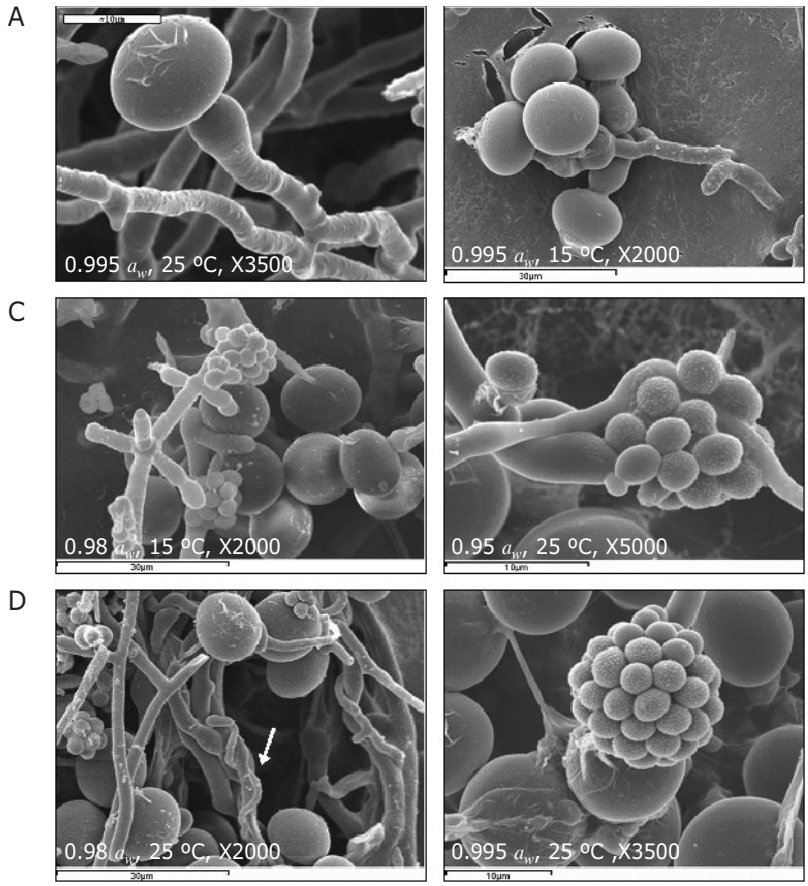

FIG. 3 - Mycoparasitic interactions between *Trichoderma harzianum* and *Nigrospora oryzae*. A and B: conidia and conidiophores of *N. oryzae,* it can be seen their morphology still undamaged; C and D: conidia and hypha of *N. oryzae* in the beginning of being attacked by *T. harzianum*; E: hypha of *T. harzianum* coiling around hypha of *N. oryzae* (arrows); F: direct penetration of *T. harzianum* into partially disintegrated conidia of *N. oryzae*.

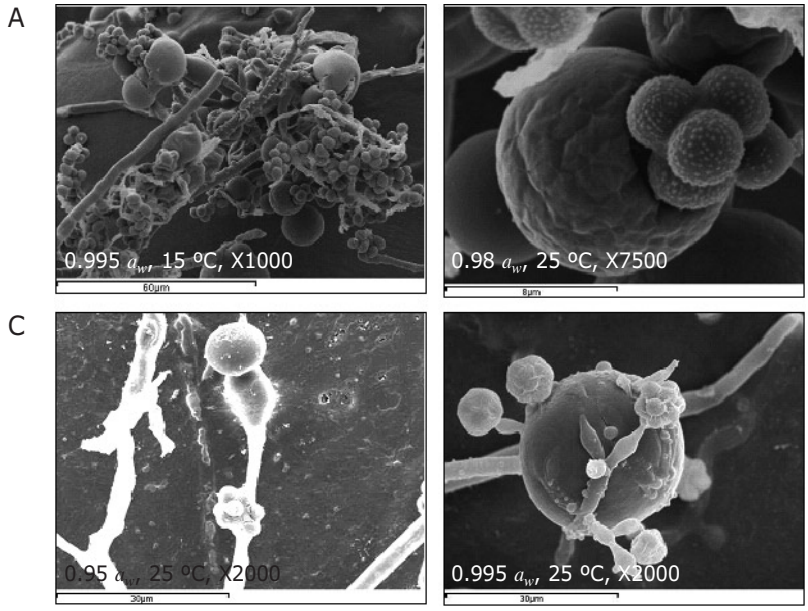

FIG. 4 - Mycoparasitic interactions between *Trichoderma harzianum* and *Nigrospora oryzae.* A: directly penetration of *T. harzianum* into partially disintegrated conidia of *N. oryzae* (arrows); B: detail of the figure A, loss of turgor and cell collapse of *N. oryzae* conidium caused by *T. harzianum*; C: conidia of *T. harzianum* coming out of a degraded hypha of *N. oryzae* (arrow); D: hypha of *T. harzianum* coiling around, penetrating and coming out a conidium of *N. oryzae.*

(Rodriguez-Kabana *et al*., 1978, Suarez *et al*., 2004). Proteolytic enzymes of *T. harzianum* reduced *Botrytis cinerea* germination and deactivated partially hydrolytic enzymes endo-PG and exo-PG produced by this strain (Elad and Kapat, 1999). Cryo-SEM study in the interaction zone, suggested that this strain produced different of these enzymes.

In addition to this parasitism, *T. harzianum* apparently produced some antifungal metabolites since morphological abnormality of the pathogen occurred.

At 0.95 a_w and 15 ºC, no contact between both species was observed. The hyphae *N. oryzae* nearest to the *T. harzianum* colony showed severe deformation. The same result was obtained when Gupta *et al*. (1999) confronted *Trichoderma viride*, *Trichoderma hamatum* and *Trichoderma pseudokoningii* against *Botryodiplodia theobromae*. Antagonism at a distance has been usually attributed to diffusible or volatile compounds (Sonnenbichler *et al*., 1994). These morphological alterations of the pathogen and its inhibition they could be due to the secretion of these substances by *T. harzianum*. In this way, species of *Trichoderma*, which are currently all registered biological control products, are known to produce several secondary metabolites inhibitory to the growth of the host with broad-spectrum antimicrobial activity (Dennis and Wester, 1971a, 1971b; Punja and Utkhede, 2003).

Cryo-SEM has been proved a useful technique to observe and describe different interactions between microorganisms. Previously the authors investigated other interactions and all studies confirmed this technique. There are few researches and the usual techniques to study fungal relations are tedious SEM preparations or light microscopical observations (Madi *et al*., 1997; Davanlou *et al*., 1999; Moussa, 2002; Zadworny *et al*., 2004).

Knowledge of the mechanism of action involved in the biocontrol process can permit establishment of optimum conditions for the interaction between the pathogen and the biological control agent and is important for implementing biological control in a given pathosystem (Cook, 1993; Handelsman and Stabb, 1996). This is the first report suggesting that *Trichoderma harzianum* antagonises *Nigrospora oryzae* through different synergistic mechanisms such as suppression of sporulation, competition for space and nutrients, mycoparasitism and possible antibiosis. Water activity and temperature are critical factors affecting the growth and metabolism of fungi but also their competitive capacity. Future researches will lead to the development of this strain for biocontrol of *Nigrospora oryzae*. Such studies are in progress in our laboratory.

REFERENCES

Abdel-Fattah G.M., Shabana Y.M., Ismail A.E., Rashad Y.M. (2007). *Trichoderma harzianum*: a biocontrol agent against *Bipolaris oryzae*. Mycophatologia, 164: 81-89.

Akai S., Kuramoto T. (1968). Microorganisms existing on leaves of rice plant and the occurrence of brown leaf spot. Annals of the Phytopathological Society of Japan, 34: 313-316.

Binod P., Sukumaran R.K., Shirke S.V, Rajput J.C., Pandey A. (1997). Evaluation of fungal culture filtrate containing chitinase as a biocontrol agent against *Helicoverpa armigera*. J. Appl. Microbiol., 103: 1845-1852.

Chakrabarti N.K., Chaudhari R. (1992). Brown spot of rice in U.S. In: Singh U.S., Mukhopadhyay N., Kumar J., Chaube H.S., Eds, Plant Diseases of International Importance, Vol. 1: Diseases of Cereals and Pulses, 2nd edn., Prentice-Hall Inc., USA, pp. 116-129.

Cook R.J. (1993). Making greater use of introduced microorganisms for biological control of plant pathogens. Annu. Rev. Phytopathol., 31: 53-80.

Davanlou M., Madsen A.M., Madsen C.H., Hockenhull J. (1999). Parasitism of macroconidia, chlamydospores and hyphae of *Fusarium culmorum* by mycoparasitic *Pythium* species. Plant Pathol., 48: 352-359.

Dennis C., Webster J. (1971a). Antagonistic properties of species groups of *Trichoderma.* II: Production of volatile antibiotics. Trans. Brit. Mycol. Soc., 57: 40-48.

Dennis C., Webster J. (1971b). Antagonistic properties of species groups of *Trichoderma.* III: Hyphal interaction. Trans. Brit. Mycol. Soc., 57: 363-369.

Dubey S.C., Suresh M., Singh B. (2007). Evaluation of *Trichoderma* species against *Fusarium oxysporum* f. sp. *ciceris* for integrated management of chickpea wilt. Biol. Control, 40: 118-127.

Elad Y., Barak R., Chet I. (1983). Possible role of lectins in mycoparasitism. J. Bacteriol., 154 (3): 1431-1435.

Elad Y., Kapat A. (1999). Role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. Eur. J. Plant Pathol., 105: 177-189.

Ezziyyani M., Pérez C., Ahmed A.S., Requena M.E., Candela M.E. (2004). *Trichoderma harzianum* como biofungicida para el biocontrol de *Phytophthora capsici* en plantas de pimiento (*Capsicum annuum* L.). Anales de Biología, 26: 35-45.

Fresco L. (2005). ''Rice is life''. J. Food Comp. Anal., 18: 249-253.

Gokulapalan C., Nair M.C. (1986). Mycoparasites of *Rhizoctonia solani* and control of sheath blight of rice, (abstract) Fourteenth Annual Meeting of the Mycological Society of India and Seminar on Applied Micology, Thanjavur, India, p. 18.

Golam Mortuza M., Ilag L.L. (1999). Potential for biocontrol of *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. in banana fruits by *Trichoderma* species. Biol. Control, 15: 235-240.

Gupta V.P., Tewari S.K., Govindaiah, Bajpai A.K. (1999). Ultrastructure of mycoparasitism of *Trichoderma*, *Gliocladium* and *Laetisaria* species on *Botryodiplodia theobromae*. J. Phytopathol., 147: 19-24.

Han J., Sun L., Dong X., Cai Z., Sun X., Yang H., Wang Y., Song W. (2005). Characterization of a novel plant growth-promoting bacteria strain *Delftia tsuruhatensis* HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens. Syst. Appl. Microbiol., 28 (1): 66-76.

Handelsman J., Stabb K. (1996). Biocontrol of soilborne plant pathogens. Plant Cell, 8: 1855-1869.

Hermosa M.R., Grondona I., Iturriaga E.A., Diaz-Minguez J.M., Castro C., Monte E., Garcia-Acha I. (2000). Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. Appl. Environ. Microbiol., 66 (5): 1890-1898.

Hervás A., Landa B., Datnoff L.E., Jiménez-Díaz R.M. (1998). Effects of commercial and indigenous microorganisms on *Fusarium* wilt development in chickpea. Biol. Control, 13: 166-176.

Hjeljord L., Tronsmo A. (1998). *Trichoderma* and *Gliocladium* in biocontrol: an overview. In: Kubicek C.P., Harman G.E., Eds, *Trichoderma* and *Gliocladium*. Taylor & Francis, Ltd., London, United Kingdom, pp. 135-151.

Inbar J., Chet I. (1994). A newly isolated lectin from the plant pathogenic fungus *Sclerotium rolfsii*: Purification, characterization and role in mycoparasitism. Microbiology, 140: 651-657.

Kapat A., Zimand G., Elad Y. (1998). Effect of two isolates of *Trichoderma harzianum* on the activity of hydrolytic enzymes produced by *Botrytis cinerea*. Physiol. Mol. Plant Path., 52: 127-137.

Khush G.S. (1997). Origin, dispersal, cultivation and variation of rice. Plant Mol. Biol., 35: 25-34.

Kim J.K., Jang I.C., Wu R., Zuo W.N., Boston R.S., Lee Y.H., Ahn I.P., Nahm B.H. (2003). Co-expression of a modified maize ribosome-inactivating protein and a rice basic chitinase gene in transgenic rice plants confers enhanced resistance to sheath blight. Transgenic Res., 12: 475-484.

Köhl J., Fokkema N.J. (1998). Strategies for biological control of necrotrophic fungal pathogens. In: Boland G.J., Kuykendall L.D., Eds, Plant-microbe interactions and biological control, M. Dekker Inc., New York, pp. 49-88.

Krishnamurthy K.S.S., Gnanamanickam S.S. (1998). Biological control of rice blast by *Pseudomonas fluorescens* strain Pf7-14: Evaluation of a marker gene and formulations. Biol. Control, 13: 158-165.

Lorito M., Harman G.E., Hayes C.K., Broadway R.M., Tronsmo A., Woo S.L., Di Pietro A. (1993a). Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. Phytopathology, 83: 302-307.

Lorito M., Di Pietro A., Hayes C.K., Woo S.L., Harman G.E. (1993b). Antifungal, synergistic interaction between chitinolytic enzymes from *Trichoderma harzianum* and *Enterobacter cloacae*. Phytopathology, 83: 721-728.

Limón M.C., Chacón M.R., Mejías R., Delgado-Jarana J., Rincón A.M., Codón A.C., Benítez T. (2004). Increased antifungal and chitinase specific activities of *Trichoderma harzianum* CECT 2413 by addition of a cellulose binding domain. Appl. Microbiol. Biotechnol., 64: 675-685.

Madi L., Katan T., Katan J., Henis Y. (1997). Biological control of *Sclerotium rolfsii* and *Verticillium dahliae* by *Talaromyces flavus* is mediated by different mechanisms. Phytopathology, 87 (10): 1054-1060.

Magan N., Lacey J. (1984). The effect of water activity, temperature and substrate on interactions between field and storage fungi. Trans. Brit. Mycol. Soc., 82: 83-93.

Massart S., Jijakli H.M. (2006). Use of molecular techniques to elucidate the mechanisms of action of fungal biocontrol agents: A review. J. Microbiol. Meth., 69: 229-241.

Morandi M.A.B., Maffia L.A., Mizubuti E.S.G., Alfenas A.C., Barbosa J.G. (2003). Suppression of *Botrytis cinerea* sporulation by *Clonostachys rosea* on rose debris: a valuable component in Botrytis blight management in commercial greenhouses. Biol. Control, 26: 311-317.

Moussa T.A.A. (2002). Studies on biological control of sugarbeet pathogen *Rhizoctonia solani* Kühn. OnLine Journal of Biological Sciences 2 (12): 800-804.

Nagarajkumar M., Bhaskaran R., Velazhahan R. (2004). Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. Microbiol. Res., 159: 73-81.

Nagarajkumar M., Jayaraj J., Muthukrishnan S., Bhaskaran R., Velazhahan R. (2005). Detoxification of oxalic acid by *Pseudomonas fluorescens* strain PfMDU2: Implications for the biological control of rice sheath blight caused by *Rhizoctonia solani*. Microbiol. Res., 160: 291-298.

Neethling D., Nevalainen H. (1996). Mycoparasitic species of *Trichoderma* produce lectins. Can. J. Microbiol., 42 (2):141-146.

Omero C., Inbar J., Rocha-Ramírez V., Herrera-Estrella A., Chet I., Horwitz B.A. (1999). G protein activators and cAMP promote mycoparasitic behaviour in *Trichoderma harzianum*. Mycol. Res., 103: 1637-1642.

Piñeiro F., García J. (2000). La sanidad en el arrozal valenciano. Enfermedades. Vida Rural, 108: 26-30.

Punja Z.K., Utkhede R.S. (2003). Using fungi and yeasts to manage vegetable crop diseases. Trends Biotechnol., 21 (9): 400-407.

Rodriguez-Kabana R., Kelley W.D., Curl E.A. (1978). Proteolytic activity of *Trichoderma viride* in mixed culture with *Sclerotium rolfsii* in soil. Can. J. Microbiol., 24: 487-490.

Rosales A.M., Mew T.W (1982). Antagonistic effects of soil microorganisms on rice sheath blight pathogen. International Rice Research Newsletter, 7:12-13.

Roy A.K., Sayre R.M. (1984). Electron microscopical studies of *Trichoderma harzianum* and *T. viride* and mycoparasitic activity of the former on *Rhizoctonia solani* f. sp. *sasakii*. Indian Pytopath., 37: 710-712.

Rush M.C., Shahjahan A.K.M., Groth D.E., Jones J.P. (1998). Biological control of sheath blight disease of rice (abstract), 27th Rice Technical Working Meeting, Reno, NV. March 1-3, pp. 112-114.

Sakthivel N., Sivamani E., Unnamalai N., Gnanamanickam S.S. (1986). Plant growth-promoting rhizobacteria in enhancing plant growth and suppressing plant pathogens. Curr. Sci., 55 : 22-25.

Sakthivel N., Gnanamanickam S.S. (1986a). Toxicity of *Pseudomonas fluorescens* towards rice sheath rot pathogen, *Acrocylindrium oryzae* Saw., Curr. Sci., 55: 106-107.

Sakthivel N., Gnanamanickam S.S. (1986b). Bacterization of rice with *Pseudomonas fluorescens* reduces sheath rot infection. Int. Rice Res. Newslett., 11: 17-18.

Santamarina P., Serna R., Asensi C., Roselló J. (2002). Evolución de la micoflora del arroz durante el periodo de almacenamiento. Phytoma, 142: 113-117.

Santamarina M.P., Roselló J. (2006). Influence of temperature and water activity on the antagonism of *Trichoderma harzianum* to *Verticillium* and *Rhizoctonia*. Crop Prot., 25: 1130-1134.

Sempere F., Santamarina M.P. (2006a). Ecofisiología de *Drechslera oryzae* Subram. & Jain en condiciones *in vitro*. Phytoma, 178: 49-50.

Sempere F., Santamarina M.P. (2006b) Microscopic and macroscopic study of the interaction between *Alternaria alternata* (Fr.) Keissler and *Nigrospora oryzae* (Berk. & Broome) Petch. Ann. Microbiol., 56: 101-107.

Sempere F., Santamarina M.P. (2007a). *In vitro* biocontrol analysis of *Alternaria alternata* (Fr.) Keissler under different environmental conditions. Mycopathologia, 163: 183-190.

Sempere F., Santamarina M.P. (2007b). Suppression of *Nigrospora oryzae* (Berk. & Broome) Petch by an aggressive mycoparasite and competitor, *Penicillium oxalicum* Currie & Thom. Int. J. Food Microbiol., Article in press. doi: 10.1016/j.ijfoodmicro.2007.11.080.

Sonnenbichler J., Jurgen D., Peipp H. (1994). Secondary fungal metabolites and their biological activities. V. Investigations concerning the induction of the biosynthesis of toxic secondary metabolites in basidiomycetes. Biol. Chem. Hoppe-Seyler, 375: 71-79.

Suarez B., Rey M., Castillo P., Monte E., Llobell A. (2004). Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. Appl. Microbiol. Biotechnol., 65: 46-55.

Sutton J.C., Peng G. (1993). Biocontrol of *Botrytis cinerea* in strawberry leaves. Phytopathology, 83: 615-621.

Sutton J.C., Li D.W., Peng G., Yu H., Zhang P., Valdebenito-Sanhueza R.M. (1997). *Gliocladium roseum*, a versatile adversary of *Botrytis cinerea* in crops. Plant Dis., 81: 316-328.

Thangavelu R., Palaniswami A., Velazhahan R. (2004) Mass production of *Trichoderma harzianum* for managing fusarium wilt of banana. Agricult. Ecosys. Environ., 103: 259-263.

Tian X.L., Cao L.X., Tan H.M., Zeng Q.G., Jia Y.Y., Han W.Q., Zhou S.N. (2004). Study on the communities of endophytic fungi and endophytic actinomycetes from rice and their antipathogenic activities *in vitro*. World J. Microbiol. Biotechnol., 20: 303-309.

Torres M.R., Ramos A.J., Soler J., Sanchis V., Marín S. (2003). SEM study of water activity and temperature effects on the

initial growth of *Aspergillus ochraceus*, *Alternaria alternata* and *Fusarium verticillioides* on maize grain. Int. J. Food Microbiol., 81: 185-193.

Viterbo A., Montero M., Ramot O., Friesem D., Monte E., Llobell A., Chet I. (2002). Expression regulation of the endochitinase chit36 from *Trichoderma asperellum* (*T. harzianum* T-203). Curr. Genet., 42: 114-122.

Whipps J.M. (2001). Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot., 52: 487-511.

Zadworny M., Werner A., Idzikowska K. (2004). Behaviour of the hyphae of *Laccaria laccata* in the presence of *Trichoderma harzianum in vitro*. Mycorrhiza, 14: 401-405.

Zimand G., Elad Y., Chet I. (1996) Effect of *Trichoderma harzianum* on *Botrytis cinerea* pathogenicity. Phytopathology, 86: 1255-1260.