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Antagonism against *Rhizoctonia solani* and fungitoxic metabolite production by some *Penicillium* isolates

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Abstract

A number of *Penicillium* isolates were recovered in association to *Rhizoctonia solani* strains pathogenic on tobacco and from soil on plates pre-colonized by the pathogen itself. Their antagonism toward *R. solani* AG-2-1 was evaluated in dual cultures *in vitro*. Inhibition of growth was evident to some extent in most pairings, while hyphal interactions referable to mycoparasitic relationships were not observed. However, the occurrence of plasmolysis and/or vacuolisation and the induction of monilioid cells were indicative of the release of bioactive compounds. Therefore, production of fungitoxic metabolites was tested by adding concentrated culture filtrates of each *Penicillium* isolate to the growth medium of *R. solani*. Complete and lasting inhibition was incited by culture filtrates of some isolates belonging to *P. brevicompactum*, *P. expansum*, and *P. pinophilum*. Three purified compounds, respectively mycophenolic acid, patulin and 3-*O*-methylfunicone, which were extracted from culture filtrates, were able to inhibit *R. solani in vitro*. Their production was also detected in dual cultures of the same *Penicillium* strains with *R. solani* prepared in sterilized soil and when the *Penicillium* strains were cultured directly on *R. solani* mycelium harvested from liquid cultures. The possible role of such metabolites in antagonism of the above-mentioned *Penicillium* species against *R. solani* is discussed.

Key words: antagonism, biological activity, culture filtrates, mycotoxins, *Penicillium, Rhizoctonia solani*

Introduction

Rhizoctonia solani Kühn [teleomorph *Thanatephorus cucumeris* (Frank) Donk] is known as the etiological agent of damping-off, sore shin and target spot of tobacco (*Nicotiana tabacum* L.). Its incidence has increased since the float-system for rearing tobacco seedlings in polystyrene containers in greenhouses has become widespread [1]. The necessity to control the pathogen in such an artificial environment incites to exploit the use of biological control agents as an alternative to fungicides; actually, a number of fungal antagonists have proved to be effective against *R. solani* on several crops [2, 3].

Species of *Penicillium* are fundamentally cosmopolitan and ubiquitous, and many of them have been thoroughly studied with regard to their ability to produce mycotoxins that can contaminate food [4–6]. Fewer investigations have been carried out on the ecological role of such compounds, particularly their implication in antagonism toward other soil fungi [7–11]. Actually, in the soil biocenosis, the ability to release toxic metabolites by many fungal species is related to competitive relationships [12]. With reference to *R. solani*, so far antagonistic activity has been observed only for a few *Penicillium* species [7, 9, 13–15]; in some cases it has been reported in relation to the production of toxic metabolites [7, 9, 15].

A number of *Penicillium* strains were recovered from colonies which occasionally developed on mycelial mat of *Rhizoctonia* isolates obtained from diseased tobacco plants. Since many known *Rhizoctonia* mycoparasites can be recovered under such circumstances [3, 16], this finding might also imply an antagonistic association. We therefore evaluated the occurrence of mycoparasitic relationships in dual cultures, and tested the biological activity of culture filtrates and some purified secondary metabolites produced by the *Penicillium* isolates on *R. solani in vitro*.

Materials and methods

Penicillium *isolates*

Isolates of *Penicillium* spp. were collected from colonies that occasionally developed in the isolation plates used to recover *Rhizoctonia* from diseased tobacco plants [17, 18]. A number of isolates also included in this study were obtained from the rhizosphere of tobacco plants with sore shin symptoms by pouring 2 mL of a soil suspension (10 g sieved soil in 100 mL sterile distilled water) directly on a *R. solani* mycelial mat developed on potato-dextrose agar (PDA) amended with 200 mg L^{-1} streptomycin sulphate in 90-mm diameter Petri dishes. The use of pre-colonized plates is believed to allow a selective isolation of fungi that are presumably antagonistic toward *R. solani* [3, 16]. *Penicillium* isolates were subcultured on several substrates, such as malt-extract agar (MEA), yeast-extract-sucrose agar (YES), Czapek-Dox agar (CDA) and, if appropriate, creatinine agar (CreA) and nitrite-sucrose agar (NSA), for taxonomic identification [4–6, 19–22]; the isolates were then stored on MEA at 4 °C.

Evaluation of antagonism

Antagonism by the *Penicillium* isolates was studied *in vitro* by evaluating growth inhibition of isolate RT27 of *R. solani* anastomosis group 2-1 (AG 2-1), that was recovered from tobacco and proved to be highly pathogenic to this plant species in a previous study [18], and by observing possible hyphal interactions. Inhibition of growth was evaluated in dual cultures which were prepared by simultaneously inoculating each *Penicillium* isolate opposite isolate RT27 in 90-mm Petri dishes containing CDA; to avoid proliferation of subcultures, the *Penicillium* isolates were inoculated from conidial suspensions prepared in semisolid water agar (0.2%), amended with 0.05% Tween-80 [5]. Cultures were kept in the dark for 1 week at 25 °C. Hyphal interactions and cytological alterations following inoculation of *Penicillium* isolates were observed in 3-day old *R. solani* cultures on 2% water agar (WA) in 90-mm Petri dishes, which were inoculated with 2 mL of a conidial suspension (1×10^6 conidia mL^{-1} distilled water) prepared from actively growing cultures on CDA of each *Penicillium* isolate. Plates were kept in the dark at 25 °C; after 3 days, 3 rectangular blocks (about 50 mm × 20 mm) from each plate were cut, mounted on glass slides, and stained with aniline blue (0.01% in lactophenol). Observation was carried out at 400× magnification.

Biological assay of culture filtrates

Liquid cultures of the *Penicillium* isolates were prepared in 500 mL-Erlenmeyer flasks containing 200 mL Czapek-Dox broth (CDB), and kept in darkness at 25 °C. After 2 weeks, cultures were filtered through 0.45 μm cellulose nitrate filters, and culture filtrates were vacuum-concentrated at 50 °C in a rotary evaporator until reduction to 1/10 of the starting volume. The residue was then sterilized by a further filtration at 0.2 μm and stored at −20 °C. Biological assays were carried out by mixing 1 mL sterile concentrated culture filtrate with 4 mL molten WA in 55-mm diameter Petri dishes. In control plates WA was added with 1 mL sterile distilled water. The plates were then inoculated at the centre with a mycelial plug from a PDA culture of isolate RT27 of *R. solani* and kept in darkness at 25 °C for 15 days. Diametric growth (mm) was measured after 5, 10 and 15 days. All assays of culture filtrates were repeated twice.

Identification and biological assay of toxic metabolites

Penicillium isolates whose concentrated culture filtrates completely inhibited growth of *R. solani* were cultured again in CDB in 500 mL-Erlenmeyer flasks, with a total of 2 L of broth prepared for each isolate. Culture filtrates were prepared

and concentrated as described above. 3-*O*-Methylfunicone was extracted and purified from *P. pinophilum* as reported in a previous paper [15]. For isolates of *P. brevicompactum* and isolate PT27 of *P. expansum*, a partially modified procedure was followed: concentrated culture filtrates were extracted 3 times with diethylether; the extract was vacuum-dried at 40 °C and the residue, recovered with chloroform, fractionated by means of silicagel thin-layer chromatography (TLC) on Kieselgel 0.5 mm thickness (Merck) developed with a mixture of chloroform–methanol (94:6 v/v). Compounds detectable at 254 nm as single bands were purified and identified by nuclear magnetic resonance (^{13}C NMR and ^{1}H NMR), and mass, ultraviolet and infrared spectrometry, as reported elsewhere [15]. Biological activity of purified metabolites dissolved in ethanol (10 mg mL^{-1}) was assayed by adding 0.1 mL of the ethanolic solution in 7.9 mL WA (final concentration: 125 μg mL^{-1}) in 55-mm diameter Petri dishes, which were inoculated at the centre with a mycelial plug of isolate RT27 of *R. solani* grown on PDA and kept in the dark at 25 °C for 15 days. Control received 0.1 mL ethanol only. The presence of toxic metabolites produced by isolates of *P. brevicompactum, P. expansum* and *P. pinophilum* was analyzed in cultures prepared directly on *R. solani* mycelium. To this purpose, liquid cultures of isolate RT27 of *R. solani* AG-2-1 were prepared in 150 mL CDB in 500 mL-Erlenmeyer flasks. After 15 days of growth in darkness at 25 °C, the mycelial cake was transferred to 90-mm Petri dishes and inoculated with 2 mL of a conidial suspension of the *Penicillium* isolates (about 1×10^5 conidia mL^{-1}, one dish per each isolate). Cultures were incubated in darkness at 25 °C. After 1 week, the mycelial mats with the overgrowing *Penicillium* colonies were extracted with 20 mL chloroform for 5 min. The chloroform extract was then vacuum concentrated at 40 °C, and the presence of mycophenolic acid, patulin and 3-*O*-methylfunicone was evaluated by TLC performed as reported above. In addition, production of mycotoxins was evaluated in dual cultures of each *Penicillium* isolate with isolate RT27 prepared in soil containing about 3.5% organic matter. A soil sample (90 g) was placed in 500 mL-Erlenmeyer flasks and sterilized at 130 °C for 1 h. The soil was brought to the field capacity with sterile distilled water, then inoculated with 10 mycelial plugs (6 mm-diameter) taken from actively growing cultures on CDA of isolate RT27, and kept in darkness at 25 °C. After 3 days, 10 mL of a conidial suspension (1×10^6 conidia mL^{-1}) of *Penicillium* isolates belonging to *P. brevicompactum*, *P. expansum* and *P. pinophilum* were added to the cultures (one flask per isolate). A control flask previously inoculated with *R. solani* received 10 mL distilled water only. Cultures were kept in the same conditions for additional 18 days, then extracted with 100 mL chloroform for 15 min, and the presence of the above-mentioned toxins in the chloroform extract was evaluated by TLC.

Observations on the cytological damage caused by some Penicillium *isolates*

Transmission electron microscopy (TEM) observations were performed for isolates belonging to *P. brevicompactum*, *P. expansum*, *P. pinophilum*, and *P. verrucosum*, for a more thorough evaluation of the cytological damage caused to *R. solani*. Dual cultures of isolate RT27 of *R. solani* and the *Penicillium* isolates were prepared in CDB in 200 mL-Erlenmeyer flasks and kept in the dark at 25 °C; a single culture of isolate RT27 was prepared in the same conditions to be used as control. After 5 days mycelium of *R. solani* from each dual culture was harvested, fixed with glutaraldehyde (2.5%) and dehydrated. Dehydrated mycelium was then included in Epon (812) resin, thin-sectioned with a Zeiss E 400 ultramicrotome, and mounted on nickel grids (100 mesh) for TEM (Philips 400) observations.

Results

A total sample of 29 *Penicillium* strains was collected (Table 1), among which 15 were recovered from *Rhizoctonia* cultures and 14 from soil. They were found to belong to the following species: *P. brevicompactum* Dierckx (3 isolates), *P. chrysogenum* Thom (7 isolates), *P. citreonigrum* Dierckx (1 isolate), *P. crustosum* Thom (9 isolates), *P. expansum* Link (2 isolates), *P. pinophilum* Hedgcock (3 isolates), *P. rugulosum* Thom (2 isolates), and *P. verrucosum* Dierckx (2 isolates). *P. citreonigrum*, *P. pinophilum*, *P. rugulosum*, and *P. verrucosum* were isolated from soil only, while all

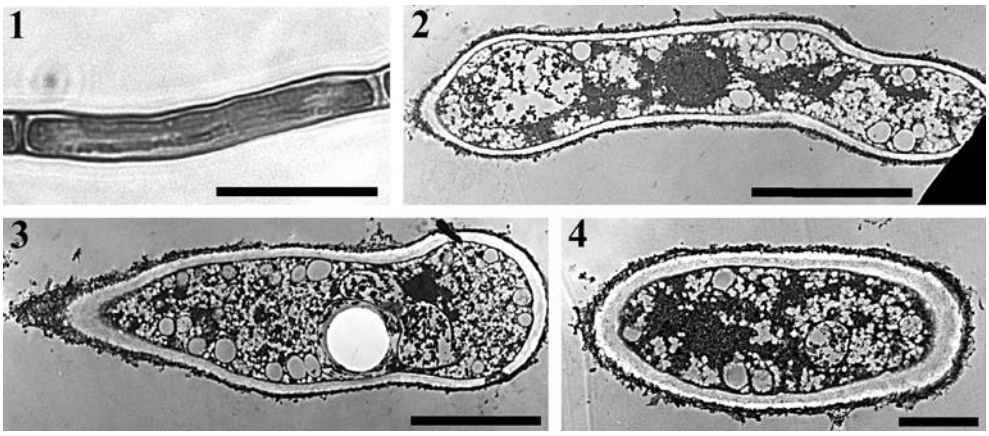
Table 1. Observations on *in vitro* antagonism of *Penicillium* isolates toward *R. solani* AG 2–1

Isolate		Dual cultures on CDA			Dual cultures on WA		
		Inhibition of growth	Formation of sclerotia	Overgrowth of mycelial mat	Formation of monilioid cells	Vacuolisation	Plasmolysis
P. brevicompactum	GV1	+	+	+	–	–	+
P. brevicompactum	PR5	+	+	+	–	–	+
P. brevicompactum	PR8	+	+	+	–	–	+
P. chrysogenum	PP7	+	–	+	–	–	–
P. chrysogenum	PP8–2	+	+	+	–	+	–
P. chrysogenum	PT20	+	+	+	+	–	–
P. chrysogenum	P714A	+	+	–	–	–	–
P. chrysogenum	PR4	+	+	–	–	–	–
P. chrysogenum	GV2	+	+	+	+	–	–
P. chrysogenum	PR7	+	+	–	+	–	–
P. citreonigrum	P714B	–	–	+	–	–	–
P. crustosum	PL10	+	+	+	+	–	–
P. crustosum	PP8–1	+	+	+	+	–	–
P. crustosum	PR1	+	+	+	+	–	–
P. crustosum	PR2	+	+	+	+	–	–
P. crustosum	PT1	+	+	+	+	–	–
P. crustosum	PT16	+	+	+	+	–	–
P. crustosum	TL9A	+	+	+	+	–	–
P. crustosum	TL9B	+	+	+	+	–	–
P. crustosum	TL9C	+	+	+	+	–	–
P. expansum	PR3	+	–	+	–	+	+
P. expansum	PT27	+	–	+	–	+	+
P. pinophilum	LT4	+	–	+	–	–	+
P. pinophilum	LT6	+	–	+	–	–	+
P. pinophilum	ST2	+	–	+	–	–	+
P. rugulosum	PL8	+	–	–	–	+	–
P. rugulosum	P822B	+	–	–	–	+	–
P. verrucosum	P822A	+	–	–	+	+	+
P. verrucosum	P12	+	–	–	+	+	+

P. brevicompactum isolates were recovered from colonies developing on *Rhizoctonia* mycelium; isolates of the other species were obtained from both sources. All the strains are stored in the mycological collection of the Tobacco Experiment Institute, Scafati. Isolate LT4 of *P. pinophilum*, patented by the Tobacco Experiment Institute, is also deposited under the Budapest Treaty in the mycological collection of the Department of Plant Biology, University of Perugia, Italy.

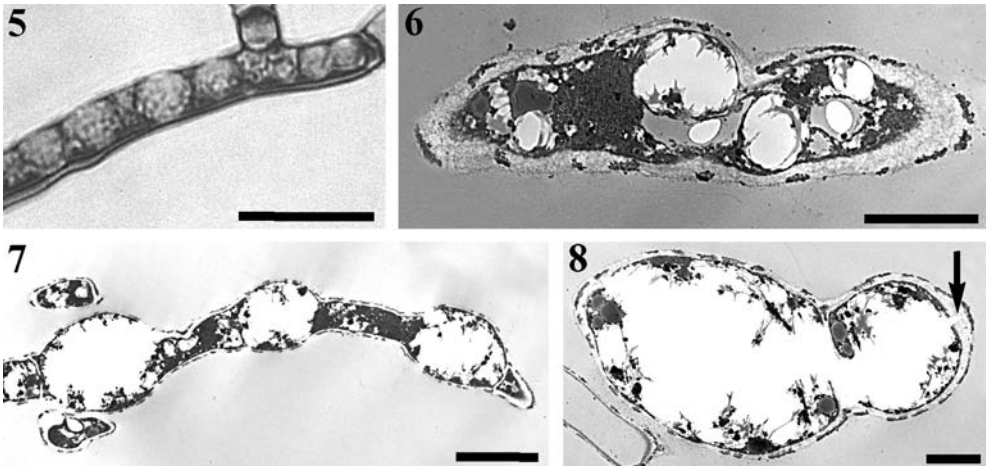
According to reports in the literature, all species except *P. verrucosum* [9] and *P. pinophilum* [13, 15] are new records as possible antagonists of *R. solani*. The isolate of *P. citreonigrum* was the only one that failed to cause inhibition to any extent in dual cultures on CDA, while, as a general feature, mycelial growth of the *R. solani* isolate appeared to be delayed as its hyphae approached the slower growing *Penicillium* colonies. In a few cases such early evidence of inhibition was overcome as growth of both the opposed isolates carried on until merging, while in most pairings the *R. solani* colony stopped developing and its mycelial mat was overgrown by the *Penicillium* isolate. Such a lasting inhibition was particularly evident for all *P. brevicompactum*, *P. pinophilum*, and *P. verrucosum* isolates, and for isolate PT27 of *P. expansum*. Within the 1-week period of observation, all the latter species but *P. brevicompactum* did not allow formation of sclerotia, which usually appear in pure *R. solani* cultures on CDA after mycelial growth has covered the available surface (3 days on average).

Dual cultures on WA allowed a more thorough appraisal of the antagonistic relationships between the paired isolates since hyphae of *R. solani* do not form a compact mat and it is possible to perform a direct observation of hyphal interactions. Pene-

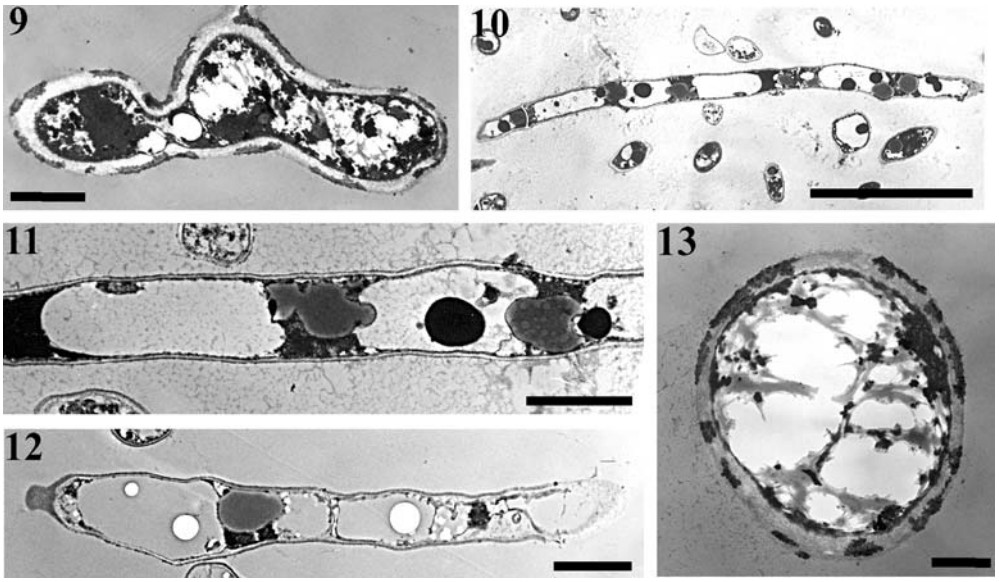


Figures 1–4. Plasmolysis of *R. solani* cells from dual cultures with isolate GV1 of *P. brevicompactum*. (1) Plasmolysed cell under light microscopy. Bar, 10 μm. (2–3) Longitudinal sections under TEM. Bars, 5 μm. (4) Transverse section under TEM. Bar, 2 μm.

tration or coiling of hyphae can be observed under these conditions with other known *R. solani* mycoparasites (e.g. *Verticillium biguttatum* and *Trichoderma* spp.), but they did not occur in all pairings with *Penicillium* isolates. However, different types of reaction by the *R. solani* isolate were observed, consisting in either unaffected growth, or formation of monilioid cells, or apparent cell plasmolysis and vacuolisation with an ensuing failure of hyphal growth (Table 1). On WA monilioid cells, which prelude formation of sclerotia, usually appear no earlier than 2 weeks after inoculation. The fact that they could be observed after just 3 days in plates co-inoculated with some *Penicillium* isolates is to be considered as a response to the presence of the latter. Formation of monilioid cells generally occurred in the same pairings that also incited an earlier formation of sclerotia on CDA, whereas plasmolysis and/or vacuolisation of the *R. solani* cells regularly occurred in pairings with isolates of *P. brevicompactum* (Figures 1–4), *P. pinophilum*, *P. verrucosum* (Figures 5–8), and *P. expansum* (Figures 9–13). To a lesser extent, vacuolisation was also observed when *R. solani* cultures were inoculated with isolates of *P. rugulosum* and isolate PP8-2 of



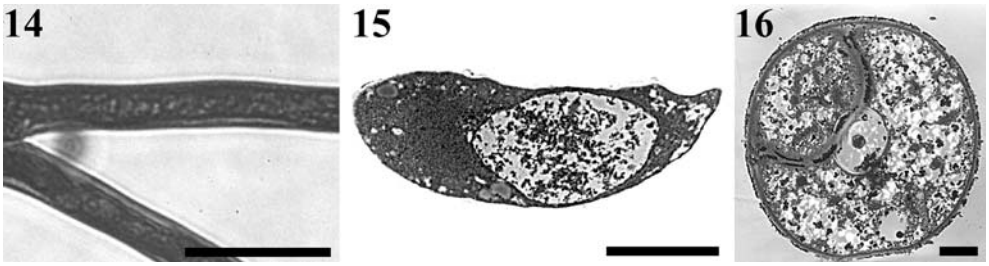
Figures 5–8. Plasmolysis and vacuolisation of *R. solani* cells from a dual culture with *P. verrucosum* isolate P12. (5) Vacuolised cell under light microscopy. Bar, 10 μm. (6) Early stage of the vacuolisation process with small vacuoles spreading in the cell protoplasm. Bar, 2 μm. (7) Coalescence of vacuoles. Bar, 5 μm. (8) Advanced stage of the vacuolisation process with ongoing ultrastructural damage (arrow). Bar, 2 μm.



Figures 9–13. Plasmolysis and vacuolisation in *R. solani* cells from a dual culture with *P. expansum* isolate PT27 under TEM. (9) Formation of small vacuoles at an earlier stage. Bar, 2 μm. (10) Coalescence of vacuoles. Bar, 10 μm. (11) Detail of the previous image. Bar, 2 μm. (12–13) Disruption of the ultrastructural integrity of the cells under longitudinal and transverse view. Bars, 1 μm.

P. chrysogenum. In the case of *P. pinophilum* and *P. brevicompactum* TEM observations confirmed the occurrence of an uniformly distributed process of plasmolysis, which was evident in both longitudinal (Figures 2–3) and transverse sections (Figure 4). Other than plasmolysis, *P. verrucosum* and *P. expansum* caused a clearly visible and ongoing process of vacuolisation (Figures 6–8 and Figures 9–13, respectively). Within the same colony, the course progressed with the coalescence of small vacuoles into bigger ones (Figures 6–7 and 9–11). At a later stage, vacuoles almost completely occupied the available space within the cell wall (Figures 8 and 12–13). As compared to the control (Figures 14–16), the ultrastructure of the cell appeared to be compromised, with swellings on the outline (Figure 8), and disruption of the membranes becoming evident (Figures 8 (arrow) and 12).

Results of evaluation of growth in WA plates added with concentrated culture filtrates are reported in Table 2. Complete inhibition of growth of *R. solani* during 15 days was observed for *P. brevicompactum* and *P. pinophilum* isolates, and *P. expansum* isolate PT27, while in most other cases the *R. solani* isolate reached the border of the plate within 5–10 days. A partial inhibition after 10 days was detected for isolates PR3 (*P. expansum*) and PP8-1 (*P. crustosum*). Although *P. verrucosum* isolates inhibited *R. solani* in dual cultures



Figures 14–16. Mycelium of *R. solani* from a control culture. (14) Light microscopy image of the *R. solani* mycelium. Bar, 10 μm. (15) Ultrastructure of a *R. solani* cell from a single culture under longitudinal view. Bar, 2 μm. (16) Ultrastructure of the same subject under transverse view. Bar, 1 μm.

Table 2. Diametric growth (mm) of *R. solani* RT27 on WA added with concentrated culture filtrate of *Penicillium* isolates (mean of 3 repeated observations)

Isolate		5 days	10 days	15 days
P. brevicompactum	GV1	0	0	0
P. brevicompactum	PR5	0	0	0
P. brevicompactum	PR8	0	0	0
P. chrysogenum	PP7	45	50	
P. chrysogenum	PP8-2	45	50	
P. chrysogenum	PT20	20	50	
P. chrysogenum	P714A	35	50	
P. chrysogenum	PR4	30	50	
P. chrysogenum	GV2	50		
P. chrysogenum	PR7	12	50	
P. citreonigrum	P714B	50		
P. crustosum	PL10	50		
P. crustosum	PP8-1	14	25	50
P. crustosum	PR1	50		
P. crustosum	PR2	50		
P. crustosum	PT1	50		
P. crustosum	PT16	50		
P. crustosum	TL9A	50		
P. crustosum	TL9B	50		
P. crustosum	TL9C	50		
P. expansum	PR3	19	45	50
P. expansum	PT27	0	0	0
P. pinophilum	LT4	0	0	0
P. pinophilum	LT6	0	0	0
P. pinophilum	ST2	0	0	0
P. rugulosum	PL8	30	50	
P. rugulosum	P822B	30	50	
P. verrucosum	P822A	50		
P. verrucosum	P12	45	50	
Control		50		

on CDA, their culture filtrate failed to produce similar results.

Extraction of secondary metabolites was carried out from culture filtrates of the isolates which had completely inhibited *R. solani*. Three known compounds were detected: 3-*O*-methylfunicone from *P. pinophilum* isolates, mycophenolic acid from *P. brevicompactum* isolates, and patulin from isolate PT27 of *P. expansum*. Their identification was carried out with reference to spectral data reported in the literature [15, 23, 24]. Mycophenolic acid was detected in TLC as a band at R_f about 0.3; crystallization from methanol solution yielded the compound in the amount of about 60 mg L^{-1} liquid culture from all isolates of *P. brevicompactum*. Patulin formed a band at R_f 0.5, and was extracted in the amount of 9 mg L^{-1} liquid culture. Finally 3-*O*-methylfunicone, detectable as a band at R_f 0.7, was extracted in the amount of 15, 11 and 8 mg L^{-1} respectively from culture filtrates of isolates LT4, ST2 and LT6 of *P. pinophilum*. In the biological assays carried out on WA, all the described metabolites were able to completely inhibit growth of *R. solani* for 15 days, thereby proving to be the toxic principles in the culture filtrates, or to provide an essential contribution to their biological activity. In addition, they all were detected by means of TLC when all isolates of *P. brevicompactum*, *P. pinophilum* and *P. expansum* were cultured directly on *R. solani* mycelium, as well as in dual cultures prepared in sterilized soil.

Discussion

In relation to the circumstances of isolation, the *Penicillium* strains obtained can be considered either mycoparasitic or fungicolous [25]. While the term fungicolous is used to describe a regular association of fungal species, mycoparasitism refers to the ability of a fungus to obtain nutrients from mycelium of another species, a condition that is difficult to demonstrate *in vitro* unless hyphal interactions between the two entities are observed. The definition of necrotrophic mycoparasitism *sensu* Barnett and Binder [26] is more appropriate to describe a relationship mediated by toxic metabolites. However, in the present paper the more general term of antagonism is preferred since the role of such compounds was not obvious for all the species considered.

On account of the results of biological assays *P. citreonigrum* is the only species which may be considered fungicolous rather than antagonistic, while in all other cases growth of *R. solani* was either inhibited or affected with the incitement of resting structures. Unlike what is reported for other mycoparasites of *R. solani*, such as *V. biguttatum* that is able to prevent sclerotial formation [27], isolates of *P. chrysogenum* and *P. crustosum* enhanced the formation of sclerotia. This finding, which is also indicative of the absence of permanent damage caused by the secondary metabolites released in the agar medium, may induce to disregard the incitement of resting structures as relevant for the evaluation of the antagonistic potential. Yet, the ability to colonize *R. solani* mycelial mat and sclerotia shown by the above-mentioned *Penicillium* strains suggests that

this mechanism plays an ecological role, since it may affect saprophytical proliferation of the pathogen in the soil environment. On the other hand, cytological damage and hyphal lysis also prevent colonization of organic substrates by *R. solani*, and influence its survival and pathogenic potential more directly [28]. In this regard, isolates of *P. brevicompactum*, *P. expansum* and *P. pinophilum*, which were definitely inhibitory in dual cultures and caused disruption of hyphal structure, displayed the most relevant antagonistic behaviour. Moreover their culture filtrates and purified metabolites also induced complete inhibition of *R. solani*.

The ability to grow in association with *R. solani* supports the hypothesis that toxic metabolites hold a significant role in the antagonistic relationship. Actually all the available isolates of these species were able to produce them both when growing on the *R. solani* mycelium and in soil in the presence of the pathogen. The latter assay also demonstrated that their production is not dependent on the nutrients contained in the synthetic growth medium. Though it is believed that fungitoxic metabolites may accumulate in soil in high local concentrations and exert their biological activity even over a small distance [29], our findings represent an evidence that mycophenolic acid, patulin and 3-*O*-methylfunicone are involved in antagonism at least when the producing strains should get in contact with *R. solani* hyphae in the soil environment. Antifungal properties of the above-mentioned compounds have been already documented. 3-*O*-Methylfunicone is a recently characterized metabolite [15], which has also revealed potent cytostatic properties [30]. Mycophenolic acid, one of the oldest known fungitoxic compounds [31], was first characterized from *P. brevicompactum*, but afterwards found in other *Penicillium* species, such as *P. roqueforti* [32], *P. echinulatum* [7], and *P. carneum* [6]. Likewise, production of patulin is widespread in the genus *Penicillium*; other than *P. expansum*, it is known in *P. carneum, P. coprobium, P. glandicola, P. griseofulvum, P. panuem, P. roqueforti, P. sclerotigenum* and *P. vulpinum* [6, 33].

Data concerning isolates of *P. verrucosum* appeared to be conflicting, since the inhibitory capacity and the cytological damage caused in dual cultures were not confirmed by observations concerning culture filtrates. However, both inhibition of growth and symptoms of cytotoxicity were so evident that it is difficult to exclude the production of fungitoxic metabolites by isolates of this species. Actually *P. verrucosum* is reported to produce a number of potent mycotoxins, such as ochratoxin A, verrucolon, citrinin, and the verrucins [33, 34], and inhibition of growth of *R. solani in vitro* by a metabolite related to citrinin extracted from culture filtrates of an isolate of this species has been reported [9]. Possible explanations may be advanced in this case, such as unstableness of the molecular structure of the toxic metabolite(s) involved which might be overcome by a continuous production in dual cultures, or the necessity of the presence of *R. solani* for their induction.

A correlation between the results of dual cultures and the inhibitory capacity of culture filtrates was observed in the case of *P. expansum*. Isolate PR3 caused a partial inhibition of *R. solani*, and patulin was found in its culture filtrate in remarkably lower concentrations than in that of isolate PT27. It is known that not all the isolates of *P. expansum* produce this toxin [33], and in fact none was detected in the culture filtrate of a further isolate recovered from a rotten apple, which also did not inhibit *R. solani in vitro*. Although based on a few strains, these preliminary observations suggest that antagonism of *P. expansum* isolates toward *R. solani* is related to their ability to produce patulin. In this regard, the importance of toxic metabolites has been already demonstrated for other mycoparasites, such as *Trichoderma virens* whose antagonistic behaviour against *Pythium ultimum* is dependent on the extent of gliovirin production [35].

Variable production of fungitoxic metabolites may also occur in other species. Particularly this could be the case for *P. crustosum*, the commonest species isolated, where some extent of inhibitory capacity was found in the case of isolate PP8-1. Further studies on differences in the production of secondary metabolites among the isolates of this species may provide additional data to evaluate relationships between toxic metabolite production and antagonism toward *R. solani*.

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References

1. Shew HD, Melton TA. Target spot of tobacco. Plant Dis. 1995; 79: 6–11.
2. Lewis JA, Kulik MM. Introduced biocontrol agents to suppress diseases caused by *Rhizoctonia*. In: Sneh B, Jabaji-Hare S, Neate S, Dijst G, eds. *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Dordrecht, the Netherlands: Kluwer Academic Publishers, 1996: 507–514.
3. Van den Boogert PHJF. Mycoparasitism and biocontrol. In: Sneh B, Jabaji-Hare S, Neate S, Dijst G, eds. *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Dordrecht, the Netherlands: Kluwer Academic Publishers, 1996: 485–493.
4. Pitt JI. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. New York, USA: Academic Press, 1979.
5. Pitt JI. A laboratory guide to common *Penicillium* species. 3rd edn. North Ryde, Australia: CSIRO Division of Food Processing, 2000.
6. Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O. Introduction to food- and airborne fungi. 6th edn. Utrecht, the Netherlands: Centraalbureau voor Schimmelcultures, 2000.
7. Anderson HA, Bracewell JM, Fraser AR, Jones D, Robertson GW, Russell JD. 5-Hydroxymaltol and mycophenolic acid, secondary metabolites from *Penicillium echinulatum*. Trans. Brit. Mycol. Soc. 1988; 91: 649–651.
8. Harrison YA, Stewart A. Selection of fungal antagonists for biological control of onion white rot in New Zealand. New Zeal. J. Exp. Agr. 1988; 16: 249–256.
9. Kharbanda PD, Dahiya JS. A metabolite of *Penicillium verrucosum* inhibitory to growth of *Leptosphaeria maculans* and *Rhizoctonia solani*. Can. J. Plant Pathol. 1990; 12: 335.
10. Melouk HA, Akem CN. Inhibition of growth of *Sclerotinia minor* and other pathogens by citrinin in the filtrate of *Penicillium citrinum*. Mycopathologia 1987; 100: 91–96.
11. Proksa B, Adamcova J, Fuska J. 2-Methylsorbic acid, an antifungal metabolite of *P. vermiculatum*. Appl. Microbiol. and Biotechnol. 1992; 37: 442–445.
12. Vey A, Hoagland RE, Butt TM. Toxic metabolites of fungal biocontrol agents. In: Butt TM, Jackson C, Magan N, eds. Fungi as Biocontrol Agents. Wallingford, England: CAB International, 2001: 311–346.
13. Alagesaboopathi C. Biological control of damping-off disease of cotton seedling. Curr. Sci. 1994; 66: 865–868.
14. Boosalis MG. Effect of soil temperature and green manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* sp. Phytopathology 1956; 46: 473–478.
15. De Stefano S, Nicoletti R, Milone A, Zambardino S. 3-*O*-Methylfunicone, a fungitoxic metabolite produced by the fungus *Penicillium pinophilum*. Phytochemistry 1999; 52: 1399–1401.
16. Mulligan DFC, Deacon JW. Detection of presumptive mycoparasites in soil placed on host-colonized agar plates. Mycol. Res. 1992; 96: 605–608.
17. Nicoletti R, Blancard D, Cailleteau B. Caractérisation et étude du puvoir pathogène de 5 isolats français de *Rhizoctonia* inféodés au tabac. Annales du Tabac, Section 2, 1998; 30: 24–29.
18. Nicoletti R, Lahoz E, Kanematsu S, Naito S, Contillo R. Characterization of *Rhizoctonia solani* isolates from tobacco fields related to anastomosis groups 2-1 and BI (AG 2-1 and AG BI). J. Phytopathol. 1999; 197: 71–77.
19. Domsch KH, Gams W, Anderson TH. Compendium of soil fungi. Vol. I. New York, USA: Academic Press, 1980.
20. Frisvad JC. Physiological criteria and mycotoxin production as aids in identification of common asymmetric *Penicillia*. Appl. Environ. Microbiol. 1981; 41: 568–579.
21. Gams W. Supplement and corrigendum to the compendium of soil fungi. Eching, Germany: IHW-Verlag, 1993.
22. Stolk AC, Samson RA, Frisvad JC, Filtenborg O. The systematics of the terverticillate *Penicillia*. In: Samson RA, Pitt JI, eds. Modern Concepts in *Penicillium* and *Aspergillus* Classification. New York, USA: Plenum Press, 1990: 121–137.
23. Scott PM. Patulin. In: Purchase LFH, ed. Mycotoxins. Amsterdam, the Netherlands: Elsevier Scientific Publishing Company, 1974: 383–403.
24. Danheiser RL, Gee SK, Perez JL. Total synthesis of mycophenolic acid. J. Amer. Chem. Soc. 1986; 108: 806–810.
25. Jeffries P, Young TWK. Interfungal parasitic relationships. Wallingford, England: CAB International, 1994.
26. Barnett HL, Binder FL. The fungal host-parasite relationship. Ann. Rev. of Phytopathol. 1973; 11: 273–292.
27. Van den Boogert PHJF, Deacon JW. Biotrophic mycoparasitism by *Verticillium biguttatum* on *Rhizoctonia solani*. Eur. J. Plant Pathol. 1994; 100: 137–156.
28. Papavizas GC, Lumsden RD. Biological control of soilborne fungal propagules. Ann. Rev. Phytopathol. 1980; 18: 389–413.
29. Sivasithamparam K, Ghisalberti EL. Secondary metabolism in *Trichoderma* and *Gliocladium*. In: Kubicek CP, Harman G, eds. *Trichoderma* and *Gliocladium*. Basic biology, taxonomy and genetics. London, England: Taylor & Francis, 1998: 139–191.
30. Stammati A, Nicoletti R, De Stefano S, Zampaglioni F, Zucco F. Cytostatic properties of a novel compound derived from *Penicillium pinophilum*: An *in vitro* study. Altern. Lab. Anim. 2002; 30: 1–7.
31. Andersen B. Consistent production of phenolic compounds by *Penicillium brevicompactum* for chemotaxonomic characterization. Antonie van Leeuwenhoek 1991; 60: 115–123.
32. Lafont P, Debeaupuis JP, Gaillardin M, Payen J. Production of mycophenolic acid by *Penicillium roqueforti* strains. Appl. Environ. Microbiol. 1979; 37: 365–368.
33. Frisvad JC, Filtenborg O. Terverticillate *Penicillia*: Chemotaxonomy and mycotoxin production. Mycologia 1989; 81: 837–861.
34. Larsen TO, Franzyk H, Jensen SR. UV-guided isolation of verrucins A and B, novel quinazolines from *Penicillium*

verrucosum structurally related to anacine from *Penicillium aurantiogriseum*. J. Nat. Prod. 1999; 62: 1578–1580.
35. Howell CR, Stipanovic RD. Gliovirin, a new antibiotic from *Gliocladium virens*, and its role in the biological control of *Pythium ultimum*. Can. J. Microbiol. 1983; 29: 321–324.

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