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Investigation on Fungal Antagonists of Root Rot Agents from the Rhizosphere of White Lupin (*Lupinus albus*)

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ABSTRACT

Crown and root rot of white lupin (*Lupinus albus*) is quite complex in aetiology as several soil-borne fungal pathogens, such as *Pythium ultimum*, *Rhizoctonia solani* and *Fusarium* spp., are usually recovered from infected plants. A severe outburst of the disease compromised the crop outcome in a farm located in Campania, southern Italy; notwithstanding, some more or less extended patches of unaffected plants were visible amidst the decaying areas. The presence of fungal antagonists was investigated in the rhizosphere of both healthy and infected plants to verify which species had been stimulated in the presence of a massive inoculum of several pathogens, and if any eventually prevailed in the unaffected patches. The ability of the strains isolated to establish mycoparasitic relationships and/or to inhibit mycelial growth of the above-mentioned pathogens was investigated *in vitro*. Besides *Trichoderma* spp. and *Clonostachys rosea*, whose active development was particularly evident on the outer surface of the roots of the infected plants, most species were recovered from both sources. *Penicillium restrictum* stood out for its prevalence in the rhizosphere of healthy plants, and showed a conspicuous mycoparasitic aptitude that is described for the first time in the present study. The occurrence of two rare *Penicillium* species, *P. glaucolanosum* and *P. sajarovii*, is also reported.

Keywords: biocontrol, mycoparasitism, *Penicillium restrictum*, suppressiveness

INTRODUCTION

Among the various factors influencing the onset of soil-borne plant diseases, microbial antagonists play a major role by regulating the accumulation of the inoculum of fungal pathogens and possibly inducing soil suppressiveness (Baker and Paulitz 1996; Borneman and Becker 2007). Based on this assumption, there is an increasing awareness that manipulation of antagonists resident in the rhizosphere may represent a viable disease management strategy (Mazzola 2004). Therefore, data concerning the composition of fungal communities inhabiting the rhizosphere of cropped plants are quite relevant both for the comprehension of their biocenotic interactions and as a tool for the selection of possible biological control agents. Actually, many soil-borne fungal diseases present a complex aetiology, and some studies have already demonstrated the useful implications of identifying antagonists with a multiple aptitude (Xue 2003; Mazzola 2004).

A severe outburst of crown and root rot of white lupin (*Lupinus albus*) occurred in a farm located in Campania, southern Italy. Isolates of *Pythium ultimum*, *Rhizoctonia solani* AG-2-1 and AG-4, and three different *Fusarium* species (*F. culmorum*, *F. oxysporum* and *F. solani*) were recovered from diseased plants. Pathogenicity assays carried out by inoculating lupin seedlings with all possible combinations of the above-mentioned pathogens showed that *Fusaria* were scantily virulent, and even able to reduce the incidence of both *P. ultimum* and *R. solani* (Nicoletti and Carella, unpublished). Moreover, the occurrence of *Clonostachys rosea* and *Trichoderma* spp., as disclosed by their heavy sporulation on the roots of decaying plants in the field, and the presence of more or less extended patches of unaffected plants were indicative of a possible implication of fungal antagonists in disease escape. Considering that spatial heterogeneity of microbial populations is a recognized factor influencing soil suppressiveness (Kirk *et al.* 2004), an investigation was carried out on their presence in the rhizosphere of healthy and diseased plants.

MATERIALS AND METHODS

Two soil samples from the rhizosphere of respectively healthy and infected plants were collected by uprooting 10 randomly-chosen plants and shaking their roots into sterile plastic bags. The soil was carefully mixed, and sieved at 2 mm. Two suspensions of 10 g in 100 ml sterile distilled water amended with 200 ppm streptomycin-sulphate were prepared. After stirring for 10 min, both suspensions were diluted at 1/10 with sterile distilled water; then 2 ml of the diluted suspensions were poured in PDA plates (Ø 90 mm) pre-colonized by isolates of *P. ultimum* (XL16P), *R. solani* AG-2-1 (XL7Rh) or *R. solani* AG-4 (XL12Rh), that had been previously recovered from diseased lupin plants. Five plates for each isolate were prepared and incubated in darkness at 25°C, and inspected daily for the development of colonies of presumptive mycoparasites, which were transferred on PDA plates. Moreover, 1 ml of each diluted suspension was poured in Petri dishes (Ø 90 mm) on melted G25N, a substrate set up for culturing *Penicillium* and *Aspergillus* species (Pitt 2000). Plate content was homogenized, and again the dishes were incubated in darkness at 25°C. After 2 days, 50 fungal micro-colonies from each plate were transferred as pure cultures on PDA.

Dual cultures were prepared by placing mycelial plugs of each collected isolate against the above-mentioned isolates of *P. ultimum* and *R. solani* AG-2-1 and AG-4 at the opposite edges of PDA plates (Ø 90 mm). The dishes were incubated in darkness at 25°C for 1 week in order to observe the eventual occurrence of inhibitory effects. Another set of dual cultures with the same isolates was prepared on special nutrient agar (SNA, for *P. ultimum*) and 2% water agar (WA, for *R. solani*) for inspecting mycoparasitic relationships. After 5 days incubation in the same conditions, rectangular agar blocks (10 × 20 mm) were cut, mounted on glass slides, stained with anilin blue in lactophenol, and observed at 600X magnification.

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Table 1 Fungal isolates recovered from lupin rhizosphere.

Species	Rhizosphere of healthy plants				Rhizosphere of diseased plants			
	G25N	*Pythium ultimum*	*R. solani* AG-2-1	*R. solani* AG-4	G25N	*Pythium ultimum*	*R. solani* AG-2-1	*R. solani* AG-4
Aspergillus niger	1				4			
Aspergillus ochraceus	1				2			
Aspergillus parasiticus	3				1			
Cladosporium sp.	7	2	1	1	8	3		1
Clonostachys rosea			1	2		4	5	7
Fusarium culmorum	1				2			
Fusarium oxysporum	3		1	1	7		1	2
Fusarium solani					1			
Penicillium glabrum		1						
Penicillium glaucolanosum	2				2			
Penicillium griseofulvum	4		1		4			
Penicillium restrictum	14				1			
Penicillium sajarovii					1			
Penicillium thomii	1				3			
Rhizopus sp.	8				9			
Trichoderma aureoviride		1	1		1	2		
Trichoderma hamatum			1	1				3
Trichoderma harzianum		2		1		3	2	1
Unidentified	5				4			

RESULTS AND DISCUSSION

The use of pre-colonized agar plates represents a well-established method for the isolation of presumptive mycoparasites from soil (van den Boogert and Deacon 1994; Knudsen *et al*. 1997; Nicoletti *et al*. 2004). In our study, isolations carried out directly on pre-colonized plates yielded a limited number of species (**Table 1**) because *C. rosea* and some *Trichoderma* spp. (*T. aureoviride*, *T. hamatum* and *T. harzianum*) seemed to be the dominant mycoparasites in the rhizosphere, particularly in the case of diseased plants. In fact, *Trichoderma* isolates generally colonized the available surface very rapidly, thereby inhibiting or concealing the development of other mycoparasites, including *C. rosea* which on the other hand, although slowly developing, often spread on the available surface with multiple colonies. Their widespread presence in the sampled soil was also attested by the almost systematic colonization of the outer surface of the roots of infected plants (**Fig. 1**). Mycoparasitism by these species against both *P. ultimum* and *R. solani*, characterized by an intense hyphal coiling, is quite common and well documented in the literature (Papavizas 1985), and was also observed in our dual cultures (**Fig. 2**). On the contrary, in the case of *F. oxysporum*, which was recovered on both *R. solani* strains from both the soil suspensions, observations of hyphal interactions were unsuccessful, despite mycoparasitism by *F. oxysporum* against *R. solani* being well known (Gupta *et al.* 1979; Arora and Dwivedi 1980). Actually, the antagonistic properties by *Fusarium* spp. resulting in the above-mentioned pathogenicity trials might rather be based on the competition for root colonization. Although detected on pre-colonized plates, *Cladosporium* isolates were not able to establish mycoparasitic relationships with both pathogens. Their ability to produce fungitoxic extrolites resulted on account of strong inhibitory properties in dual cultures, and actually effective compounds, such as cladosporol, have been characterized by several *Cladosporium* species inhabiting the rhizosphere (Sakagami *et al.* 1995; Assante *et al.* 2004). Failure to establish mycoparasitic relationships against both *R. solani* and *P. ultimum* also resulted in the case of *Penicillium griseofulvum* and *P. glabrum*, two typical soil species which were recovered in single isolates from the rhizosphere of healthy plants on pre-colonized plates. However, substantial inhibition of mycelial growth in dual cultures supports previous evidences of their possible ecological role as fungal antagonists. In fact, *P. glabrum* (syn. *P. frequentans*) is known for its rhizosphere-competence that has introduced some consideration as a biological control agent against damping-off of *Picea glehnii*, that is also incited by *Fusarium*, *Pythium* and *Rhizoctonia* spp. (Yamaji *et al*. 2005). On the other hand, antagonistic properties of *P. griseofulvum* have been reported against several soil-borne fungal pathogens (Zazzerini and Tosi 1985; Dewan and Sivasithamparam 1988), including *R. solani* (Nicoletti *et al*. 2003) and *P. ultimum* (Gravel *et al*. 2005).

Fig. 1 Massive sporulation of *C. rosea* on an infected lupin taproot.

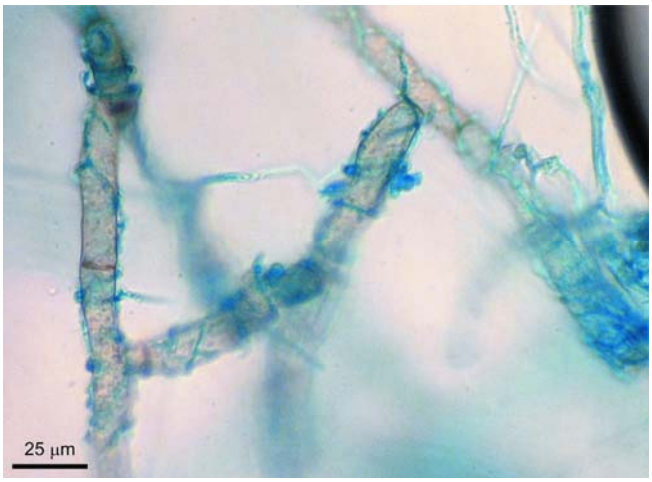

Fig. 2 Coiling of *R. solani* AG-2-1 hyphae by *C. rosea*.

As only a limited number of species was obtained by using pre-colonized plates, the isolation of fungal antagonists from the soil suspensions was also performed on agar media directly. Isolations were initially carried out on PDA, but results were unsatisfactory since the fast growth of *Trichoderma* sp. and/or *P. ultimum* did not allow sub-isolations of other fungi. Therefore as an alternative we used G25N, a medium where the initial development of fungal colonies is quite restricted. The use of this substrate did not allow to re-

cover any fungal species actually represented in the soil samples, as it particularly stimulates the growth of fungi able to use glycerol as the main carbon source. In fact, *C. rosea* is unable to grow on this medium, and *Trichoderma* spp. themselves seem to be strongly inhibited, as just one *T. aureoviride* isolate was recovered by this method. However G25N was particularly useful for the detection of *Penicillium* and *Aspergillus* species, some of which are typical soil fungi influencing the composition of fungal communities by the production of antibiotics (**Table 1**). Besides the unidentified isolates and *Rhizopus* sp. that showed neither inhibitory nor mycoparasitic properties, most species recovered on G25N were somehow able to affect mycelial growth of the tested pathogens. Three species of the genus *Aspergillus* (*A. niger*, *A. ochraceus* and *A. parasiticus*), well known as mycotoxin producers, were found in both soil samples, and all isolates showed remarkable inhibitory capacities toward the pathogens under investigation. Mycoparasitism has been previously documented for *A. niger* only, particularly against *R. solani* (Venkatasubbaiah and Safeeulla 1984), but no clear evidence other than adpressed hyphal growth was found in our dual cultures. Occurrence of the above-mentioned *Cladosporium* sp. was similar in both soil samples, while isolation of *Fusarium* spp. was more consistent from the rhizosphere of diseased plants, most likely in relation to their occurrence on the infected roots. As above reported for *F. oxysporum*, *F. culmorum* and *F. solani* also did not establish mycoparasitic relationships, while growth inhibition in dual cultures was quite evident in the case of *F. culmorum*, particularly against *R. solani*. Besides *P. griseofulvum*, four more *Penicillium* species with some degree of inhibitory properties were also isolated on G25N: *P. sajarovii* was recovered from the rhizosphere of diseased plants, while *P. thomii*, *P. glaucolanosum* and *P. restrictum* were obtained from both soil samples. Interestingly, the latter was much more common in the rhizosphere of healthy plants, a circumstance deserving to be more accurately considered for its possible implications with disease escape. Antagonism by *P. restrictum* has been reported against some soil-borne fungal pathogens (Mekwatanakarn and Sivasithamparam 1987), including *Pythium* spp. (Gravel *et al*. 2005). However, these properties were rather ascribed to the production of antifungal compounds, which in fact have been characterized from this species (Hensens *et al.* 1991; Jackson *et al.* 1993). Besides a notable inhibitory capacity, *in vitro* assays evidenced the occurrence of hyphal interactions with the pathogens under investigation, consisting of adpressed mycelial growth with hyphal penetration by haustorium-like structures produced at regular intervals and sketches of hyphal coiling (**Fig. 3**), which demonstrated unequivocally the capacity of *P. restrictum* to behave mycoparasitically. Occasional evidence of hyphal penetration was also observed on *R. solani* AG-4 in the case of a *P. thomii* isolate (XLT3M), but further observations are necessary to draw up a more conclusive frame in this regard. *P. thomii* has been reported as an antagonist of several plant pathogenic fungi (Manandhar *et al.* 1987), and has been successfully applied as a biological control agent against *P. ultimum* (Carisse *et al.* 2003). Inhibitory capacities without mycoparasitic clues were also noticed on isolates of *P. glaucolanosum* and *P. sajarovii*, two closely related species in the subgenus *Furcatum* whose species status is uncertain by reason of the reduced number of isolates available (Arthur de Cock, pers. comm.). However, it cannot be excluded that their real occurrence in nature has been underestimated so far in consequence of a possible confusion with the morphologically related species *P. cremeogriseum* and *P. simplicissimum*, with the latter also known for its antagonistic properties against *P. ultimum* (Gravel *et al.* 2005).

As it is known that the proportion of soil-inhabiting microorganisms that can be isolated and cultured is very little (Mazzola 2004), our investigation did not aim to describe or quantify the fungal community of the lupin rhizosphere, which would be rather a considerable challenge (Borneman and Becker 2007). Actually, it is quite likely that additional microbial species may be effective in determining disease escape in the unaffected patches. Nevertheless, it is also evident that disease control strategies based on the management of resident microbial communities imply the capacity to initially detect and identify the biological determinants of suppressiveness (Weller *et al.* 2002). On the other hand, a general assumption to be considered in the selection of biocontrol agents of soil-borne fungal pathogens is that they must be able to colonize the rhizosphere (Whipps 2001). In this sense, it is to be remarked that even a simple isolation method can detect the presence of several antagonists and mycoparasites that are possibly involved in plant protection, together with some differences in the composition of microbial communities inhabiting the rhizosphere of diseased or unaffected plants. Particularly this is the case of *P. restrictum*, whose relative abundance in the rhizosphere of healthy plants may be indicative of an active role in plant protection. *P. restrictum* is generally quite difficult to isolate due to its very slow growth on agar media, which could also explain its uneven spread in the sampled soil and the unsuccessful isolation attempts on pre-colonized plates. To our knowledge this is the first report of a mycoparasitic aptitude by this *Penicillium* species. Further studies will be carried out on its host range and its ability to suppress soil-borne plant pathogens *in vivo*.

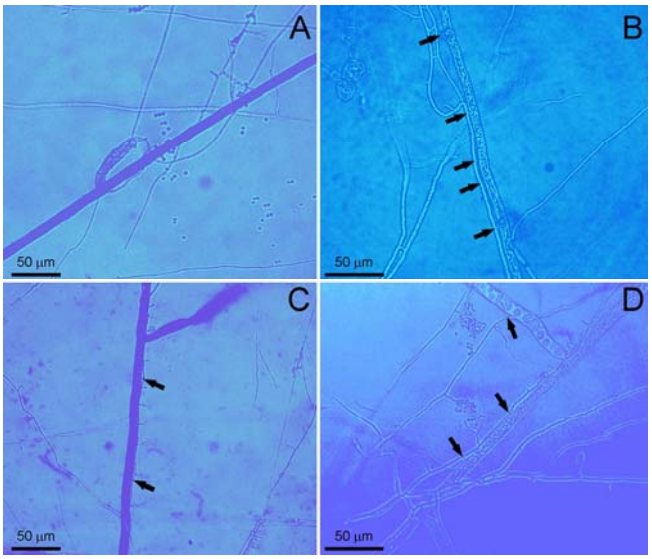

Fig. 3 Mycoparasitic interactions of *P. restrictum* with *R. solani* AG-2-1 (a), *P. ultimum* (b) and *R. solani* AG-4 (c-d).

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