In vitro Evaluation of Fungal Antagonists of *Phytophthora nicotianae*

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Abstract

As tobacco black shank epidemics caused by *Phytophthora nicotianae* occurred in central Italy in the late 1990s, fungal antagonists of the pathogen were searched in the rhizosphere of tobacco plants. Isolates of *Aspergillus sydowii*, *Fusarium chlamydosporum, Gliocladium roseum, Penicillium brevicompactum*, *P. chrysogenum, Scopulariopsis candida* and *Trichoderma harzianum* were recovered. Antagonism of these isolates toward *P. nicotianae* was evaluated *in vitro*: even if no hyphal interactions were observed in dual cultures, aberration in mycelial growth and morphology of sporangia occurred in most cases. Unlike those of *T. harzianum*, concentrated culture filtrates of *A. sydowii, F. chlamydosporum, G. roseum, P. brevicompactum, P. chrysogenum*, inhibited growth of all *P. nicotianae* isolates tested, while culture filtrates of *S. candida* caused aberrant mycelial growth.

Keywords: *Phytophthora nicotianae*; tobacco; fungal antagonists; *in vitro* assays

INTRODUCTION

Unlike other countries, economic damage due to black shank has never been recorded on tobacco in Italy, even though the disease is known since a long time and the causal agent, *Phytophthora nicotianae* Breda de Haan, is widespread on a number of crops. Epidemics occurred in the dark fire-cured tobacco growing-area of Pontecorvo, central Italy, in the late 1990s have been found to be related to the widespread use of the susceptible cultivar Ky171, and high water supplies (NICOLETTI *et al.* 2001). The use of tolerant genotypes and a more convenient crop management proved to be able to bring disease incidence under an economic threshold; however a natural retrogression of pathogen attacks was observed even in farms where no substantial adjustments in the crop management had been made. On that account we tried to evaluate if the acquired suppressiveness in those soils might be referred to a possible role of fungal antagonists of *P. nicotianae* as biological control agents.

Unlike other soil-borne fungal pathogens, little is known about antagonists of *P. nicotianae*. In a review on the subject, MALAJCZUK (1983) listed 22 species of fungal antagonists against species in the genus *Phytophthora*, but none with reference to *P. nicotianae*. However, since then there have been several reports on a number of crop plants (Table 1).

MATERIAL AND METHODS

Fungal antagonists were recovered either directly from colonies developing onto *P. nicotianae* isolates from diseased tobacco plants or from soil samples from tobacco fields in the Pontecorvo area. Isolation from soil was carried out by plating 1 ml of a soil suspension (5 g sieved soil in 50 ml distilled water amended with 12 mg streptomycine sulphate, stirring for 1 hour) onto colonies of *P. nicotianae* developing on corn meal agar (CMA, Oxoid).

Antagonism was evaluated *in vitro* in dual cultures on CMA in 60-mm diameter Petri dishes: mycelial plugs of *P. nicotianae* were placed opposite a mycelial plug of the fungal antagonist; for *Penicillium* and *Aspergillus* isolates inoculation was operated with a needle previously dipped in a spore suspension prepared in 0.2% water agar amended with 0.05% Tween 80, in order to avoid culture proliferation. Plates were kept in the dark at 25°C; after 1 week inhibition was evaluated in comparison with a control culture inoculated with *P. nicotianae* only. The assay was repeated twice using different *P. nicotianae* isolates.

Liquid cultures of the fungal antagonists were prepared on Czapek-Dox broth (150 ml in 500-ml Erlenmayer flasks) and kept in the dark at 25°C. After 2 weeks cultures were filtered and concentrated at 50°C in a rotary evaporator until reduction to 1/10 of the starting volume. After sterilization through 0.45 μm polisulfone filters (Acrodisc, Gelman), 1 ml concentrated culture filtrate (CCF) was added to 4 ml melted CMA in 60-mm diameter Petri dishes (20% v/v); mycelial plugs of *P. nicotianae* isolates grown on CMA were inoculated at the center of the plates, which were kept in the dark at 25°C for 1 week. This assay was also repeated twice using different *P. nicotianae* isolates.

Observation concerning hyphal growth and interactions, and sporangial morphology was carried out at 200× and 400× magnifications.

RESULTS AND DISCUSSION

In the Pontecorvo area isolates of the following fungal species were recovered, which may be presumptively considered antagonistic toward the pathogen on account of isolation conditions: *Aspergillus sydowii* (Bain. & Sart.) Thom & Church (isolate APh-1), *Fusarium chlamydosporum* Wollenw. & Reinking (isolate FP1R), *Gliocladium roseum* Bainier (isolate GRF), *Penicillium brevicompactum* Dierckx (isolate PPh-2), *Penicillium chrysogenum* Thom (isolates PPh-1 and PPh-3), *Scopulariopsis candida* (Guéguen) Vuill. (isolate SP1), and *Trichoderma harzianum* Rifai (isolates TP1 and TP2). Isolates of *F. chlamydosporum* and *G. roseum* were recovered from soil samples, while the other ones were recovered directly from *P. nicotianae* colonies. Although already known as fungal antagonists, all the above-mentioned species except *T. harzianum* are new records with reference to *P. nicotianae*.

Antagonism in dual cultures was not evaluated in quantitative terms since the extent of growth of the isolates tested varied substantially; in fact isolates of *F. chlamydosporum*, *G. roseum* and *T. harzianum* grew quickly and were able to overgrow *P. nicotianae* in a few days, while isolates of *A. sydowii*, both *Penicil-*

Table 1. Known fungal antagonists of *Phytophthora nicotianae*

Species	Evidence on:	Reference
Aspergillus fumigatus	Carnation	MIGHELI *et al.* (1993)
Chaetomium globosum	*In vitro* assay	HELLER & THEILER-HEDTRICH (1994)
Gliocladium virens	*In vitro* assays	HELLER & THEILER-HEDTRICH (1994), GHOSHI (2000)
Glomus fasciculatus	Tobacco	SREERAMULU *et al.* (1998)
Glomus mossae	Tomato	VIGO *et al.* (2000)
Penicillium canescens	Carnation	MIGHELI *et al.* (1993)
Penicillium puberulum	Carnation	MIGHELI *et al.* (1993)
Pythium nunn	Azalea, sweet orange	FANG *et al.* (1995)
Pythium oligandrum	*In vitro* assay	PICARD *et al.* (2000)
Rhizoctonia sp. (BNR)	Tobacco	CARTWRIGHT & SPURR (1998)
Trichoderma hamatum	*In vitro* assay	ANJU *et al.* (1994)
Trichoderma harzianum	Tobacco	FISHER & MPOFU (1992), SREERAMULU *et al.* (1998)
	Carnation	MIGHELI *et al.* (1993)
	Betelvine	MAHANTY *et al.* (2000)
	In vitro assays	BELL *et al.* (1982), ANJU PURI *et al.* (1994), GHOSHI (2000)
Trichoderma piluliferum	*In vitro* assay	ANJU *et al.* (1994)
	Cassava	BEDOYA *et al.* (1999)
Trichoderma sp.	*Citrus* sp.	MAY & KIMATI (1999)
	Tobacco	WANG *et al.* (2001)
Trichoderma viride	*In vitro* assays	ANJU PURI *et al.* (1994) HELLER & THEILER-HEDTRICH (1994), GHOSHI (2000)

lium species and *S. candida* grew at a slower rate and did not overgrow *P. nicotianae* within 1 week. In all pairings no hyphal interactions such as penetration or coiling were observed as the opposed colonies merged, thereby excluding mycoparasitic relationships. The most interesting observation concerns induction of formation of sporangia, which occurred in all pairings but those with *T. harzianum*. Sporangia are not usually produced by *P. nicotianae* on CMA, and it is believed that their formation occurs to allow the fungus to escape from unfavourable environmental conditions (MALAJCZUK 1983); therefore their production in the dual cultures has to be regarded as a reaction to the presence of the antagonist. However, in pairings with isolates of *A. sydowii*, both *Penicillium* species and *S. candida* they were formed in a comparatively lower number and appeared to be aberrant morphologically (e.g. smaller, distorted, vacuolated and/or empty). In the case of *S. candida*, sporangia mostly germinated directly with multiple germ tubes, and, as the antagonist overgrew *P. nicotianae* hyphae, they were colonized and filled by its conidial structures. Isolate SP1 also stimulated aberrant mycelial growth with a proliferation of branching which gave the *P. nicotianae* mycelium a peculiar dendritic or coral-like appearance. Such effects are currently under investigation by means of electron microscopy for an evaluation under the ultrastructural viewpoint.

Results concerning biological activity of culture filtrates are summarized in Table 2. Complete inhibition was observed with CCFs of isolates FP1R (*F. chlamydosporum*), PPh-2 (*P. brevicompactum*) and PPh-1 (*P. chrysogenum*), which demonstrated they are able to produce toxic metabolites possibly involved in the expression of antagonism. Isolates GRF (*G. roseum*), APh-1 (*A. sydowii*), PPh-3 (*P. chrysogenum*) and SP1 (*S. candida*) were only able to reduce the growth rate at some extent. The latter isolate caused the lowest degree of inhibition, but again the aberrant mycelial growth and the coral-like appearance were evident. Moreover, only CCF of isolate SP1 stimulated the production of sporangia, thereby showing a biological activity similar to that exhibited by the fungus itself. Finally, CCF of both isolates of *T. harzianum* again failed to inhibit *P. nicotianae*.

Table 2. Biological activity of culture filtrates of the isolates tested

Isolate	% inhibition	Sporangia	Mycelial growth
APh-1 (*A. sydowii*)	78	absent	normal
PF1R (*F. chlamydosporum*)	100	–	–
GRF (*G. roseum*)	88	absent	normal
PPh-2 (*P. brevicompactum*)	100	–	–
PPh-1 (*P. chrysogenum*)	100	–	–
PPh-3 (*P. chrysogenum*)	56	absent	normal
SP1 (*S. candida*)	35	present	aberrant
TP1 (*T. harzianum*)	0	absent	normal
TP2 (*T. harzianum*)	0	absent	normal

The absence of an inhibitory aptitude by isolates of *T. harzianum* observed in our study was somehow unexpected, since so far this species is the antagonist of *P. nicotianae* with a higher number of records. However, there is an experimental evidence that the efficacy of *T. harzianum* as an antagonist of plant pathogens pertains to selected isolates rather than to the species as a whole (BELL *et al.* 1982); moreover, in one of the cited references treatment of tobacco plants with *T. harzianum* failed to reduce significantly the disease incidence in the field (FISHER & MPOFU 1992).

Other than providing evidence of a natural occurrence of fungal antagonists of *P. nicotianae* in tobacco fields, the present study showed that a preliminary screening *in vitro* may allow a selection of species and isolates to be assayed *in vivo* against a disease which appears to be manageable by means of biological control.

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