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ORIGINAL ARTICLE

Clonostachys rosea BAFC3874 as a *Sclerotinia sclerotiorum* antagonist: mechanisms involved and potential as a biocontrol agent

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Abstract

Aims: To establish the modes of action of the antagonistic fungal strain *Clonostachys rosea* BAFC3874 isolated from suppressive soils against *Sclerotinia sclerotiorum* and to determine its potential as a biocontrol agent.
Methods and Results: The antagonistic activity of *C. rosea* BAFC3874 was determined *in vitro* by dual cultures. The strain effectively antagonized *S. sclerotiorum* in pot-grown lettuce and soybean plants. Antifungal activity assays of *C. rosea* BAFC3874 grown in culture established that the strain produced antifungal compounds against *S. sclerotiorum* associated with secondary metabolism. High mycelial growth inhibition coincided with sclerotia production inhibition. The *C. rosea* strain produced a microheterogeneous mixture of peptides belonging to the peptaibiotic family. Moreover, mycoparasitism activity was observed in the dual culture.
Conclusions: *Clonostachys rosea* strain BAFC3874 was proved to be an effective antagonist against the aggressive soil-borne pathogen *S. sclerotiorum* in greenhouse experiments. The main mechanisms involve peptaibiotic metabolite production and mycoparasitism activity.
Significance and Impact of the Study: *Clonostachys rosea* BAFC3874 may be a good fungal biological control agent against *S. sclerotiorum*. In addition, we were also able to isolate and identify peptaibols, an unusual family of compounds in this genus of fungi.

Introduction

Sclerotinia sclerotiorum is a cosmopolitan pathogen affecting many economically important crops (Boland and Hall 1994). It can produce mycelia or sclerotia (anamorph) or apothecia, asci and ascospores (teleomorph) (Kohn 1979). Sclerotia are viable for more than 5 years (Le Tourneau 1979). During myceliogenic germination, the hyphae grow towards host roots and hypocotyls, causing shoot wilt (Adams and Ayers 1979). The absence of host-resistant cultivars and an increased resistance to fungicides in populations of this pathogen, together with certain limitations and environmental disadvantages associated with the use of chemical fungicides, have increased demand for alternatives to synthetic fungicides in agriculture and horticulture (Whipps 2001). One of these alternatives is the use of beneficial micro-organisms or biocontrol agents, which may promote plant growth and provide disease control (Zahir *et al.* 2004). The biological control of plant pathogens by micro-organisms has been considered a more natural and environmentally acceptable alternative (Whipps 2001).

One fundamental aspect in determining the potential of a fungal strain as a control agent is to identify its

antagonistic effects against target pathogens and establish the main mechanisms involved to address the most appropriate biocontrol management strategies.

Clonostachys rosea (Sch.) Schroers and Samuels is a common world-wide soil saprophyte (Domsch *et al.* 1980). In addition, *C. rosea* is a potentially useful biocontrol agent against several economically important plant pathogens, including *S. sclerotiorum* (Ervio *et al.* 1994), *Verticillium dahliae* (Keinath *et al.* 1991), *Fusarium culmorum* (Knudsen *et al.* 1995) and *Botrytis cinerea* (Nobre *et al.* 2005). Some of its modes of antagonism against pathogens are nutrient competition, mycoparasitism and induced resistance (Sutton *et al.* 1997; Roberti *et al.* 2008). Also, its production of secondary metabolites may exert an antibiotic effect, similar to that found in other biocontrol fungi (Roberti *et al.* 2001; Innocenti *et al.* 2003). Several studies have used this natural advantage to develop effective biological control strategies (Butt *et al.* 2001; Whipps 2001).

The aim of this study was to establish the modes of action of the antagonistic strain BAFC3874 of *C. rosea* isolated from a suppressive soil against *S. sclerotiorum* and determine its potential as a biocontrol agent.

Materials and methods

Fungal strains

Clonostachys rosea BAFC3874 (Sch.) Schroers and Samuels isolate was selected for the present experiments based on its superior performance against *S. sclerotiorum* in screening tests of microbes recovered from a soil suppressive to the pathogen (Rodríguez 2004). The strain was morphologically identified (Schroers *et al.* 1999) as BAFC3874 and deposited in the Buenos Aires Fungal Collection (BAFC), University of Buenos Aires, Argentina.

Dual cultures

Sclerotinia sclerotiorum (BAFC225 and BAFC2232) and *C. rosea* were confronted in dual cultures in Petri dishes in malt extract agar (MEA) and potato dextrose agar (PDA) (Whipps 1987). Two assays were conducted: *C. rosea* was inoculated either 48 h before or at the same time as the pathogen strains. In both assays, two plugs (4 mm diameter) excised from the leading edge of an actively growing colony of each fungus (Whipps 1987) were positioned 4·5 cm apart in 9-cm-diameter Petri dishes. Three replicates were included for each confrontation. Control dishes were inoculated only with the pathogen strain in each of the media being assessed. All Petri dishes were kept at 25°C in darkness.

The width of the inhibition zone (Iz) was determined, and the percentage of radial growth inhibition (%RGI) was calculated as: $\%RGI = (rc - rd)/(rc) \times 100$, where rc is the radius of the control pathogen colony, and rd is the radius of the pathogen colony in the dual culture (Melgarejo *et al.* 1985).

To establish and describe the existence of alterations in the morphology of the mycelium, squares of 10×10 mm were removed from the pathogen colonies in contact with the inhibition zone on MEA 10 days post-inoculation. Each square was stained with Cotton Blue (0·05 g of Cotton Blue, 20 g of phenol crystals, 40 g of glycerine, 20 g of lactic acid and 20 ml of distilled water) and observed by light microscopy (Nikon OPTIPHOT-2 light binocular microscope equipped with a Nikon Coolpix950 digital camera; Nikon, Tokyo, Japan).

Production of nonvolatile metabolites

Sterilized cellophane (50 mm diameter) was placed on the surface of MEA medium in Petri dishes and a plug (4 mm diameter) from the *C. rosea* colony was centrally inoculated on the cellophane. The plates were incubated at 25°C in darkness. After 3 days, the cellophane and the colony were removed. A 4-mm plug of pathogen, excised from a 4-day-old culture, was placed at the centre of the plate. As a control, the same treatment was repeated but without inoculation of *C. rosea*. The plates were incubated at 25°C for 15 days, and the radii of the pathogen colonies were measured every day (Whipps 1987). Three replicates were performed for each treatment.

The assay was repeated again under the same conditions, testing for fungicidal or fungistatic activity. After 4 days of incubation, *S. sclerotiorum* plugs, which had not grown in the presence of *C. rosea* exudates, were reinoculated onto fresh MEA and the percentage of viable plugs was determined (Dennis and Webster 1971a; Jackson *et al.* 1991). This procedure was carried out in triplicate.

Production of volatile metabolites

Clonostachys rosea was tested for inhibitory volatile metabolites using the following method: Petri dishes were inoculated centrally with agar discs cut from a 4-day-old culture of this strain and kept at 25°C in darkness. After 4 days, the lid of each dish was replaced by a bottom with MEA inoculated with a plug cut from a 4-day-old culture of the pathogen. Then, Petri dish bottoms were attached and sealed with Parafilm® (Chicago, IL). The lids of the control plates in which only the pathogen plug was inoculated were also replaced in the same way (Dennis and Webster 1971b). The plates were incubated at 25°C in darkness, and the diameters of the pathogen colonies were measured every day for 10 days. Three replicates were made.

Antifungal activity during the growth of the antagonist

The antagonist strain of *C. rosea* was cultured in malt extract broth (MEB) under static conditions at 25°C, and the presence of nonvolatile antifungal metabolites in the medium was studied (Fuhrmann 1994). A 4-mm plug of *C. rosea*, taken from the growing edge of the culture on MEA, was used to inoculate 50-ml Erlenmeyer flasks containing 20 ml of MEB. Activity of broth from MEB cultures of *C. rosea* against the growth of *S. sclerotiorum* was estimated as a function of the duration of incubation and of the mycelial growth of the antagonist in the broth at 1, 3, 5, 7 and 10 days after inoculation. Individual 20-ml cultures were vacuum-filtered (Whatman® no. 1 filter paper; Maidstone, UK), and the mycelium was oven-dried at 70°C for 48 h. Petri dishes were prepared with the broth (filtered using a 0·2 μm filter; Millipore, Bedford, MA) incorporated into fresh MEA (10% broth in MEA, v/v). This procedure was carried out in triplicate and repeated at least twice with similar results. Control dishes were prepared using filtered culture medium (10% MEB in MEA, v/v). A 4-mm pathogen plug was centrally inoculated in each Petri dish and incubated at 25°C in darkness.

After 4 days of incubation, the percentage of growth inhibition of the pathogen colony (%GI) was calculated as: $\%GI = (D1 - D2)/(D1) \times 100$, where $D1$ is the diameter of the control pathogen colony and $D2$ is the diameter of the pathogen colony when the antagonist exudates were added. Morphological changes in the colonies were also described.

Extraction and isolation of the active compound

A 5-mm plug of the *C. rosea* colony on MEA was used to inoculate 250-ml Erlenmeyer flasks containing 100 ml of MEB. After 1 week, this culture was used to inoculate a 4-l Erlenmeyer flask containing 1 l of MEB. Incubation was carried out at 25°C for 21 days (stationary growth phase) under static conditions. This procedure was carried out in triplicate and repeated at least twice with similar results.

The broth (2 l) was filtered and Amberlite XAD-16 (150 g l^{-1}) was added to the filtrate. After 18 h, the suspension was filtered and the Amberlite phase was washed with distilled H_2O and then eluted with MeOH (2 l). The MeOH eluate (6·5 g) was evaporated and subjected to vacuum chromatography on RP-C18 using H_2O and mixtures of H_2O–MeOH of decreasing polarity. The active fractions, eluted with 8 : 2 MeOH/H_2O (named fraction Crm), were analysed by NMR and MS, and subjected to HPLC (column: YMC C18, 5 μm, 22·5 × 2·5 cm; eluent: 8 : 2 MeOH/H_2O; detection: UV 220 nm and refractive index). The purification process was guided by antimicrobial assays of the collected fractions against an *S. sclerotiorum* colony (Hadacek and Greger 2000).

NMR spectra were recorded on a Bruker Avance II instrument (Bruker Daltonics, Billerica, MA) at 500·13 MHz for ^{1}H and at 125·13 MHz for ^{13}C NMR. Electrospray ionization (ESI) MS and ESI MS/MS were performed on a Bruker MicrOTOF-Q II (Bruker Biospin, Rheinstetten, Germany).

Effectiveness of *Clonostachys rosea* against *Sclerotinia sclerotiorum* in soybean and lettuce

Two greenhouse experiments were conducted using *Glycine max* (soybean) and *Lactuca sativa* (lettuce). Pregerminated seeds were planted in 250-ml pots containing 200 ml of steam-pasteurized soil inoculated with the antagonist strain. The *C. rosea* inoculum was added to steam-pasteurized soil as a mass of mycelium and spores growing on boiled rice at a concentration of $1{\cdot}3 \times 10^6$ (colony-forming units per gram of soil: CFU g^{-1}). After 3 and 6 days, respectively, the soybean and lettuce plants with all roots and soil were transplanted to 600-ml pots containing 300 ml of soil inoculated with 15%, w/v, *S. sclerotiorum* BAFC225, containing pathogen mycelia and sclerotia. This inoculum was produced in sterile polythene bags containing rice/bran/water (20/20/100; v/v/v) as the substrate that were inoculated with 5-mm pathogen plugs (6 per 350 g of substrate) and incubated for 20 days at 24–28°C in darkness. A completely randomized design was used. Four treatments with four replicates each were performed: pathogen only (treatment S), antagonist only (treatment Cr), pathogen + antagonist (treatment S + Cr) and control (C) with neither antagonist nor pathogen. The control plants received the same substrates at the same proportion, but without the inoculum. The experiment was repeated with five replicates per treatment. The percentage of surviving plants and the dry weight of roots and shoots were recorded. Shoot lengths and fresh weights were also recorded in soybean plants and lettuce plants, respectively.

Assessment of antagonistic activity against other fungal pathogens

The antagonistic activity against other phytopathogenic fungi was evaluated. *Clonostachys rosea* and phytopathogenic strains were confronted in dual cultures in Petri dishes on MEA. *Clonostachys rosea* was inoculated for 48 h before *Macrophomina phaseolina* and *Rhizoctonia solani* were added to the dish. Similarly, *Fusarium solani* and *Colletotrichum truncatum* were added to a dish at the same time of *C. rosea*. These experiments took into account the growth rate of each strain. Three replicates

were performed for each confrontation. Control dishes were inoculated with the pathogen strain on MEA. All Petri dishes were kept at 25°C. The width of the Iz was determined, and the %RGI was calculated for each strain.

Experimental design and statistical analysis

Each experiment was repeated at least twice with similar results. One way analysis of variance was performed at the significance level of $P < 0{\cdot}05$. When appropriate, means were separated using Tukey's test ($P < 0{\cdot}05$).

Results

Dual cultures

When *C. rosea* and *S. sclerotiorum* were grown in dual culture, radial growth of the pathogen was reduced by more than 25% compared to control in both media tested. When the strains were inoculated simultaneously, the %RGI on MEA (over 30%) was significantly higher than that obtained on PDA (over 25%). A larger inhibition halo on MEA than on PDA was also found (Table 1). *Clonostachys rosea* profusely colonized the sclerotia 20 days after confrontation in both pathogen strains evaluated, but the infectivity (%IS) was significantly higher against strain BAFC2232.

The inoculation of *C. rosea* 48 h before the pathogen allowed for the establishment of the inhibitory effects over pathogen growth, independently of its growth rate. This assay resulted in a greater antagonistic effect. The %RGI was higher than 42% for all strains tested, but the results varied according to the medium and pathogen strain used. Inhibition halos were observed only on MEA and were significantly larger against strain BAFC2232 (Table 1). Sclerotia colonization by the antagonistic strain was as profuse as in the simultaneous inoculation. The infectivity was also higher against strain BAFC2232 in both media. In addition, the highest %IS was found on PDA. In all the assays, the proportion of infected sclerotia inversely correlated with their viability (Table 1).

Pathogen hyphae in contact with the inhibition halos showed protoplasm collapse, an increase in the number and a shortening of the hyphal branches and/or melanization in comparison with hyphae in the control (Fig. 1). Cell lysis of the pathogen hyphae was observed sporadically. Melanization of the hyphae in contact with the inhibition halo was observed 20 days after confronting the strains, a response that was more evident on MEA.

Production of nonvolatile and volatile metabolites

Figure 2a shows the growth curve of the pathogen on MEA in the presence of *C. rosea* exudates in the cellophane test. The *C. rosea* strain inhibited pathogen growth completely during the 15-day experiment. When the experiment was repeated and the plug was transplanted to a dish with medium without exudates, only 50% (50·0 ± 16·6%) recovered their growth, on average, 8 days (8 ± 0·5) after the transplant.

In the assessment of volatile metabolite production, the *C. rosea* strain did not show inhibitory effects (Fig. 2a). The broth medium in which *C. rosea* was grown, however, did suppress *S. sclerotiorum* when incorporated into the agar medium used to assay pathogen growth. The suppression was greatest when the broth was sampled during the stationary growth phase of *C. rosea*. Figure 2b shows the percentage of pathogen growth inhibition (%GI) in the presence of *C. rosea* culture broth on different days of harvest. Particularly, a significantly higher activity in the culture on day 7 (stationary growth phase) was found, where the %GI was 73·3%. Besides a marked

Table 1 Summary of simultaneous and deferred inoculation assay results from dual cultures of the antagonistic strain *Clonostachys rosea* BAFC3874 against *Sclerotinia sclerotiorum* strains BAFC225 and BAFC2232 evaluated by percentage of radial growth inhibition (%RGI), inhibition zone (Iz) width, percentage of colonized sclerotia (%CS), percentage of infected sclerotia (%IS) and percentage of viable sclerotia (%VS)

	Simultaneous inoculation				Deferred inoculation of *S. sclerotiorum*			
	MEA		PDA		MEA		PDA	
	BAFC225	BAFC2232	BAFC225	BAFC2232	BAFC225	BAFC2232	BAFC225	BAFC2232
%RGI	30·7 ± 1·6^{a}	31·1 ± 2·4^{a}	25·5 ± 2·3^{b}	27·7 ± 2·3^{b}	51·8 ± 1·8^{a}	45·5 ± 2·3^{b}	42·9 ± 1·8^{d}	44·8 ± 3·2^{c}
Iz (mm)	4·8 ± 0·7^{a}	4·0 ± 0·6^{a}	0·0 ± 0·0^{c}	2·1 ± 0·7^{b}	3·8 ± 0·9^{b}	5·5 ± 0·5^{a}	0·0 ± 0·0^{a}	0·0 ± 0·0^{c}
%CS	86·7 ± 15·7^{a}	91·1 ± 14·4^{a}	82·2 ± 14·7^{a}	86·1 ± 22·1^{a}	95·7 ± 6·6^{a}	88·0 ± 17·8^{a}	90·2 ± 9·9^{a}	86·5 ± 15·1^{a}
%IS	20·0 ± 12·6^{a}	72·7 ± 33·3^{b}	40·0 ± 12·6^{a}	86·1 ± 22·1^{b}	13·3 ± 10·3^{d}	47·3 ± 24·4^{b}	43·3 ± 23·3^{c}	86·1 ± 22·1^{a}
%VS	86·6 ± 10·3^{a}	32·2 ± 26·3^{b}	70·0 ± 20·9^{a}	27·7 ± 22·7^{a}	90·0 ± 0·9^{a}	58·3 ± 13·7^{c}	60·0 ± 25·3^{b}	9·7 ± 15·2^{d}

PDA, potato dextrose agar; MEA, malt extract agar.

Values represent the means for each treatment with the standard error.

Different letters indicate significant differences between treatments (ANOVA Tukey's test $P < 0{\cdot}05$).

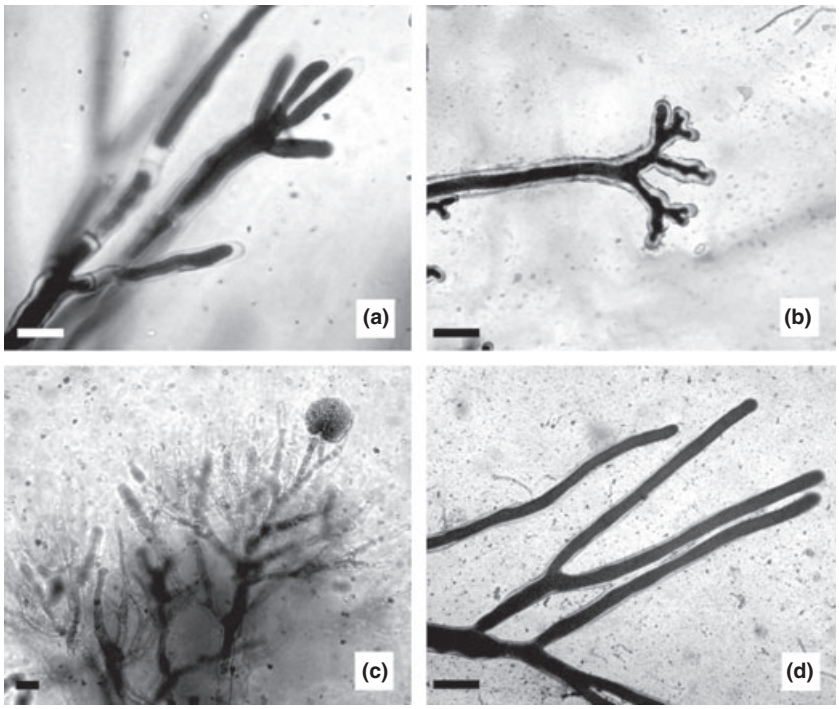

Figure 1 Alterations of the *Sclerotinia sclerotiorum* mycelium in contact with the *Clonostachys rosea* BAFC3874 inhibition zone, including branched hyphae and collapsed cytoplasm (a, b) and lysed hyphae (c). The edge of a colony in the control is also shown (d). Scale bar: 10 μm.

inhibition of sclerotia formation, changes in the colony morphology, such as the presence of irregular edges and an increase in the density of hyphae around the plug inoculation site, were found in comparison with the control colony.

Extraction and isolation of the antifungal compound

After purification of the *C. rosea* culture broth, the active fraction showed complex NMR spectra, which indicated the presence of a mixture. The ^{1}H NMR spectra showed many signals between δ 7 and 8, suggesting the presence of *N*H amide protons. This assumption was confirmed by ^{13}C NMR in which the spectra showed more than 25 signals from carbonyl carbons from amide groups. In these spectra, typical C-α amino acid signals between 50 and 65 ppm, and eight signals between δ 7·5 and 8·8 were also observed. These latter unusual chemical shifts are typical of the terminal methyl groups of the nonproteinogenic amino acid isovaline. Several signals at 70 ppm, characteristic of the methine carbon attached to oxygen in hydroxyproline, were also observed. The ESI MS showed four main peptides in the active fraction with molecular weights of about 2000. This information indicated that the Crm fraction contained a mixture of peptaibiotics composed of 17–21 amino acids, with a high content of isovaline. The compositions of the main peptides were determined by ESI MS/MS and are shown in Fig. 3.

Effectiveness of *Clonostachys rosea* against *Sclerotinia sclerotiorum* in soybean and lettuce

The assays in soybean showed a bioprotective effect of *C. rosea* against *S. sclerotiorum*. All the plants survived when inoculated with *C. rosea* alone or with *C. rosea* in combination with *S. sclerotiorum* (100·0 ± 0·0%), whereas only 22·5% (22·5 ± 2·5%) survived when inoculated only with the pathogen. The shoots in all the treatments were slightly shorter than those in the control experiments, but the differences were only significant in the plants inoculated with both micro-organisms (treatment S + Cr). Shoot and root dry weights did not differ significantly among treatments (Fig. 4a).

In the assays carried out with lettuce plants the biocontrol effect was smaller than in soybean plants. In fact, 50% (50·0 ± 10·0%) of the plants inoculated with both *S. sclerotiorum* and *C. rosea* survived, whereas none (0·0 ± 0·0%) of the plants inoculated only with the pathogen (treatment S) survived. All the plants in control (C) and only with the antagonist (Cr) treatments survived (100·0 ± 0·0%). The surviving lettuce plants inoculated only with *C. rosea* presented the highest shoot fresh weight values, but only was significantly higher ($P > 0·05$) than the S + Cr treatment. Also, shoot dry weight was significantly higher ($P > 0·05$) in the Cr treatment. Root dry weight was higher ($P > 0·05$) in control treatments, whereas root fresh weight did not show significant differences between them (Fig. 4b).

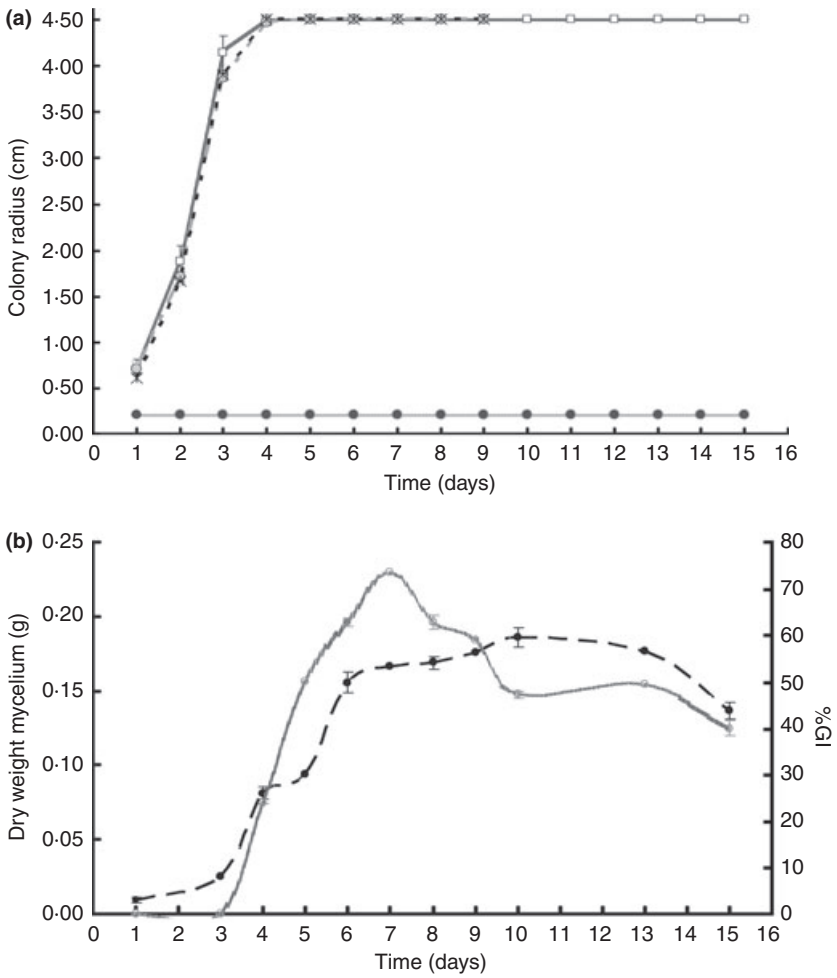

Figure 2 Growth curve of the pathogen colony growing in medium with nonvolatile metabolites (–●–) or without exudates (–□–) from *Clonostachys rosea* BAFC3874 culture (cellophane test) and with volatile metabolites (–○–) or without volatile metabolites (-ж-) (a). Percentages of pathogen colony growth inhibition (%GI) growing in medium with 10%, v/v, *C. rosea* BAFC3874 exudates (—○—), collected on the day indicated, associated with *C. rosea* BAFC3874 growth evaluated as dry weight of mycelium (—●—) (b).

AcN-Ala-Iva-Aib-Leu-Ala-Aib-Aib-Gly-Ser-Leu-Iva-Aib-Aib-Hyp-Leu-Iva-Aib-Hyp-Iva-Iva-Ala
AcN-Ala-Iva-Iva-Ala-Leu-Iva-Aib-Gly-Ser-Leu-Aib-Aib-Aib-Hyp-Leu-Iva-Aib-Hyp-Iva-Iva-Ala
AcN-Ala-Iva-Aib-Ala-Leu-Iva-Aib-Gly-Ser-Leu-Iva-Aib-Iva-Hyp-Leu-Iva-Aib-Hyp-Iva-Iva-Ala

Figure 3 Sequences of the main peptides produced by *Clonostachys rosea* BAFC3874.

Activity against other phytopathogens

The dual cultures on MEA showed that *C. rosea* is able to inhibit other pathogens, including the root pathogens *R. solani*, *F. solani* and *M. phaseolina* and the shoot pathogen *C. truncatum*. Table 2 shows that the %RGI values varied according to the pathogen, being higher against *F. solani* and lower against *C. truncatum*. Inhibition halos were observed particularly against *F. solani*, *C. truncatum* and *M. phaseolina*.

Discussion

Dual cultures are routine assays used to evaluate fungal strains as antagonists against different plant pathogens (Barakat *et al.* 2006; Zafari *et al.* 2008; Gromadzka *et al.* 2009) and are particularly useful in the case of soil-borne fungi (Shoresh *et al.* 2010). In addition, these assays can help identify the mechanisms by which antagonists reduce crop damage (Mejía *et al.* 2008).

Although confrontation assays by others have shown mutual inhibition between *C. rosea* and phytopathogens (Whipps 1987; Mejía *et al.* 2008), our results highlight *C. rosea* BAFC3874 as a potent antagonist against *S. sclerotiorum*, as shown by the %RGIs greater than 25%. In addition, substances produced by *C. rosea* BAFC3874 inhibited the mycelia growth of both *S. sclerotiorum* strains tested, albeit to different degrees. These results are in line with previous studies that showed that fungal susceptibility to inhibitory substances can differ between species and

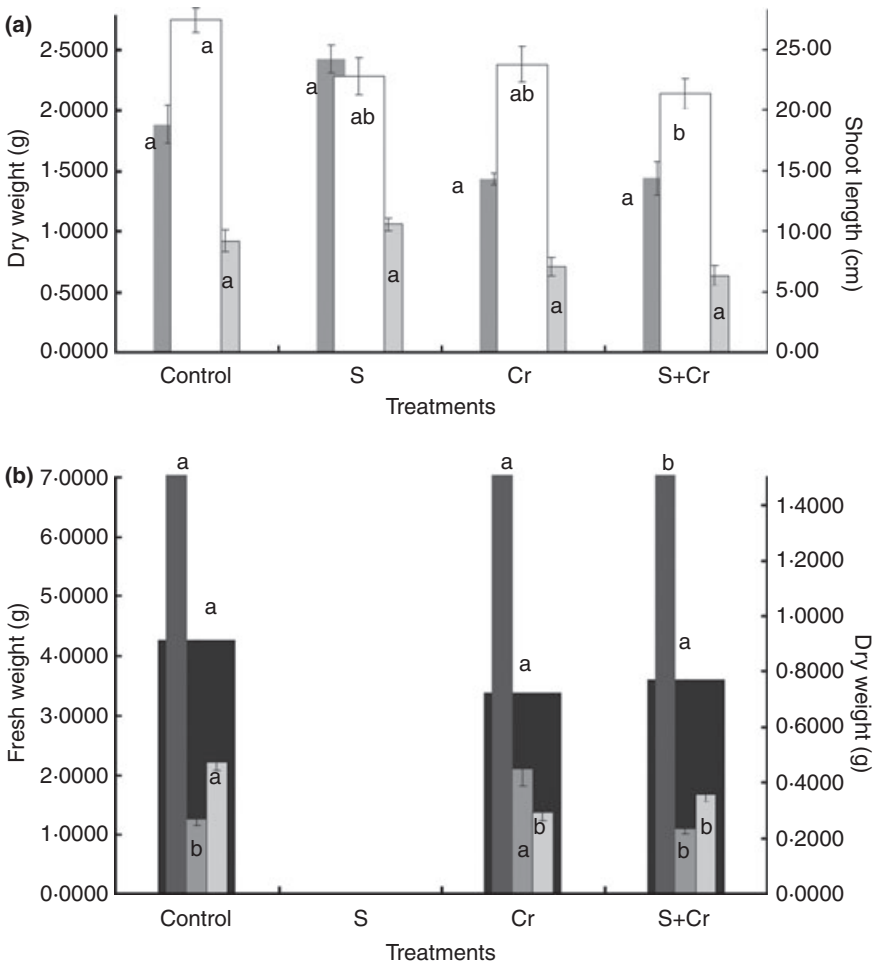

Figure 4 Biocontrol experiments with *Clonostachys rosea* BAFC3874 confronting *Sclerotinia sclerotiorum* in soybean (a) and lettuce (b) greenhouse experiments using four treatments: plants inoculated with *C. rosea* BAFC3874 (Cr); *S. sclerotiorum* (S); *C. rosea* BAFC3874 and *S. sclerotiorum* (S + Cr) and control (without *C. rosea* BAFC3874 or *S. sclerotiorum* inoculation). Growth parameters in the soybean greenhouse experiments (a): length □; shoot dry weight ■ and root dry weight ■ of surviving plants. Growth parameters in the lettuce greenhouse experiments (b): shoot fresh weight ■; shoot dry weight ■; root fresh weight ■ and root dry weight ■ of surviving plants. Different letters (anova Tukey's test $P < 0{\cdot}05$) indicate significant differences between treatments. Bars indicate standard error.

isolates of the same species (Dennis and Webster 1971a). Also, mycelial morphological alterations were observed. Different metabolites could be involved in growth inhibition and induction of abnormal morphology in fungal colonies. Such alterations have been found in dual cultures in which *S. sclerotiorum* and other pathogens were tested against different strains of *Trichoderma* spp., *Fusarium* sp. and bacteria (Zazzerini and Tosi 1985; Harish *et al.* 1998; Vey *et al.* 2001; Aryantha and Guest 2006).

Melanin, which can be both toxic and protective, defends cells against metabolites, enzymatic lysis and autolysis (Butler *et al.* 2001). In our assays, melanization of the *S. sclerotiorum* hyphae, protoplasm collapse and increases in branching hyphae were observed in the presence of *C. rosea* exudates. These responses were more evident on MEA than on PDA, suggesting that the nutritional conditions could be involved either in the protection response of the pathogen (Nosanchuk and Casadevall

Table 2 Summary of dual culture results of antagonistic strain *Clonostachys rosea* BAFC3874 with *Rhizoctonia solani*, *Fusarium solani*, *Colletotrichum truncatum* and *Macrophomina phaseolina* evaluated by percentage of radial growth inhibition (%RGI) and inhibition zone (Iz) width

	R. solani	*F. solani*	*C. truncatum*	*M. phaseolina*
%RGI	48·8 ± 1·2	62·9 ± 0·7	41·4 ± 1·3	53·3 ± 1·2
Iz (mm)	0·6 ± 0·3	4·0 ± 0·5	3·6 ± 0·3	3·3 ± 0·3

Values represent the means for each treatment with the standard error.

2003) or in the behaviour of the antagonist, regardless of whether it involves metabolite production (Whipps and Magan 1986) or fungal overgrowth (Hermosa *et al.* 2000).

In addition, both strains of *S. sclerotiorum* showed sclerotia colonization by *C. rosea* and a reduction in the viability of the sclerotia 20 days after inoculation in dual cultures. Thus, our findings suggest that decreased culture medium nutrient levels cause the trophic ability of *C. rosea* to change from saprotrophic to parasitic (Schroers *et al.* 1999; Whipps 2001).

On the other hand, antifungal activity assays related to the growth curve of *C. rosea* established that the compound or group of compounds that contributed to this activity are diffusible, nonvolatile and associated with secondary metabolism. High mycelium growth inhibition and sclerotia production inhibition were observed, indicating that metabolites present in the exudates of this strain of *C. rosea* caused these inhibitory effects. This inhibition may be linked to both the nature and the concentration of the metabolites involved (Hadacek and Greger 2000). Moreover, the concentration of metabolites in soil may be less than that in rich culture media (Whipps 1987). Together with the sclerotia viability reduction observed in dual culture, these results showed the potential of *C. rosea* BAFC3874 to reduce this important pathogen inoculum source.

Our *C. rosea* strain produced a microheterogeneous peptide mixture containing novel linear sequences belonging to the family of peptaibiotics. Interestingly, the C-terminal end of these peptides resembles that of integramides isolated from *Dendrodochium* sp. (Singh *et al.* 2002). These kinds of metabolites are often found in antagonistic fungal strains, and their activity is linked to their three-dimensional structure. In fact, because of the high percentage of aminobutyric acid (Aib) and hydroxyproline (Hyp) residues, the molecule becomes helical. This allows it to insert into the plasma membrane, causing alterations in the osmotic balance of the cell (Degenkolb *et al.* 2006). Such effects may explain some of the changes observed in the mycelium of the pathogen, including cell lysis of the hyphae and melanization, and have been described in association with the parasitic activity of different strains of *Trichoderma* spp. (Daniel and Rodrigues Filho 2007; Viterbo *et al.* 2007). These peptides interrupt fungal growth, causing the fungus to parasitize the rest of the mycelium (Schirmböck *et al.* 1994). The genus *Trichoderma*, which is taxonomically related to *C. rosea*, is the main source of these metabolites (Vey *et al.* 2001). However, peptaibiotics have been isolated very infrequently in *Clonostachys* (Chikanishi *et al.* 1996; Jaworski and Brückner 2000; Degenkolb *et al.* 2007). Thus, this study is the first to report a *C. rosea* strain with antifungal activity against plant pathogens whose activity has been attributed to peptaibiotics. The purification and identification of the metabolites could improve our understanding of the mechanism involved in this system.

Important advances have been made in recent years on the biosynthesis, biological activity, and structural determination of peptaibols produced by *Trichoderma* spp. (Daniel and Rodrigues Filho 2007). Recently, these types of compounds produced by *Trichoderma virens* have been shown to induce a systemic response (Viterbo *et al.* 2007).

In the greenhouse assays, under the conditions indicated, *C. rosea* prevented mortality of soybean plants caused by *S. sclerotiorum* and partially reduced mortality caused by the pathogen in the lettuce plants. Previous studies have found that *C. rosea* is an adequate competitor in the rhizosphere (Roberti *et al.* 2001; Bennett and Whipps 2008). As *C. rosea* inocula were applied before the pathogen in sterile soil, this facilitated its establishment and probably reduced *S. sclerotiorum* damage by competence.

In addition, our assays showed that *C. rosea* BAFC3874 caused positive effects on lettuce plants and resulted in significant increases in shoot dry weights. Harman *et al.* (2004) also showed that *Trichoderma harzianum*, using similar conditions in sterile soil, promoted growth of maize plants, suggesting a direct mechanism through plant response. Micro-organisms and their positive interaction with plants may involve a molecular recognition system, such as the well-reported interaction of *Trichoderma* species with plants or symbiotic mycorrhizal association, which promote plant growth (Altomare *et al.* 1999; Yedidia *et al.* 2001; Roberti *et al.* 2008).

Also, it is important to highlight that *C. rosea* BAFC3874 was able to inhibit other root pathogens such as *R. solani*, *F. solani* and *M. phaseolina* and the shoot pathogen *C. truncatum*, suggesting the high potential of this strain as an active biocontrol agent. To assess the full potential of *C. rosea*, it is necessary to do experiments in the field.

Clearly, no single mechanism can explain the effect of a potential biocontrol fungus. In fact, fungi also have the ability to ameliorate a wide range of abiotic and biotic stresses, particularly, by enhancing nutrient uptake in plants (Shoresh *et al.* 2010). These abilities may thus be more important to agriculture than disease control (Whipps 2001).

In summary, *C. rosea* protected *S. sclerotiorum*-infected crops through the production of novel peptaibiotic metabolites that have antifungal activity. A plant-induced defence response in the host that involves peptaibiotic production should not be dismissed.

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References

Adams, P.B. and Ayers, W.A. (1979) Ecology of *Sclerotinia* species. *Phytopathology* **69**, 896–899.

Altomare, C., Norvell, W.A., Björkman, T. and Harman, G.E. (1999) Solubilization of phosphates and micronutrients by the plant-growth promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295–22. *Appl Environ Microbiol* **65**, 2926–2933.

Aryantha, I.N.P. and Guest, D.I. (2006) Mycoparasitic and antagonistic inhibition on *Phytophthora cinnamomi* rands by microbial agents isolated from manure compost. *Plant Pathol J* **5**, 291–298.

Barakat, R.M., Al-Mahareeq, F. and AL-Masri, M. (2006) Biological control of *Sclerotium rolfsii* by using indigenous *Trichoderma* spp. isolates from Palestine. *Hebron Univ Res J* **2**, 27–47.

Bennett, A.J. and Whipps, J.M. (2008) Beneficial microorganism survival on seed, roots and in rhizosphere soil following application to seed during drum priming. *Biol Control* **44**, 349–361.

Boland, G.J. and Hall, R. (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Can J Plant Pathol* **16**, 93–108.

Butler, M.J., Day, A.W., Henson, J.M. and Money, N.P. (2001) Pathogenic properties of fungal melanins. *Mycologia* **93**, 1–8.

Butt, T.M., Jackson, C. and Magan, M. (2001) Introduction-fungal biological control agents: progress, problems and potential. In *Fungi as Biocontrol Agents: Progress, Problems and Potential* ed. Butt, T., Jackson, C. and Magan, N. pp. 1–8. Southampton and Cranfield, UK: University of Wales.

Chikanishi, T., Hasumi, K., Harada, T., Kawasaki, N. and Endo, A. (1996) Clonostachin, a novel peptaibol that inhibits platelet aggregation. *J Antibiot* **50**, 105–110.

Daniel, J.F. and Rodrigues Filho, E. (2007) Peptaibols of *Trichoderma*. *Nat Prod Rep* **24**, 1128–1141.

Degenkolb, T., Gräfenhan, T., Nirenberg, H.I., Gams, W. and Brückner, H. (2006) *Trichoderma brevicompactum* complex: rich source of novel and recurrent plant-protective polypeptide antibiotics (peptaibiotics). *J Agric Food Chem* **54**, 7047–7061.

Degenkolb, T., Kirschbaum, J. and Brückner, N. (2007) New sequences, constituents, and producers of peptaibiotics: an updated review. *Chem Biodivers* **4**, 1052–1067.

Dennis, C. and Webster, J. (1971a) Antagonistic properties of species-groups of *Trichoderma* I. Production of non-volatile antibiotics. *Mycol Res* **57**, 25–39.

Dennis, C. and Webster, J. (1971b) Antagonistic properties of species-groups of *Trichoderma*: II. Production of volatile antibiotics. *Mycol Res* **57**, 41–48.

Domsch, K.H., Gams, W. and Anderson, T.H. (1980) *Compendium of Soil Fungi*, vol. **1**. London, UK: Academic Press.

Ervio, L.R., Halkilahti, A.M. and Pohjakallio, O. (1994) The survival in soil of *Sclerotinia* species and their ability to form mycelia. *Adv Front Plant Sci* **8**, 121–133.

Fuhrmann, J.J. (1994) Isolation of microorganisms producing antibiotics. In *Methods of Soil Analysis. Part 2. Microbiological and Biochemical Properties*. Soil Science Society of America Book Series ed. Weaver, R.W., Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A. and Wollum, A. pp. 379–403. Madison, WI, USA: Soil Science Society of America; pp: 1121.

Gromadzka, K., Chelkowski, J., Popiel, D., Kachlicki, P., Kostecki, M. and Golinski, P. (2009) Solid substrate bioassay to evaluate the effect of *Trichoderma* and *Clonostachys* on the production of zearalenone by *Fusarium* species. *World Mycotoxin J* **2**, 45–52.

Hadacek, F. and Greger, H. (2000) Testing of antifungal natural products: methodologies, comparability of results and assay choice. *Phytochem Anal* **11**, 137–147.

Harish, S., Manjula, K. and Podile, A.R. (1998) *Fusarium udum* is resistant to the mycolytic activity of a biocontrol strain of *Bacillus subtilis* AF 1. *FEMS Microbiol Ecol* **25**, 385–390.

Harman, G., Petzoldt, R., Comis, A. and Jie Chen, J. (2004) Interactions between *Trichoderma harzianum* strain T22 and maize inbred line Mo17 and effects of these interactions on diseases caused by *Pythium ultimum* and *Colletotrichum graminicola*. *Phytopathology* **94**, 147–153.

Hermosa, M.R., Grondona, I., Iturriaga, E.A., Diaz-Minguez, J.M., Castro, C., Monte, E. and Garcia-Acha, I. (2000) Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. *Appl Environ Microbiol* **66**, 1890–1898.

Innocenti, G., Roberti, R., Montanari, M. and Zakrisson, E. (2003) Efficacy of microorganisms antagonistic to *Rhizoctonia cerealis* and their cell wall degrading enzymatic activities. *Mycol Res* **107**, 421–427.

Jackson, A.M., Whipps, J.M. and Lynch, J.M. (1991) *In vitro* screening for the identification of potential biocontrol agents of *Allium* white rot. *Mycol Res* **95**, 430–434.

Jaworski, A. and Brückner, H. (2000) New sequences and new fungal producers of peptaibol antibiotics antiamoebins. *J Pept Sci* **6**, 149–167.

Keinath, A.P., Fravel, D.R. and Papavizas, G.C. (1991) Potential of *Gliocladium roseum* for biocontrol of *Verticillium dahliae*. *Phytopathology* **81**, 644–648.

Knudsen, I.M.B., Hockenhull, J. and Jensen, D.F. (1995) Biocontrol of seedling diseases of barley and wheat caused by *Fusarium culmorum* and *Bipolaris sorokiniana*: effects of selected fungal antagonists on growth and yield components. *Plant Pathol* **44**, 467–477.

Kohn, L.M. (1979) Delimitation of the economically important plant pathogenic *Sclerotinia* species. *Phytopathology* **69**, 873–886.

Le Tourneau, D. (1979) Morphology, cytology and physiology of *Sclerotinia* species in culture. *Phytopathology* **69**, 887–890.

Mejía, L.C., Rojas, E.I., Maynard, Z., Van Bael, S., Arnold, A.E., Hebbar, P., Samuels, G.J., Robbins, N. *et al.* (2008) Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. *Biol Control* **46**, 4–14.

Melgarejo, P., Carrillo, R. and Sagasta, E.M. (1985) Mycoflora of peach twigs and flowers and its possible significance in biological control of *Monilia laxa*. *Mycol Res* **85**, 313–317.

Nobre, S.A.M., Maffia, L.A., Mizubuti, E.S.G., Cota, L.V. and Dias, A.P.S. (2005) Selection of *Clonostachys rosea* isolates from Brazilian ecosystems effective in controlling *Botrytis cinerea*. *Biol Control* **34**, 132–143.

Nosanchuk, J.D. and Casadevall, A. (2003) The contribution of melanin to microbial pathogenesis. *Cell Microbiol* **5**, 203–223.

Roberti, R., De Vero, L., Pisiand, A. and Cesari, A. (2001) Biological control of wheat foot rot by antagonistic fungi and their modes of action. *IOBC/WPRS Bull* **24**, 13–16.

Roberti, R., Veronesi, A.R., Cesari, A., Cascote, A., Di Berardino, I., Bertini., L. and Caruso, C. (2008) Induction of PR proteins and resistance by the biocontrol agent *Clonostachys rosea* in wheat plants infected with *Fusarium culmorum*. *Plant Sci* **175**, 339–347.

Rodríguez, M.A. (2004) *Hongos del suelo antagonistas de Sclerotinia sclerotiorum. Selección y estudio de potenciales agentes de biocontrol*. Tesis Doctoral. Buenos Aires, Argentina: Departamento de Biodiversidad y Biología Experimental, FCEyN, UBA.

Schirmböck, M., Lorito, M., Wang, Y.L., Hayes, C.K., Arisan-Atac, C., Scala, F., Harman, G.E. and Kubicek, C.P. (1994) Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Appl Environ Microbiol* **60**, 4364–4370.

Schroers, H.J., Samuels, G.J., Seifert, K.A. and Gams, W. (1999) Classification of the mycoparasite *Gliocladium roseum* in *Clonostachys* as *C. rosea*, its relationship to *Bionectria ochroleuca*, and notes on other *Gliocladium*-like fungi. *Mycologia* **91**, 365–385.

Shoresh, M., Harman, G.E. and Mastouri, F. (2010) Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu Rev Phytopathol* **48**, 1–23.

Singh, S.-B., Herath, K., Guan, Z., Zink, D.L., Dombrowski, D.A., Polishook, J.D., Silverman, K.C., Lingham, R.B. *et al.* (2002) Integramides A and B, Two novel non-nibosomal linear peptides containing nine Cr-Methyl amino acids produced by fungal fermentations that are inhibitors of HIV-1 integrase. *Org Lett* **4**, 1431–1434.

Sutton, J.C., Li, D.W., Peng, G., Yu, H., Zhang, P. and Valdebeneito-Sanhueza, R.M. (1997) *Gliocladium roseum*: a versatile adversary of *Botrytis cinerea* in crops. *Plant Dis* **81**, 316–328.

Vey, A., Hoag, I.R.E. and Butt, T.M. (2001) Toxic metabolites of fungal biocontrol agents. In *Fungi as Biocontrol Agents: Progress, Problems and Potential* ed. Butt, T.M., Jackson, C. and Magan, N. pp. 311. Bristol, USA: CAB International Publishing.

Viterbo, A., Wiest, A., Brotman, Y., Chet, I. and Kenerley, Ch. (2007) The 18mer peptaibols from *Trichoderma virens* elicit plant defence responses. *Mol Plant Pathol* **8**, 737–746.

Whipps, J.M. (1987) Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. *New Phytol* **107**, 127–142.

Whipps, J.M. (2001) Microbial interactions and biocontrol in the rhizosphere. *J Exp Bot* **52**, 487–511.

Whipps, J.M. and Magan, N. (1986) Effects of nutrient status and water potential of media on fungal growth and antagonist–pathogen interactions. *EPPO Bulletin* **17**, 581–591.

Yedidia, I., Srivastva, A.K., Kapulnik, Y. and Chet, I. (2001) Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant Soil* **235**, 235–242.

Zafari, D., Koushki, M.M. and Bazgir, E. (2008) Biocontrol evaluation of wheat take-all disease by *Trichoderma* screened isolates. *Afr J Biotechnol* **7**, 3653–3659.

Zahir, Z.A., Arshad, M. and Frankenberger, W.T. (2004) Plant growth promoting rhizobacteria: applications and perspectives in agriculture. *Adv Agron* **81**, 97–168.

Zazzerini, A. and Tosi, L. (1985) Antagonistic activity of fungi isolated from sclerotia of *Sclerotinia sclerotiorum*. *Plant Pathol* **34**, 415–421.