Antagonistic activity of *Penicillium oxalicum* Corrie and Thom, *Penicillium decumbens* Thom and *Trichoderma harzianum* Rifai isolates against fungi, bacteria and insects *in vitro*

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**Summary**

The antibiotic activity of 70 isolates belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Trichoderma* was tested as preliminary screening. The highest activity was obtained with three *Penicillium oxalicum* isolates, one *Penicillium decumbens* isolate and the *Trichoderma harzianum* isolate. After that, we chose these five isolates in order to carry out other studies with bacteria, fungi and insects. Extracts from these isolates were obtained. The extracts were tested for antibiotic activity with positive results, which implies that metabolite production is involved in this antagonistic effect. The highest activity was shown by *T. harzianum* and *P. oxalicum* extracts, but there was high variability among *P. oxalicum* isolates.

**Key words**

Bactericidal and fungicidal activity, Secondary metabolites, *Penicillium oxalicum*, *Penicillium decumbens*, *Trichoderma harzianum*

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**Resumen**

En el presente trabajo se realizó un estudio preliminar de la actividad antibiótica de 70 cepas fúngicas pertenecientes a los géneros *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* y *Trichoderma*. Los mejores resultados se obtuvieron con tres cepas de *Penicillium oxalicum*, una de *Penicillium decumbens* y la cepa de *Trichoderma harzianum*, por lo que estas cinco cepas se seleccionaron para llevar a cabo otros estudios frente a bacterias, hongos e insectos. Con los extractos obtenidos de cada una de las cepas seleccionadas, se realizaron ensayos para la detección de la actividad bactericida, fungicida e insecticida de los mismos, obteniéndose resultados positivos, lo que implica que la producción de metabolitos activos está involucrada en el efecto antagonista de estos agentes. Los extractos de *T. harzianum* y *P. oxalicum* mostraron los mejores resultados; sin embargo, se observó una gran variabilidad entre las distintas cepas ensayadas de *P. oxalicum*.

**Palabras clave**

Actividad bactericida y fungicida, Metabolitos secundarios, *Penicillium oxalicum*, *Penicillium decumbens*, *Trichoderma harzianum*
Pesticides play an important role in the stabilization and increase of agricultural yield, but are accused of being a possible source of atmospheric pollution, with residual toxicity to mammals and wildlife.

Microbial products with antimicrobial activity are now being applied in every sphere of pesticide use. Thus, some antifungal, antibacterial, insecticidal and herbicidal products used in crop protection have been obtained from microorganisms [1].

Some species of fungi can secrete substances or metabolites that have very specialized activity, being lethal to a particular group of life forms. Since 1963, fungi have received great attention as biocontrol organisms against pests [2-5].

Extensive work has been carried out in order to identify fungi with potential capabilities as pesticides, thus many screenings for antagonistic activities of fungi can be found in the literature [5-11].

Several new disease biocontrol agents have become commercially available in the recent years, including Gliocladium virens, Streptomyces griseoviridis, Trichoderma harzianum and vesicular-arbuscular mycorrhizal fungi. Each has a different pathogen/host target but all are registered for use in protected crops rather than in the field, emphasizing the significance of stable environmental conditions for achieving reproducible biological disease control [12]. Recent advances on soil-borne disease control include: a) development of biocontrol formulation systems for delivery, b) genetic manipulation of biocontrol agents to increase their efficacy; and c) the use of biocontrol in integrated pest management [13].

Research in agriculture has been directed to fungi as biocontrol agents against other fungi and insects. Most of fungal antagonists, however, have been used because of their antifungal properties [8,14,15].

The purpose of this paper was to study the activity of different Penicillium, Aspergillus, Fusarium, Alternaria and Trichoderma isolates from cereals and their metabolic broths against both bacteria and fungi, in order to look for some antibacterial and antifungal and insecticidal products.

MATERIALS AND METHODS

Microorganisms. The 70 isolates tested in this study were isolated from cereal samples obtained from the retail market in Valencia (Spain). The distribution of the isolates was: Penicillium (32), Aspergillus (30), Fusarium (7) and Trichoderma (1). The reference isolates used in the different tests were obtained from Spanish collections: CECT (Colección Española de Cultivos Tipo), CR (Reus Medicine Faculty Collection) and MTAL (University of Lleida, Food Technology Dept. Collection). All the isolates were maintained in potato dextrose agar (PDA).

Preliminary assay. Wickerham’s test was performed on solid media following the classical procedure and the lack of growth in a 1-2 cm width area around the fungal colonies was recorded as the result of an inhibitory effect. The bacterial strains used belonged to the CECT gal colonies was recorded as the result of an inhibitory effect. The bacterial strains used belonged to the CECT Collection (CECT 472), E. coli (Migula) Castellani and Chalmers (CECT 943), P. solanacearum (Smith) Smith (CECT 125), X. campestris (Pammel) Dowson (CECT 97), S. marcescens Bizio (CECT 977). They were incubated at 37 °C and the reading was taken 24 h later, by measuring the inhibition halo produced.

Fungicidal capacity. The method consists of determining the inhibition of fungal growth, on impregnating the fungal extracts on discs, using in the test, solid media (Mueller-Hinton agar). Bacteria were grown both in Wickerham and potato dextrose broth were grown in a broth medium and 0.5 ml of this broth (10^8 UFC/ml) inoculated by inclusion, then impregnated paper disks (5 mm diameter) were placed on the agar surface. For each tested bacteria, two different assays were carried out, one containing 50µg of extracts in each disk, and another containing 100µg of extracts in each disk. The following bacteria were used in this test: A. tumefaciens (Smith and Townsend) Conn (CECT 472), E. coli (Migula) Castellani and Chalmers (CECT 943), P. solanacearum (Smith) Smith (CECT 125), X. campestris (Pammel) Dowson (CECT 97), S. marcescens Bizio (CECT 977). They were incubated at 37 °C and the reading was taken 24 h later, by measuring the inhibition halo produced.

Insecticidal capacity. Maturation induction activity against Oncopeltus fasciatus. Oncopeltus fasciatus used in this test, came from a colony kept in the laboratory at 29 °C, with a relative humidity between 65-70%.

For this test, extracts were evaporated to dryness and dissolved in cyclohexane, acetone or a mixture of both depending on solubility until reaching a concentration of 250 µl/ml. Then 130 µl of this dilution were placed in a Petri dish plus two additional ml of solvent, giving...
a residual deposit of 500 µg/cm². Once the dish was dry, 15 individuals in the nymph stage were placed in along with appropriate feed. Incubation was carried out at relative humidity of 60-70%, at 29 ºC and with a constant photoperiod for 14 days, the toxicity of the extracts was studied, by determining the percentage of dead individuals after three, seven, and 14 days.

An additional experiment was carried out by transferring survivors after three days to containers under suitable conditions in order to observe the ones that reach adult stage as well as precocious adults.

RESULTS AND DISCUSSION

Preliminary test. Out of 70 isolates assayed, 37 (52.11%) were able to exert an inhibitory effect against at least one of the bacteria tested. The percentages depended on the genus they belonged to: Aspergillus (17 out of 30), Penicillium (24 out of 32), Fusarium (one out of three), and Trichoderma (one out of one). Most of the isolates with positive results belonged to Penicillium genus, which is well known because of the ability of its species to produce antibiotics, some of them at an industrial scale. Moreover, Penicillium species inhibited a wider range of bacteria. P. griseofulvum has been reported to be an important inhibitor of bacterial growth; this ability has been associated with its griseofulvin and patulin production [16].

Aspergillus isolates showed an inhibitory activity mainly against S. aureus, one E. coli isolate and S. albus, while Penicillium isolates were effective mainly against B. subtilis, S. albus, S. aureus and S. pyogenes. Previously, Penicillium isolates from dry-cured ham had shown wide antibiotic effects when tested against both bacteria and yeast, similarly they observed a high sensitivity in E. coli, B. subtilis and S. aureus isolates, while S. marcescens displayed a weak sensitivity. By contrast, little sensitivity was shown by S. marcescens, S. aureus and B. subtilis against Aspergillus species, concluding as in the present study that Penicillium spp. beard wider antibiotic capabilities [17]. Among Penicillium and Aspergillus species isolated from vineyard soils, P. oxalicum was one of the species with a broader spectrum of activity against S. aureus, B. subtilis, B. cereus, P. mirabilis and Candida albicans [9].

The only T. harzianum isolate tested showed a wide spectrum of inhibition. Fungi of this genus have been successfully applied for seed treatments [18]. T. harzianum has also been applied in soil, and T. koningii on cowpea leaves, as a biocontrol agent against Rhizoctonia solani on cotton in a greenhouse environment [19,20], and against wood degrading fungi [21]. Both T. viride and T. harzianum are recognized by biopesticides mainly against Rhizoctonia, Sclerotinia and Botrytis [13]. In in vitro studies, Rhizoctonia solani, Pythium ultimum and Chalara elegans were strongly inhibited by Trichoderma viride, T. harzianum, T. pseudokoningii and T. koningii, both when the pathogen and antagonist were grown in pairs in the same agarized medium and when they were grown on separate media in a confined environment. These results indicate that the biocontrol efficacy of Trichoderma seems to perform not only at medium, but also at atmosphere level [15]. Effect of volatiles produced by T. hamatum on growth of phytopathogen fungi from soil has been shown [22].

The best results were obtained by three Penicillium oxalicum isolates, one isolate of P. decumbens, and one T. harzianum isolate, which were used in the following sections. All these species are distributed world-wide. P. oxalicum is especially common in soil and upon various organic materials undergoing slow deterioration or decay. It is undoubtedly one of the most ubiquitous of all the Penicillia. P. decumbens is also abundant in nature and regularly occurs in soil dilution plates. T. harzianum is commonly isolated from soil, grains, pecans, paper and textiles. Although most of the screenings for biocontrol agents have been carried out involving the microorganism itself; it would be interesting to know if the metabolic broth where these fungi have been grown are also active. Mycoparasitism involving lytic enzymes has been already described as the mechanism of action of Trichoderma isolates in the biological control of commercially important plant pathogens [23]. Trichoderma employs a variety of antagonistic mechanisms for combating other fungi. The simplest one is probably competition for non-structurally-bound nutrients, however volatile and soluble antifungal metabolites are also involved [24].

Study of the antibiotic activity of extracts. Two different extracts were obtained from each fungus because they were grown on two different media (Wickerham (WB) and potato dextrose (PDB) broth). Results suggest that in general the extracts from potato dextrose broth have higher effectiveness.

None of the extracts showed bactericidal capacity at a concentration of 50 µg/disk, however this changed with a 100 µg/disk concentration. Extracts 1, 2, 3, 4 and 9 showed the widest inhibitory effect (Table 1).

Referring to fungicidal activity (Table 2), results were generally better with a 100 µg extract/disk concentration. None of the extracts showed activity against B. cinerea. However, trichoderex, a preparation from T. harzianum T39 controls diseases caused by Botrytis cinerea in greenhouse crops and vineyards [25]. This might indicate that there is an intraspecific difference, or that the antagonistic activity against B. cinerea is not due to the metabolite production but to the fungus itself and volatiles release.

The more active extracts were produced by T. harzianum (1 and 2) and P. oxalicum (3, 4 and 5). Among them, to date, only T. harzianum has been investigate for antibiotic metabolites production. Then, culture extracts from nine antagonistic Trichoderma spp. have been tested.

Table 1. Bactericidal activity of extracts: halos of inhibition (mm).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Extract No</th>
<th>100 µg extract/disk</th>
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<tbody>
<tr>
<td>Pseudomonas solanearum</td>
<td>1</td>
<td>5 12 8 12 11</td>
</tr>
<tr>
<td>Xanthomonas campestris</td>
<td>2</td>
<td>5 10 8 12 11</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>3</td>
<td>10 9 11 9 9</td>
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<tr>
<td>Escherichia coli</td>
<td>4</td>
<td>11 9 11 9 9</td>
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<tr>
<td>Senata marcescens</td>
<td>5</td>
<td>5 5 5 9 9</td>
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<tr>
<td>P. oxalicum 1</td>
<td>6</td>
<td>5 5 5 9 9</td>
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<tr>
<td>P. oxalicum 2</td>
<td>7</td>
<td>5 5 5 9 9</td>
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<td>P. oxalicum 3</td>
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<td>5 5 5 9 9</td>
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<td>P. oxalicum 4</td>
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<td>5 11 9 10 9</td>
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<td>P. oxalicum 5</td>
<td>10</td>
<td>5 5 5 9 9</td>
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<tr>
<td>Control</td>
<td>11</td>
<td>5 5 5 5 5</td>
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</tbody>
</table>

* 1, 3, 5, 7, 9, extracted from cultures grown in potato dextrose broth of T. harzianum, P. oxalicum 1, P. oxalicum 2, P. decumbens and P. subtilis, respectively.
* 2, 4, 6, 8, 10, extracted from cultures grown in Wickerham broth of T. harzianum, P. oxalicum 1, P. oxalicum 2, P. decumbens and P. oxalicum 3, respectively.
* 11, extracted from P. oxalicum 3 cultures grown in Wickerham broth plus maize flour.
on Geotrichum candidum for antibiotic activity, and all produced antifungal metabolites [26]. These antifungal metabolites could only be detected after conidogenesis [24].

One of these substances has been identified as 3-(2-hydroxypropyl)-4-(2-hexadienyl)-2(5-hydroxyl)-furaneone, a new natural product [27]. Trichodermin, a commercial product obtained from T. koningii has been used for control of root rots on greenhouse-grown tomato and cucumber [28], for control of Curvularia leaf spot of yam [29], and for treatment of Capsicum plants against Verticillium dahliae [30].

Very different activities were shown by P. oxalicum isolates, which is in accordance with the great variability found among the isolates of the different Penicillium species in toxigenic capacity of the secondary substances or metabolites excreted [31]. P. oxalicum has been reported to be a biocontrol agent for Fusarium oxysporum f. sp. lycopersici [32]. Leaf spot severity (Cercospora canescens) on Vigna mungo was reduced by spraying with P. oxalicum before inoculation of C. canescens [33]. A study by Pandey et al. [11] suggested that volatiles produced from P. oxalicum inhibited the growth of Glomerella cingulata, while volatiles from T. harzianum inhibited the growth of Pestalotia psidii. Application of a spore suspension of each test fungus inhibited lesion development of guava leaves caused by the test pathogens in vitro. However, metabolite extracts from this species have not been tested for inhibitory activity.

P. decumbens has been investigated for the obtaining of enzymes industrially applied such as cellulases [34] and naringinases [35], but not for antibiotic metabolites. On the whole, extracts from one isolate of P. oxalicum Corrie and Thom, presented the maximum activity both in terms of bactericidal and fungicidal activity, higher than the well-known Trichoderma species. In general, PDB was a more suitable medium for the production of antibiotic compounds, than WB was.

Study of the insecticidal activity. Table 3 shows the results obtained in the insecticidal activity test with the assayed extracts. In general, oppositely to previous section, higher activities were shown by extracts from cultures on WB than those on PDB. High mortalities were reported after three days of contact, moreover, mortality increased from three to seven days, but then remained constant, with no differences between seven and 14 days of treatment. Extract 4 led to a 100% mortality, while extracts 1, 2, 10 and 11 provoked the death of 80% of the individuals, and even a higher percentage. These effective extracts came from P. oxalicum and T. rifai cultures. Extracts from cultures of Penicillium funiculosum have been reported to induce 80-100% mortality of the same insect [3].

Penicillium and Trichoderma species have been isolated from Glossina palpalipes, however, their were only mildly pathogenic for adult Glossina, compared to bacteria isolates, and were not pathogenic for pupae [8]. By contrast, fungal genera associated with Stomatium fulvum larvae were isolated and pathogenicity test done; Trichoderma and Penicillium were among the genera recommended as biological control agents for S. fulvum larvae [7].

A laboratory method was described by Jassim et al. (1990) for the rearing of larvae of Scolytus multistriatus

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**Table 2.** Fungicidal activity of extracts: halos of inhibition (mm).

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<tr>
<th>Fungi</th>
<th>Extract Nº</th>
<th>50 µg</th>
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<td>Penicillium griseofulvum</td>
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<td>Aspergillus candidus</td>
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<td>Curvularia trifolii</td>
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<td>Botrytis cinerea</td>
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**Table 3.** Percentages of mortality induced by fungal extracts on Oncopeltus fasciatus individuals.

<table>
<thead>
<tr>
<th>N. extract</th>
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<th>10</th>
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<tr>
<td>After 3 days treatment</td>
<td>66.6</td>
<td>40</td>
<td>0</td>
<td>66.6</td>
<td>13.3</td>
<td>60</td>
<td>13.3</td>
<td>20</td>
<td>46.7</td>
<td>80</td>
<td>73.3</td>
<td>0</td>
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<tr>
<td>After 7 days treatment</td>
<td>73.3</td>
<td>73.3</td>
<td>60</td>
<td>100</td>
<td>46.6</td>
<td>60</td>
<td>33.3</td>
<td>46.7</td>
<td>46.7</td>
<td>86.7</td>
<td>80</td>
<td>0</td>
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<tr>
<td>After 14 days treatment</td>
<td>80.3</td>
<td>80</td>
<td>60</td>
<td>100</td>
<td>46.6</td>
<td>60</td>
<td>33.3</td>
<td>46.7</td>
<td>46.7</td>
<td>86.7</td>
<td>80</td>
<td>0</td>
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<tr>
<td>After 3 days treatment+ 3 days normal conditions</td>
<td>-</td>
<td>-</td>
<td>60</td>
<td>100</td>
<td>-</td>
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<td>13.3</td>
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and S. scolytus on an artificial diet following exposure to cultures of microorganisms. In control colonies, the natural mortality for both species was 21.2 and 17.6%, respectively. Inoculation of Trichoderma harzianum caused more than 80% larval mortality.

Referring to retarded activity, it was only shown by extracts 3 and 4 (P. oxalicum). Insects treated with extract 3 showed an initial resistance (0% mortality after three days), but once the treatment finished mortality increased up to 60%.

P. oxalicum has been reported to be a pathogenic fungus of the aphid Ceratovacuna lanigera, an insect pest on sugarcane [29].

Out of the five isolates tested, P. decumbens showed the least bactercidal, fungicidal and insecticidal activity.

The results showed in this paper are a preliminary study to find species with capacity to control harmful fungi and insects. In a future other studies will be necessary to know the possibility to use these fungi as biological pesticides.

References