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STUDY OF THE INTERACTIONS BETWEEN *PENICILLIUM OXALICUM* CURRIE & THOM AND *ALTERNARIA ALTERNATA* (FR.) KEISSLER

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ABSTRACT

The aim of this research was the analysis of the possible antagonistic effect of *Penicillium oxalicum* over the pathogen rice fungus *A. alternata* under different conditions of temperature, water activity and culture media. The macroscopic study of the dual growth revealed that according to the Index of Dominance *P. oxalicum* was more competitive that *A. alternata* at 25ºC whereas at 15ºC was this species. Microscopic analysis showed that *P. oxalicum* was a mycoparasite of *A. alternata* at all conditions tested. The antagonist penetrated into *A. alternata* and disintegrated its conidiophores and conidia. The results suggests that *P. oxalicum* may be a possible biological control agent of the rice pathogens in a future.

Key words: *Alternaria alternata*, *Penicillium oxalicum*, rice, water activity, mycoparasitism.

INTRODUCTION

Alternaria alternata (Fr.) Keissler is a pathogen fungus of several crops causing losses of different consideration. Also this species is known to produce several mycotoxins in foods: alternariol, alternariol monomethyl ether, etc.

Rice grains are frequently contaminated with various *Alternaria* species, particularly *A. alternata* and its presence has been reported in different worldwide areas (1, 12, 13, 18). In this cereal the strain produces a reduction of the initial quality: undesirable caryopsides' colorations, brittle grains, weight loss, reduction of nutritional constituents, etc.

Application of chemical fungicides is a common management strategy to combat the pathogens on rice (5, 20). But it is essential the application the respectful alternatives with the environment. The success of biological control agents for the control of others crops pathogens fungi require researches of new organisms for a possible future control of these strains on the different production phases. Few studies have been carried for the determination of the biological control agents of rice fungi. Currently no available biocontrol agents of these important cereal pathogens exist for its commerzialitation.

The aim of this research was the analysis of the possible antagonistic effect of *Penicillium oxalicum* 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

Microscopic fungi

The fungus *Alternaria alternata* was isolated from samples of rice grains collected from different rice fields and cooperatives of the main rice producing areas in Valencia.

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The fungus *Penicillium oxalicum* was obtained of the fungal collection of the Department of Agroforest Ecosystems in the School of the Rural Environment and Enology.

Substrates

The substrates used in this study were Rice Extract Agar and rice of Valencia. The synthetic medium Rice Extract Agar was obtained from grains of rice. The water activity (a_w) of this basal medium was modified by the addition of different amounts of glycerol to obtain five different levels of water activities (0.995, 0.98, 0.95, 0.90 and 0.85).

The sterilized rice grains with a solution of sodium hypochlorite were deposited 48 hours in the different solutions for set the water activity (0.995, 0.98 and 0.95 a_w: 2.5, 11 and 23.5 g glycerol / 100 mL distilled water) (15).

Ecophisiological study

The ecophisiological study of the two fungi was carried in Rice Extract Agar at the different water activities. Eight millimetres diameter disks of *Penicillium oxalicum* and *Alternaria alternata*, 45 mm apart, obtained from the growing margins of the fungus colonies grown in PDA at 25 ºC for 5 days were inoculated in the Petri Plates. The samples were incubated at two temperatures (15ºC and 25ºC).

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).Each treatment was repeated four times.

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. Water activity was checked using an Aqualab (Decago, Inc., Pullman, WA, USA).

Daily the diameters of the growing colonies were measured in two directions at right angles to each other during a period of five days. A linear regression of increase in radius (in mm) against time (in days) was used to obtain the growth rates (mm day^{-1}) for each set of treatment conditions. The computer software used was Microsoft Excel 2003.

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

Macroscopic study of the interaction

Rice Extract Agar at three water activities (0.995, 0.98 and 0.95) was the substratum used for the macroscopic study of the interaction. After the ecophisiological study the Petri plates were incubated a period of eight weeks for the determination of the interactions.

According to method proposed by Magan and Lacey (9), the interactions were determined and numerical scores were assigned for *P. oxalicum* and *A. alternata* with the objective to obtain the Index of Dominance at the water activities and temperatures assayed. 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 [4for the dominant species, 0 for the inhibited species]; dominance at a distance [5 for the dominant species, 0 for the inhibited species].

Microscopy study of the fungi relations

A scanning microscope JEOL JSM 5410 and a light microscope Olympus PM-10AK3 were used in this study. Microscopic analysis was performed on Rice Extract Agar and full rice grains. The water activities experimented were 0.95, 0.98 and 0.995 at both temperatures

Previously the preparation of the samples for its microscopical observation was described. For the analysis in the synthetic medium, both fungal species were inoculated in REA squares at different a_w levels 5 mm apart, mounted on glass slides in glass rods inside Petri plates of 90 mm under conditions of total asepsis. Coverslips were placed on the films. To maintain the levels of water activity, filter paper disks impregnated with different solutions (0.995, 0.98 and 0.95 aw: 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 sterilized with a solution of sodium hypochlorite and were deposited 48 h in the solutions described above for setting the water activities. The study was conducted in the same conditions that the analysis in the synthetic medium, except for the strains that were inoculated 3 mm apart. For cryo-scanning electron analysis no coverslips were deposited on the substrates (16).

The plates with the *dual microculture* in Rice Extract Agar and rice grains were incubated a period between 7-30 days depending on both temperature and water activity.

RESULTS

Growth at different environmental conditions

Figure 1 shows the growth rates of *Penicillium oxalicum* and *Alternaria alternata* at different a_w levels and temperatures. Maximum growth rate in both fungal species occurred at a temperature of 25ºC and water activities of 0.995 for *A. alternata* and 0.98 for *P. oxalicum*, with values of 6.18 mm day^{-1} and of 4.16 mm day^{-1} respectively.

At 0.995 a_w *A. alternata* exhibited higher growth rates that *P. oxalicum*. Minimal differences between the growth rates of both isolates at the others water activities and both temperatures were observed. For example at 0.98 and 25ºC, 4.75 mm day^{-1} was the numerical value registered for *A. alternata* and 4.16 mm day^{-1} for *P. oxalicum*.

The minimum amount of water activity producing growth in Rice Extract Agar occurred at 0.90 a_w for *A. alternata* and at 0.85 a_w for *P. oxallicum*. Although initially development was not registered in these conditions, by the end of the eight weeks it was observed.

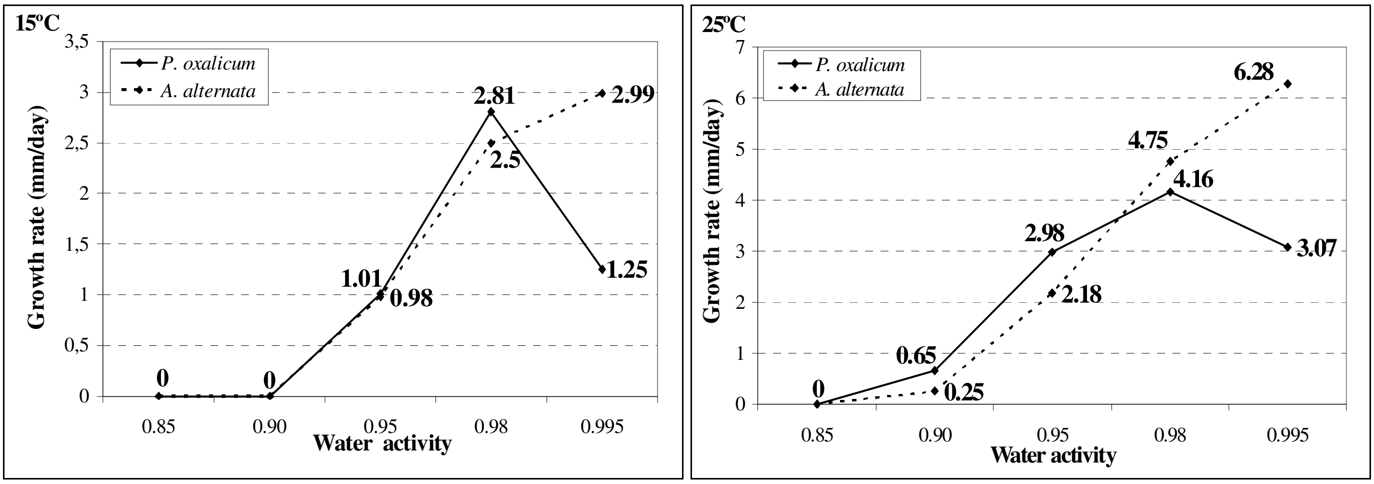

Figure 1. Ecophisiological study of *Penicillium oxalicum* and *Alternaria alternata* dual culture in Rice Extract Agar at different temperatures and water activities.

Dual growth of *P. oxalicum* and *A. alternata* was significantly affected by the simple factors: water activity, temperature, species and the interactions (a_w x T, a_wxE) (P <0.01). The dual factor TxE had a significant effect (P <0.05) (Table 1).

Initially the inoculation of both isolates in the same substrate not affected their growth. Later the colonies came into contact, the interaction significantly influenced their development.

Interactions according to the method of Magan and Lacey

When *P. oxalicum* and *A. alternata* were inoculated on Rice Extract Agar, three type of interactions were registered at the different conditions experimented (Figure 2). At 0.98 a_w

and both temperatures *A. alternata* inhibited *P. oxalicum* on contact. Both species continued growing until both colonies came into contact. Later, *A. alternata* grew over *P. oxalicum*. The same type of interaction was registered at 0.995 a_w and 15ºC.

Mutual antagonism on contact of both isolates at 0.95 a_w and 15ºC was observed. At the rest of conditions *P. oxalicum* inhibited *A. alternata* on contact.

Table 1. Anaysis of variance of the growth rate of *P. oxalicum* and *A. alternata*; significance of water activity (a_w), temperature (T), species (E) and their interaction. (DF) Degrees of freedom. (MS) Mean squares. **Indicates that the factor elicited a significant effect (P<0.01). *Indicates that the factor elicited a significant effect (P<0.05).

FACTOR	DF	MS	F-ratio	P-value
a_w	4	1636.63	115.39	0.0000**
T	1	2263.38	159.58	0.0000**
E	1	332.151	23.42	0.0000**
a_w x T	4	358.826	25.30	0.0000**
a_w x E	4	233.133	16.44	0.0000**
TxE	1	52.2006	3.68	0.0558*

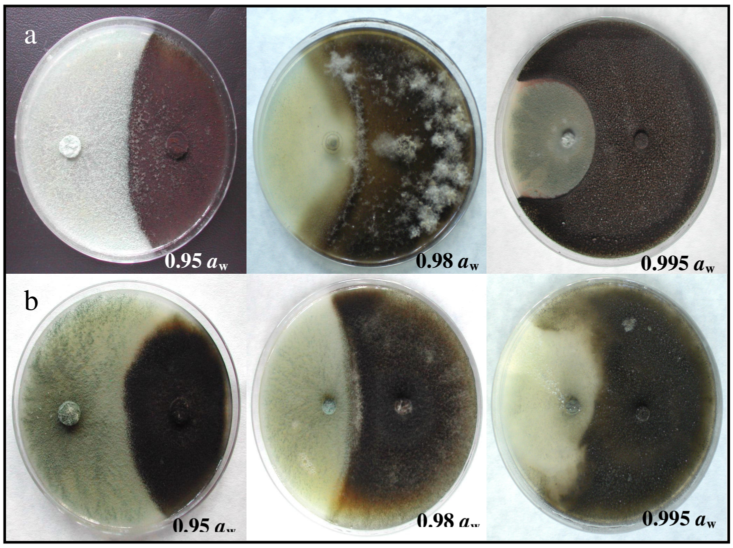

Figure 2. Dual cultures between *P. oxalicum* and *A. alternata* after 8 weeks on Rice Extract Agar at different temperatures and water activities. Row a: 25ºC. Row b: 15ºC. Left: *P. oxalicum*. Right: *A. alternata*.

According to the Index of Dominance, *P. oxalicum* was a species more competitive that *A. alternata* at 25ºC (Table 2).

Macroscopically, in all abiotic factors tested, no apparent changes of *P. oxalicum* and *A. alternata* colonies were observed. Cultural characters appeared similar to that of the fungi in singles cultures at the different temperatures and water activities.

Table 2. Index of Dominance (I_D). I_D refers to sum of scores at 25ºC and 15ºC for *P. oxalicum* competing with *A. alternata* based on the interaction scores for each species. Dominance on contact (4 for the dominant species, 0 for the inhibited species). (X) Analysis of interaction was discarded.

Temperature	Fungus species	0.995 a_w	0.98 a_w	0.95 a_w	0.90 a_w	I_D
25 ºC	*P. oxalicum*	4	0	4	X	8
	A. alternata	0	4	0	X	4
15 ºC	*P. oxalicum*	0	0	2	X	2
	A. alternata	4	4	2	X	10

Efficacy of *P. oxalicum* isolate against *A. alternata* on different substrates

Microscopic analysis of the interactions on Rice Extract Agar and full rice grains revealed that *P. oxalicum* was a mycoparasite of *A. alternata* at all conditions of temperatures and water activities.

Alternaria alternata sporulated at the same environmental conditions that when this fungus were inoculated individually (0.995 a_w to 0.95 a_w at both temperatures), but *P. oxaliucm* parasited the different reproductive structures of the pathogen when the isolates were grown dually. This rice pathogen species in REA medium and full rice grains presented septate hyphae and pluricellular conidia with transverse and, in some cases, longitudinal septa irregularly distributed, ovoid, pyriform, ellipsoidal or oval-shaped, brownish in colour, with rough surface ornamentation, which were born from simple walled conidiophores with smooth walls, or from the previous spore, giving rise in this case to a chain that tends to get branched if the spore generates more than one sprout.

Penicillium oxalicum in both substrates jointly *A. alternata* and individually sporulated at all temperatures and temperatures assayed (0.995 a_w to 0.85 a_w at 15 and 25º). Terverticillate *Penicillium* with smooth conidiophores (stipe, metulae, branches and phialides), ampulliformes to cylindrical phialides with a conical apical portion. Spherical to ellipsoidal conidia, unicellular and hyaline. Smooth to slightly rough cell wall conidia in no branched long chains originated from the phialides.

In the figures 3A, 3B, 3C, 4A, 4B and 4C it can be seen the initial contact and recognition between the mycoparasite *P. oxalicum* and its host *A. alternata*. Later, *P. oxalicum* penetrated into *A. alternata* and disintegrated its conidiophores and conidia (Figures 3D and 4D).

The attack of the *A. alternata* hypha not were observed in the study at the different conditions.

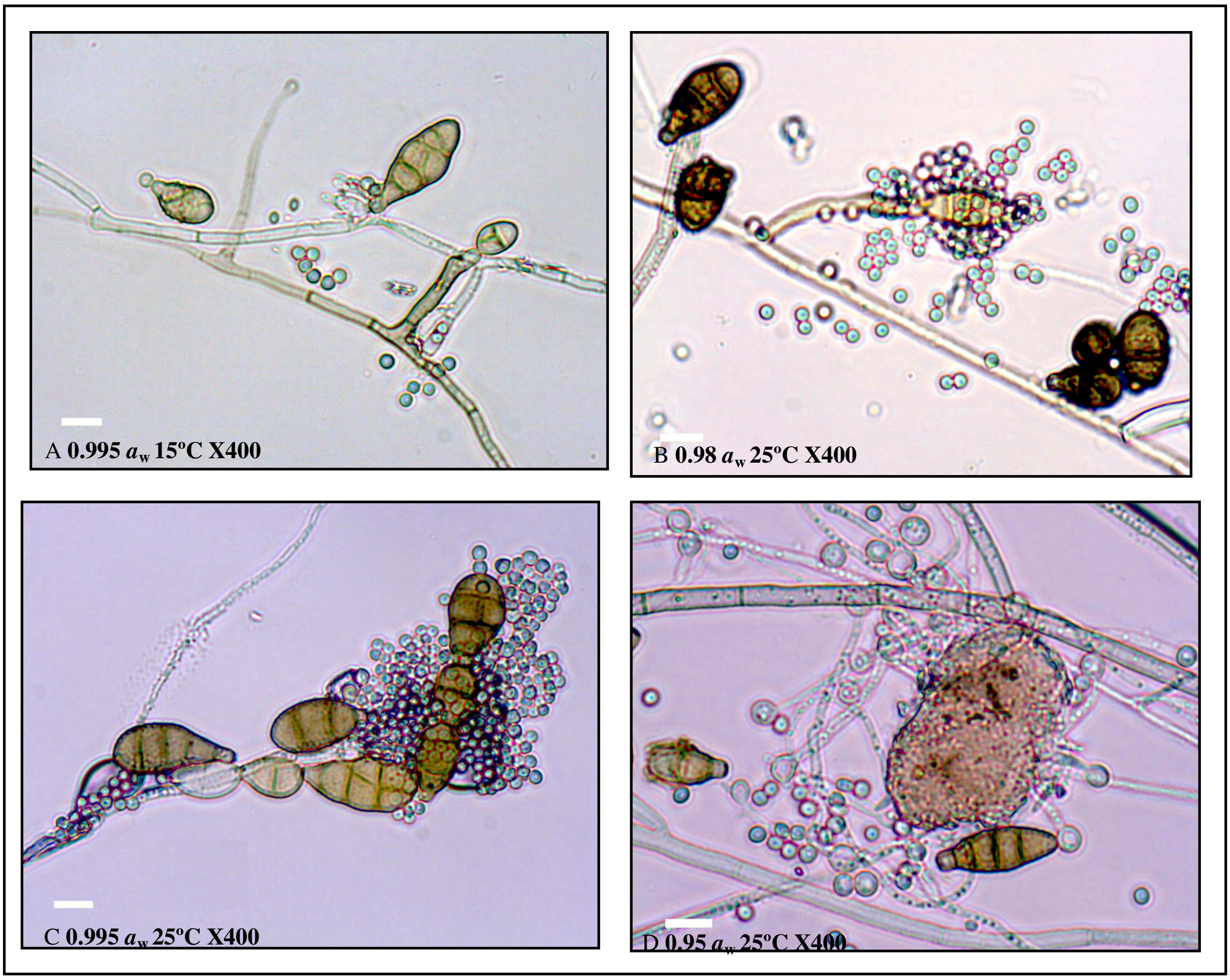

Figure 3. Light micrographs of the dual microculture of *P. oxalicum* and *A. alternata* at different temperatures and water activities. A: Physical examination and contact between *P. oxalicum* and the conidiophores and conidia of *A. alternata*. B, C: Antagonist attack on the conidia of *A. alternata*. D: Completely disintegrated conidia of *A. alternata* by the action of *P. oxalicum*. ▬ ≈ 5 µm.

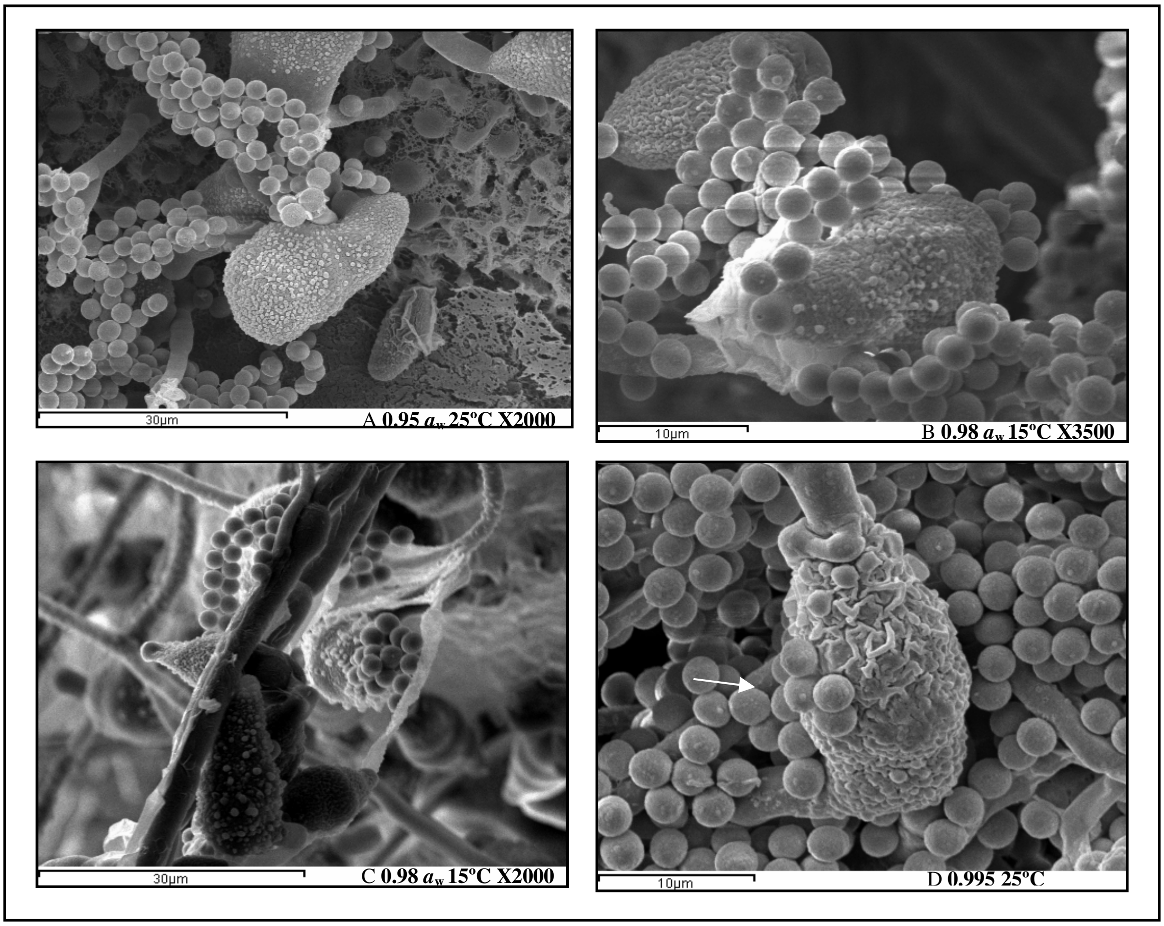

Figure 4. Cryo-scanning electron micrographs showing mycoparasitism of *A. alternata* by *P. oxalicum* at different temperatures and water activities. A, B, C: Initial contact, attack and penetration of *P. oxalicum* into *A. alternata*. D: Disintegrating conidium of *A. alternata* by the action of *P. oxalicum*. It can be seen the conidia of *P. oxalicum* coming out of a conidium of *A. alternata* (see arrow). A, B: The fungi were inoculated on Rice Extract Agar. C, D: Full rice grain.

DISCUSSION

This study is the first detailed the mycoparasitism of *P. oxalicum* over *A. alternata*. The analysis performed in this work reveals that the mechanisms by which *P. oxalicum* antagonize *A. alternata* vary depending on the conditions of temperature and water activity. At 0.995 a_w 25ºC and at 0.95 a_w and both temperatures *P. oxalicum* exerted synergistically two mechanisms of antagonism: mycoparasitism and competition for space and nutrients.

Previously *Trichoderma* species has been tested over *A. alternata*. *Trichoderma* isolates antagonized *A. alternarta* through different mechanisms such as competition for space and nutrients, mycoparasitism and possible antibiosis (10, 11, 16).

Different researches described the antagonistic mechanism of induction of resistance for *Penicillium oxalicum*. In this sense, *Penicillium oxalicum* is a promising fungal agent for biological control of tomato diseases, such as those caused by *Fusarium oxysporum* f. sp. *lycopersici* (2, 3), *Verticillium dahliae* (6, 7, 14). Also this antagonistic agent has been tested over strawberry and pea fungal strains and others crop pathogens with good results (4, 19).

When this species was confronted with other species belonging to the dominant mycobiota of Valencia rice: *Nigrospora oryzae*, *P. oxalicum* antagonized this rice pathogen by mycoparasitism and competition for space and nutrients in all conditions assayed (17).

The degradation and penetration of cell walls of *A. alternata* conidiophores suggests that *P. oxalicum* produce antifungal components and extracellular metabolites like cell wall degrading enzymes chitinases, glucanases and proteases. Ma et al. (8) reported the potential of *Penicillium striatisporum*, as a biological control agent of soil-borne diseases caused by plant pathogens such as *Phytophthora* spp., *Cladosporium cucumerium*, and *Sclerotinia sclerotiorium* and the authors suggested that the suppression of these pathogens may be due to the production of toxic metabolites by this biological control agent. In this study no mycoparasitism was observed.

Alternaria alternata species can contaminate rice from its cultivation to harvest, during its transportation and storage, and in various production phases causing loss of different consideration. The results of this study are the first to describe the parasitism of *A. alternata* by *P. oxalicum* in rice grains and Rice Extract Agar in vitro conditions. *P. oxalicum* may be a good biological control agent of the rice pathogens fungi in a future but more researches is necessary for this consideration.

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