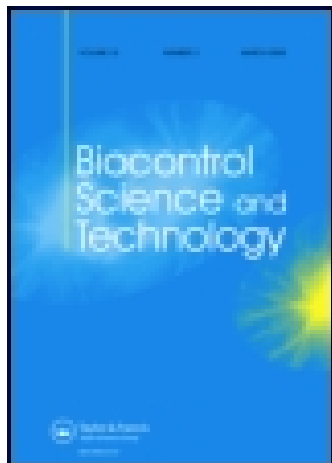


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Suppressive soil against *Sclerotinia sclerotiorum* as a source of potential biocontrol agents: selection and evaluation of *Clonostachys rosea* BAF1646

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Suppressive soil against *Sclerotinia sclerotiorum* as a source of potential biocontrol agents: selection and evaluation of *Clonostachys rosea* BAFC1646

The fungal diversity structures of soils that are suppressive and non-suppressive to *Sclerotinia sclerotiorum* were characterized and screened for fungal strains antagonistic to the *S. sclerotiorum* pathogen. Soil suppressiveness was associated with a particular fungal diversity structure. Principal component analysis (PCA) showed that antagonism by fungal species in suppressive soils was associated with the occurrence of *Fusarium oxysporum*, *F. solani*, *Talaromyces flavus* var. *flavus*, and *Clonostachys rosea* f. *rosea*. In particular, *C. rosea* f. *rosea* occurred exclusively in suppressive soil samples, suggesting that this morpho-species plays an important role in suppression of *S. sclerotiorum* diseases. One strain of *C. rosea* f. *rosea* (BAFC1646) was selected for further experiments. Dual-culture assays confirmed the antagonistic behaviour of *C. rosea* f. *rosea* BAFC1646 against three different *S. sclerotiorum* strains. Antifungal activity was corroborated by diffusion assays with metabolite extracts. Greenhouse assays with soybean plants showed that the selected *C. rosea* f. *rosea* strain reduced the percentage of dead plants when co-inoculated with *S. sclerotiorum*. In addition, inclusion of *C. rosea* f. *rosea* alone increased shoot lengths significantly. In this work, we established the involvement of fungal species in soil suppressiveness and in further assays confirmed that *C. rosea* f. *rosea* BAFC1646 exhibits a bioprotective effect against *S. sclerotiorum* in soybean plants.

Keywords: biological control; *Sclerotinia sclerotiorum*; antagonism; *Clonostachys rosea*

Introduction

The fungal plant pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary affects many economically important crops worldwide (Boland & Hall, 1994). This fungus survives in soil in the form of sclerotia, which germinate myceliogenically or carpogenically depending on environmental conditions (Bardin & Huang, 2001). Recurrent fungicide application has led to fungicide resistance in *S. sclerotiorum* populations (Gossen, Rimmer, & Holley, 2001; Kuang, Hou, Wang, & Zhou, 2011). Consequently, the approach of biological control has become an attractive potential means of reducing the incidence of Sclerotinia diseases (e.g. Sclerotinia wilt and stem rot). Biocontrol involves selection and evaluation of microorganisms that can promote plant growth or reduce infection by phytopathogens (Weller, Raaijmakers, Gardener, & Thomashow, 2002).

Biocontrol has become an area of primary interest in plant pathology research in recent last years (Butt, Jackson, & Magan, 2001). If microorganisms are introduced to help control soilborne fungal plant pathogens, it should be done as part of an integrated pest management plan that is both efficient and environmentally friendly (Garbeva, Hol, Termorshuizen, Kowalchuk, & De Boer, 2011). In this context, suppressive soils represent a promising source for isolating new biological control agents (Garbeva et al., 2011).

Suppressiveness of soilborne plant pathogens has been reported worldwide and suppressive levels can be attributed to the microbial community composition of particular soils (Borneman & Becker, 2007; de Boer, Verheggen, Klein Gunnewiek, Kowalchuk, & van Veen, 2003). All soils have some level of suppressive activity, which can be modified by management practices. Two types of suppressiveness have been defined: general suppressiveness, which owes its activity to the total soil microbial community and is not transferable between soils, and specific suppressiveness, which owes its activity to the effects of individual or selected groups of microorganisms and is transferable between soils (Weller et al., 2002). For most suppressive soils, however, the consortia of microorganisms and mechanisms involved in pathogen control are unknown. Studies have shown that plant roots can exploit microbial consortia from the soil for

protection against disease (Mendes et al., 2011). Soilborne plant diseases could be attenuated through the management of resident soil microbial antagonists. The identification of fungal species that favor disease suppression has led to the development of biocontrol agents for suppression of soilborne plant pathogens.

The aims of this study were threefold. Firstly, we compared the fungal species diversity structures of suppressive and non-suppressive soybean soil samples, employing a morphological species concept (Kirk, Cannon, David, & Stalpers, 2001). That is, we used morphological characters to identify and differentiate taxa among the fungal species observed. Second, we isolated and cultured potential biocontrol agents that were differentially represented in suppressive versus non-suppressive samples. Finally, we evaluated a selected potential agent for its capacity to reduce *S. sclerotiorum* infection, with the long-term goal improving management of diseases caused by *S. sclerotiorum*.

Materials and Methods

Sampling site

Fungal strains were isolated from an argiudol vertic soil obtained from a *Glycine max* (L.) Merrill field (Salto, Argentina) with parcels of Sclerotinia-suppressive and non-suppressive soils. Soybean fields with dead or wilted plants showing signs of *S. sclerotiorum* infestation were found in non-suppressive soils in parcels that were intercalated with similar sized disease-free parcels (~100 m²). Mycelium of the pathogen was observed on the roots and stems of plants in affected fields. In non-suppressive soils, no evidence of apothecia was observed, but myceliogenic germination was detected. Three soil samples were collected from suppressive soils parcels and two from non-suppressive soils parcels. The climate of the region is temperate, with an annual precipitation of ~900 mm and an annual average temperature of about 17 °C (Gómez, 2008).

Isolation and identification of fungi

Active saprotrophic fungal strains were isolated from suppressive and non-suppressive soil parcels by a simple soil-particle washing method wherein fungal hyphae are isolated from soil samples in an automated washing machine. Each soil sample was placed in a separate sterile box containing a 0.2-mm sieve. They were washed vigorously for about 2 min and in 35 steps with sterile water. The washing action was achieved by passing sterile air through the system. This method removes nearly all fungal spores from soil particles (Parkinson, 1994).

Soil particles were cultured on malt extract agar (MEA) with streptomycin (0.5% v/v) and chlorotetracycline (0.25% v/v) at 25 °C in the dark. Most of the isolated fungi were identified based on culture characteristics and spore morphology (Domsch, Gams, & Anderson, 1980). *Sclerotinia sclerotiorum* strain BAFC225 (Buenos Aires Fungal Collection, Universidad de Buenos Aires) isolated from a sclerotium found in a non-suppressive soil sample was used for antagonism and biocontrol evaluation assays.

Characterization of fungal diversity structure

The diversity of culturable and morphologically identifiable species in the two soil types studied was characterized according to the following parameters:

Fr (frequency of occurrence of each species) = no. species occurrences \times 100 / total no. inoculated soil particles

Ar (relative abundance of each species) = no. species isolates \times 100 / total no. isolates obtained

SR (species richness) = no. species found at each site \times 100 / total no. inoculated soil particles

The Shannon-Wiener Diversity Index (H) was used to calculate the diversity of filamentous fungi in each site as follows (Krebs, 1994):

$$H = -\sum_{i=1}^s p_i \times \ln p_i$$

where s is the number of isolates of the i^{th} species and p_i is the proportion of the total sample belonging to the i^{th} species. This function incorporates two components of species diversity: the number of species and the proportion of individuals of each species (Donnison, Griffith, Hedger, Hobbs & Bardgett, 2000). The obtained frequencies of occurrence of each species (Fr) were used to examine trends in species diversity structure in the two soils by principal component analysis (PCA), a classical method of data analysis for synthesis of information (Kenkel & Booth, 1992).

Characterization and selection of antagonistic soil fungi

Three different *in vitro* assay methods were used to evaluate the antagonistic ability of each fungal isolate. Their effects on pathogen growth and sclerotia formation in dual culture and volatile and non-volatile metabolite production were evaluated in 90-mm Petri dishes at 25 °C in the dark, as detailed below:

Dual culture

All of the soil fungal isolates obtained were tested against the pathogen *S. sclerotiorum* BAFC225 on MEA in a dual culture (Whipps, 1987). For each confrontation, two plugs (diameter, 4 mm) were used, one from the potential antagonist (i.e. the target isolate) and the other from *S. sclerotiorum*. The plugs, excised from the edge of an actively growing MEA culture of each colony, were planted 4.5 cm apart in 90-mm-diameter Petri dishes (Whipps, 1987) as shown in Figure 1. Each dual culture plate was grown in parallel with two controls plates: a pathogen alone plate and a target isolate alone plate.

The interaction observed for each isolate was characterized according to the following four parameters: type of interaction (types [TIs] defined in Table 1), index of dual culture growth inhibition, effect on *S. sclerotiorum* sclerotia formation, and inhibition halo width (Ih) (Whipps, 1987). Production of sclerotia was determined in four colony zones after 6 days and after 13 days in dual culture (Figure 1). To assess effects on sclerotium formation capacity, the number of sclerotia

encountered in dual culture was compared with a dual culture system based only on BAFC225 (Jackson, Whipps, & Lynch, 1991). Each condition was examined in triplicate for each fungal isolate evaluated. The parameters considered in the dual cultures are summarized in Table 2.

Production of non-volatile metabolites

Plugs (4 mm diameter) retrieved from isolate colonies were placed on a 6 cm diameter cellophane membrane (Sterlitech) in MEA-containing Petri dishes. After 3 days, the membrane and the fungus were removed. Then, a single *S. sclerotiorum* BAFC225 colony plug (4 mm diameter) was placed in the center of each MEA plate. The control treatment consisted of the same steps but without isolate inoculation of the cellophane membrane, as described by Whipps, 1987. Incubation was carried out at 25 °C; *S. sclerotiorum* colony diameters were measured periodically. Growth inhibition was measured by the Index of Growth Inhibition in the cellophane membrane assay (IGIc). It was calculated as the diameter of the pathogen colony on medium with membranes pre-inoculated with a potential antagonist compared with the diameter of the control colony (Table 2). IGIc values were determined on the day that the pathogen reached the Petri dish edge in the control treatment. Each treatment condition was examined in triplicate for each fungal isolate evaluated. To confirm that the fungal isolates did not penetrate the membrane, the experiment included a control condition in which a membrane was treated with the antagonist, with subsequent removal of the membrane, but without *S. sclerotiorum* inoculation.

Production of volatile metabolites

Modifications of compartmentalized cultures were used to establish each fungal isolate's capacity to produce inhibitory volatile metabolites (Dennis & Webster 1971). Two MEA-containing Petri dishes (90-mm diameter) were employed: one was inoculated with a pathogen plug and the other with the isolate being evaluated. Then, the bottoms of the two Petri dishes were joined and sealed hermetically with Parafilm®. In the control condition, only the pathogen plug was inoculated. The

incubation was performed at 25 °C and the colony diameter was recorded periodically. Growth inhibition was evaluated through the Index of Growth Inhibition in the volatile metabolites assay (IGIV), with the IGIV value being determined when the control colony reached the Petri dish edge (Table 2). The experiment included three replicates of each of the evaluated isolates.

Analysis of antagonism

The antagonistic capacity of each soil was evaluated through the behavior of the isolates obtained from each one and analyzed using a matrix of antagonism and PCA. The parameters considered in the matrix of antagonism were the aforementioned parameters for each *in vitro* assay (summarized in Table 2). Different isolates of the same species in each soil sample were grouped as a function of their morphotype and their antagonistic behavior and were considered to be members of the same taxon (strain) in the PCA. Also percentage (%) of antagonistic isolates was determined for each soil as:

$$\% \text{ Ant (percentage of antagonistic isolates)} = \frac{\text{number of antagonistic isolates of a species} \times 100}{\text{Total isolates evaluated.}}$$

Selection of an antagonistic strain

The PCA results (based on frequency of occurrence of each species and antagonistic behavior) were then used to associate observed soil suppressiveness with particular fungal taxa and to select the most promising antagonistic strains. The species whose presence and antagonistic activity showed the best separation between suppressive and non-suppressive soils were selected for further evaluation in subsequent anti-*S. sclerotiorum* antagonism assays.

Identification and characterization of the selected antagonistic strain

Identification

A highly promising strain of fungus was identified with the aid of DNA barcodes in polymerase chain reaction (PCR) experiments, and it was incorporated in the Buenos Aires Fungal Collection (Universidad de Buenos Aires) as BAFC1646. The strain was cultured on 20% (w/v) malt extract broth (MEB) at 25 °C for 1 week. Genomic DNA was extracted from the harvested mycelium (\approx 80 mg dried weight) using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, CA). The rDNA ITS region was amplified by PCR with specific primers, namely ITS1 and ITS4 (White, Bruns, Lee, & Taylor, 1990). PCR amplification was performed in a 50- μ l volume of a mixture containing 1 μ l of 10 mM dNTP, 10 μ l of 10 \times Iproof buffer, 0.5 μ l of 100 μ M primers, 0.5 μ l of 1.5 U iProof™ High Fidelity DNA Polymerase (BIO-RAD Laboratories, Hercules, CA), and 2 μ l of genomic DNA. The thermal cycler parameters were programmed for 1 cycle of denaturation at 98 °C for 3 min, 35 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 30 s, extension at 72 °C for 20 s, and a final extension at 72 °C for 10 min. The PCR-amplified products were purified with an UltraClean® PCR Clean-up DNA Purification Kit (MO BIO) and sequenced by our institutional genotyping service (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) with the ITS1 and ITS4 primers. Sanger sequencing was performed on an ABI 3130XL 16-capillary sequencer using big dye 3.1 sequencing chemistry. Taxon affinities were confirmed by BLAST analysis using Sequencher software (Altschul et al., 1997).

Antagonistic strain characterization

P-solubilization. A 4-mm diameter mycelial inoculum plug of *C. rosea* f. *rosea* BAFC1646 was used to inoculate Petri dishes containing NBRIP (National Botanical Research Institute Phosphate's growth) medium consisting of (L^{-1}): glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g; $(NH_4)_2SO_4$, 0.1 g; and agar, 15 g. (Nautiyal, 1999). The inoculated samples were incubated at 25 °C in the dark. The appearance of a clear zone around the colony after

5 days was considered a positive sign of P-solubilization ability. The experiment included three replicates.

Hydrolytic enzyme production. Enzyme activities were measured qualitatively by means of the halo generated by the degradative activity of the produced enzymes. The enzyme activities evaluated included cellulolytic, xylanolytic, pectinolytic, amylolytic, lipolytic, and proteolytic activities. The culture media used included carboxymethylcellulose (CMC-Sigma, St. Louis, MO), oat xylan (Sigma, St. Louis, MO), apple pectin (Sigma, St. Louis, MO), soluble starch (Sigma, St. Louis, MO), Tween20 (Sorbitan Monolaurate), and gelatin as substrates. Cellulases and xylanases were revealed with Congo Red dye (Pointing, 1999), pectinases with Ruthenium Red (Hankin & Anagnostakis, 1977), and amylases with I₂-KI (Gessner, 1980). Lipases were detected by the presence of a precipitate around the fungal colonies caused by the formation of lauric acid calcium salt crystals (Abdel-Raheem & Shearer, 2002). Proteolytic activity was evidenced by visualization of a precipitate, which results in a more opaque agar and an enhanced clear zone around the colonies (Hankin & Anagnostakis, 1977). Three replicates were used for each enzyme assay.

IAA production. BAFC1646 was inoculated in a slightly acidic (pH 6.1) broth containing (L⁻¹): glucose, 2.5 g; sodium succinate 2.5 g; K₂HPO₄ 6 g; KH₂PO₄ 4 g; KOH 2,1 g; NH₄Cl 1 g; MgSO₄·7H₂O 0.2 g; NaCl 0.1 g; CaCl₂·2H₂O 0.02 g; FeCl₃ 0.01 g; and Na₂MoO₄·2H₂O 0.002 g. The samples were supplemented with tryptophan (100 mg ml⁻¹) and incubated with shaking for 7 days at 25 °C (Fuentes- Ramírez, Jiménez-Salgado, Abarca-Ocampo, & Caballero-Mellado, 1993). Three replicates were used. Mycelia were filtered and the exudates present in the medium were used to detect production of indole-3-acetic acid (IAA) with Salkowski's chromogenic reagent (Ehmann, 1977). Absorbance was measured at 530 nm and compared with a standard curve of commercially available IAA (Merck).

Antagonism of C. rosea f. rosea BAFC1646

In vitro assays

The antagonistic behavior of *C. rosea f. rosea* strain BAFC1646 was evaluated against three *S. sclerotiorum* strains (BAFC225, BAFC2232, and BAFC217) in dual culture assays on MEA and potato dextrose agar (PDA) (Whipps, 1987). A 4-mm colony plug was used for BAFC1646 inoculation. After 2 days, the *S. sclerotiorum* strain colony plugs were inoculated at a distance of 4.5 cm (Whipps, 1987). Three replicates were used for each confrontation. Control dishes were inoculated only with the pathogen strain in each of the media being assessed. All cultures were incubated at 25 °C in the dark. The width of the inhibition halo was determined and the percentage of radial growth inhibition (%RGI) was calculated as:

$$\%RGI = (rc - rd) (rc)^{-1} \times 100$$

where rc is the radius of the control *S. sclerotiorum* colony and rd is the radius of *S. sclerotiorum* in a dual culture colony (Whipps, 1987).

Antifungal activity

MEB (100 ml) was inoculated with a 4-mm colony plug excised from the edge of an actively growing MEA culture of BAFC1646. After 7 days of incubation, the culture (mycelium plus spores) was used to inoculate 1 L of MEB (in a 4-L Erlenmeyer flask). Incubation was performed at 25 °C for 21 days (stationary growth phase) under static conditions. This procedure was done in duplicate. Amberlite XAD-16 (150 g L⁻¹) was then added to 2 L of filtered broth. The suspension was filtered 18 h later. The Amberlite was washed with distilled water and then eluted with MeOH (2 L). The MeOH eluate was evaporated to dryness and subjected to vacuum chromatography on RP-C18 using water and mixtures of water and MeOH of decreasing polarity (90:10 H₂O:MeOH; 80:20 H₂O:MeOH; 70:30 H₂O:MeOH; 60:40 H₂O:MeOH; 50:50 H₂O:MeOH; 40:60 H₂O:MeOH; 30:70 H₂O:MeOH; 20:80 H₂O:MeOH; 10:90 H₂O:MeOH; 100% MeOH). Each fraction was evaluated by antimicrobial diffusion assays against *S. sclerotiorum* BAFC225 colonies (Hadacek &

Greger, 2000). Dried extract samples (100 µg) were used to impregnate filter paper discs (4 mm diameter) placed on MEA in the center of a Petri dish confronting four *S. sclerotiorum* colonies at 25°C in the dark. The pathogen was inoculated at four equidistant locations as 4-mm-diameter colony plugs excised from the edge of an actively growing MEA culture. The distance between the impregnated filter papers and the colony plugs was 2.5 cm. Filter paper with each solvent mixture, but without organic extract, was employed in control treatments. Each mixture and control sample was evaluated in triplicate. The %RGI was evaluated after 4 days and observed over a period of 10 days.

Bioprotective capacity of BAFC1646 in soybean plants

A glass-house experiment was conducted using *G. max* (soybean). Pathogen infection through mycelium and myceliogenic germination of the sclerotia was employed to simulate the type of infection observed in the field (in non-suppressive soil parcels). Soybean seedlings were sown in 200 ml of steam-pasteurized soil inoculated with the antagonistic strain added at a concentration of 1.2×10^6 colony-forming units per gram of soil (cfu g⁻¹; determined by the method of soil dilution plate). The antagonist inoculum was incorporated into the soil as a mass of boiled and autoclaved rice previously inoculated with mycelium plugs to full colonization (100 g substrate/10 days). The amount (i.e. mass) of this substrate was incorporated to reach the above mentioned concentration (1.2×10^6 cfu per g of soil). The seedlings were planted in 200-ml plastic pots (one seedling per pot). After 3 days, the plants and soil were transferred to 600-ml pots by breakage of the 200-ml pots and the addition of 300 ml of soil colonized by *S. sclerotiorum* BAFC225 containing mycelia and sclerotia at the bottom and around the sides of the pot (final concentration: 15%, w/v of pathogen inoculum).

The pathogen-infected soil was prepared in sterile polypropylene bags containing rice:bran:water (20:20:100; v/v/v) as substrate, and was inoculated with 5-mm pathogen plugs (six per 350 g of substrate) and then incubated in the dark for 20 days at 24–28 °C (Rodríguez, Cabrera,

Gozzo, Eberlin, & Godeas, 2011). A completely randomized design was employed. Four treatments with five replicates each were used: *S. sclerotiorum* only, *C. rosea* f. *rosea* only, *S. sclerotiorum* + *C. rosea* f. *rosea* together, and control without any fungus inoculated (i.e. plants received only substrate). Four replicates per treatment were used in the assay repetition. The percentage of alive plants, shoot lengths, and the dry weights of roots and shoots were assessed. Harvested plants were dried in an oven at 80 °C until at a constant weight.

Results

Identification and characterization of the fungal diversity structure

A total of 146 isolates belonging to 30 different morpho-species were isolated from suppressive and non-suppressive soybean soils (Table 3). *Fusarium oxysporum*, *Clonostachys rosea* f. *catenulata*, *Humicola grisea*, *Talaromyces helicus* var. *helicus*, *Trichoderma harzianum*, and *Trichoderma koningii* were common in both soils.

The five species with the highest frequency of occurrence in suppressive soils were *F. oxysporum*, *H. grisea*, *T. harzianum*, *T. koningii*, and *C. rosea* f. *rosea*, with the latter species being recovered exclusively from suppressive soil. *Trichoderma koningii*, *F. oxysporum*, *H. grisea*, and *Phoma exigua* were the most frequent species found in non-suppressive soils. Among the species found to occur exclusively in non-suppressive soils, *Trichoderma viride* and *P. exigua* were noteworthy for their high frequency. Greater SR and H values were observed for non-suppressive soils (SR = 0.33 and H = 3.77) than for suppressive soils (SR = 0.24 and H = 2.38), indicating that there was greater species richness and diversity (i.e. according to the Shannon-Wiener index) in the non-suppressive soils.

PCA based on frequency of occurrence (Fr) revealed distinctive patterns differentiating samples from suppressive versus non-suppressive soils. As shown in Figure 2a, there was separation along the ordination axes as a function of components 1 and 5, which showed the best separation between samples. Component 1 segregated suppressive soil samples due to the presence of *Fusarium solani*, *F. dimerum*, and *F. equiseti*, whereas component 5 segregated suppressive soil

samples due to the presence of *C. rosea* f. *rosea*, *Trichoderma harzianum*, and *Fusarium semitectum*. Discriminant analysis showed that 92% of the samples were well grouped.

Characterization and selection of antagonistic fungal strains

It was determined that 71% of the isolates from suppressive soil samples were antagonistic against *S. sclerotiorum*. Among the isolates with antagonistic effects, 82% showed evidence of volatile or non-volatile metabolites inhibiting the pathogen. Antagonistic isolates exhibited TI5 and TI3b types of interaction (Table 1) or another TI combined with observed growth inhibition in antifungal metabolite assays. Antagonism against *S. sclerotiorum* was observed for 100% of the *F. oxysporum*, *T. harzianum*, and *C. rosea* f. *rosea* isolates, which represent three of the five species most frequently isolated from suppressive soils.

Antagonistic activity against *S. sclerotiorum* was observed in only 51% of strains isolated from non-suppressive soils. Isolates of *T. koningii*, *F. oxysporum*, and *H. grisea*—the species most frequently isolated from non-suppressive soils—exhibited antagonistic activity in 69%, 71%, and 0% of trials, respectively (Table 3). About three quarters (76%) of the antagonistic isolates from non-suppressive soils showed growth inhibition indicative of the presence of inhibitory metabolites. That is, they exhibited inhibition types TI5 or TI3b, or another TI combined with observed growth inhibition in antifungal metabolite assays (see Table 1).

The PCA of antagonistic behavior showed that the suppressive and non-suppressive soil samples were best segregated by representation of component 3 in the functions of components 4 and 5 (Figure 2b and c). Component 4 segregated the samples mainly according to the antagonism of *F. oxysporum* (strains 1 and 5) and *F. solani*, whereas component 5 segregated suppressive soil samples characterized mainly by the antagonistic behavior of *T. flavus* var. *flavus* and *C. rosea* f. *rosea* (strain 2).

Identification and characterization of C. rosea BAFC1646

The strain BAFC1646 was identified as being *C. rosea* f. *rosea* based on its molecular barcode and morphological characteristics. Alignment of the generated sequence in Sequencher software and comparisons with deposited sequences in BLAST searches indicated that the ITS sequenced from the strain (Query coverage 97%) was 99% similar to AF358235 from strain CBS 710.86, the ex-neotype strain of *C. rosea* (Schroers, et al., 1999; Schroers, 2001). The GenBank accession number of the newly generated nucleotide sequence is KF765504.

The BAFC1646 strain did not show P-solubilization ability as evidenced by the absence of halos in NBRIP medium. Enzyme activity assays indicated that this fungal strain produces cellulases, amylases, lipases, and xylanses, but not pectinases, and no evidence of proteolytic activity was observed. Production of IAA was detected.

Antagonism of C. rosea f. rosea BAFC1646

In vitro assays

All three *S. sclerotiorum* strains tested were inhibited by *C. rosea* f. *rosea* BAFC1646 (Figure 3a and b). Inhibition halos were observed with all three *S. sclerotiorum* strains on MEA and against *S. sclerotiorum* strain BAFC225 on PDA (classified as TI3b, Table 1). The antagonistic *C. rosea* f. *rosea* strain BAFC1646 stopped growth of *S. sclerotiorum* strains BAFC2232 and BAFC217 on PDA upon colony contact with a width ≤ 2 mm (classified as TI2b, Table 1). As shown in Table 4, inhibition of *S. sclerotiorum* radial growth was more effective on PDA (%RGI range 47~54%) than on MEA (43~50%) in dual culture assays with all three pathogen strains. On MEA, BAFC225 exhibited significantly more growth inhibition than the other two strains. Meanwhile, on PDA, both BAFC225 and BAFC2232 exhibited significantly more growth inhibition than BAFC217 (Table 4). *C. rosea* f. *rosea* BAFC1646 produced inhibition halos with all three *S. sclerotiorum* strains on MEA, but only with BAFC225 on PDA. The inhibition halos observed for the three *S. sclerotiorum* strains on MEA did not differ significantly from one another (Table 4). Changes in the growth of

pathogen colony hyphae in contact with inhibition halos were observed under an optical microscope; the observed changes included increased branching, shortening of branches, and collapsed cytoplasm (Figure 4).

Antifungal activity

Organic extracts of *C. rosea* f. *rosea* strain BAFC1646 (eluted with 10:90 and 0:100 of H₂O-MeOH in the C18 fractionation) produced significant inhibition zones (halo > 5 mm) in diffusion assays. The pathogen showed morphological abnormalities of the mycelium, similar to those observed in the dual culture assays, as well as melanization of the colony in the zone of contact with crude extract (Figure 3c and d). The %RGI values for the pathogen in contact with the extract were >20%.

Bioprotective capacity of BAFC1646 in soybean plants

Disease symptoms of plants (e.g. wilting, root rot) and the presence of mycelium on the roots and stem base were reduced in *G. max* in the presence of *C. rosea* f. *rosea* BAFC1646. Sclerotia were observed only on diseased plants 20 days after the appearance of disease signs. All plants in *C. rosea* f. *rosea* treatment and control conditions survived. Only 20.0% (\pm 20.00%) of the plants in the *S. sclerotiorum* only condition survived, whereas most plants ($87.5 \pm 6.25\%$) in the *C. rosea* f. *rosea* and *S. sclerotiorum* co-inoculation condition survived (symptoms of disease was not observed in surviving plants). Plants in the *C. rosea* f. *rosea* BAFC1646 only condition had significantly longer shoots than plants in the other conditions ($P < .05$), however shoot and root dry weight did not differ significantly between the treatment groups (Figure 5). Values are the mean from two assays. Re-isolation of *S. sclerotiorum* from the roots and stems of plants exhibiting symptoms of disease allowed us to confirm that it was the causal agent of disease.

Discussion

This study focused on the role of fungi in suppressive soils in inhibiting the growth of the phytopathogenic fungus *S. sclerotiorum*. Knowledge of the fungal species that inhabit suppressive

and non-suppressive soils is important for understanding soil suppressiveness. It is also essential for isolating and identifying the specific microorganisms responsible for effective biocontrol (Borneman & Becker, 2007). Here, we found that *F. oxysporum*, *C. rosea* f. *catenulata*, *H. grisea*, *T. helicus* var. *helicus*, *T. harzianum*, and *T. koningii* were present in both suppressive and non-suppressive soils, but with different frequencies. We observed higher frequencies of *F. oxysporum*, *H. grisea*, *T. helicus* var. *helicus*, and *T. harzianum* in suppressive soils, but higher frequencies of *C. rosea* f. *catenulata* and *T. koningii* in non-suppressive soils. These results support Dix and Webster's (1995) hypothesis that primary differences in community composition are related more to variations in species frequencies than fundamental composition.

The soil washing method used had the benefit of ensuring that we were sampling active fungi (Parkinson, 1994). Our diversity analyses showed that suppressive soils tended to have a less diverse fungal structure than non-suppressive soils (see SR and H data), which indicates that antagonism against *S. sclerotiorum* is attributable to particular species. Indeed, some taxa (e.g. *C. rosea* f. *rosea* and *F. solani*, both with 100% of isolates being antagonistic) were found exclusively in suppressive soils, and some (e.g. *F. oxysporum*, *T. harzianum*, and *T. koningii*) were present in both suppressive and non-suppressive soils, but were found in higher proportions in antagonistic isolates of suppressive soils. Some authors have suggested that changes in frequency mask changes in relative levels of various subpopulations (Hunter et al., 2006).

Our experimental results were confirmed by PCA. The PCA results indicated that antagonism differences between suppressive and non-suppressive soils could be attributed to *C. rosea* f. *rosea* and *F. solani* in suppressive soils. In fact, *C. rosea* f. *rosea*, among the five most frequently encountered morpho-species, was the only taxon that was observed exclusively in suppressive soils. All recovered isolates of this species displayed antagonistic activity against the pathogen.

Our results support the notion that naturally occurring soil microorganisms are important for both natural and induced disease suppressiveness. The suppressiveness of soils may be

characterized by high microbial population diversity (Ghorbani, Wilcockson, Koocheki, & Leifert, 2008; Weller et al., 2002). Although the present study did not address this question directly, our findings are consistent with the hypothesis that suppressive soils should have a high frequency and inoculum density of specific antagonistic fungal taxa (Bonanomi, Antignani, Capodilupo, & Scala, 2010). In fact, when we evaluated antagonism, we found that 71% and 51% of all the isolates obtained from suppressive and non-suppressive soils, respectively, showed some kind of antagonism. All isolates of three of the five species most frequently found in suppressive soils (*F. oxysporum*, *T. harzianum*, and *C. rosea* f. *rosea*) showed antagonistic abilities.

Most of the antagonistic isolates from suppressive soil samples produced antifungal metabolites and inhibited growth of the pathogen. A low proportion of the isolates overgrew pathogen colonies. This is the case of *Trichoderma* spp. *Talaromyces flavus* var. *flavus* and *Clonostachys rosea* f. *catenulata*. Relationships among organisms are critical aspects of the fungal diversity structure (Dix & Webster, 1995). In this regard, our results establish that soybean soil suppressiveness was associated with the antagonistic activity of certain fungal strains, with the inhibitory effect due to antifungal metabolites. Other studies have shown that both soil microbial community composition and antifungal metabolite production can be major determinants of soil fungistasis (de Boer et al., 2003; Garbeva et al., 2011; Vey, Hoagland, & Butt, 2001). De Boer et al. (2003) consider that although the nutrient status of the soil can exert a role in the production of antifungal compounds, it is the microbial community composition and the interactions between their members that determine the quality and quantity of these antifungal compounds.

The ordination analysis (i.e. PCA) based on the antagonistic behavior of different species and/or species-specific mechanisms indicated that *F. oxysporum*, *F. solani*, *T. flavus*, and *C. rosea* f. *rosea* were the most likely sources of suppressiveness. This conclusion is supported by prior work (Rodríguez et al., 2006) in which a strain of *F. oxysporum* was found to protect soybean plants against *S. sclerotiorum* through the production of antifungal secondary metabolites. It is noteworthy that the occurrence frequencies of *F. solani* and *C. rosea* f. *rosea* were determinant components in

the separation between suppressive and non-suppressive soils and that *C. rosea* f. *rosea*, in particular, was present at high frequencies in suppressive samples. Indeed, *C. rosea* f. *rosea* meets the desirable biocontrol agent criterion of being ecologically adapted to the environment of its target pathogen (Butt et al., 2001). However, *C. rosea* is used already in commercially available formulations. In this context, our findings suggest that the BAFC1646 strain of *C. rosea* may be a promising biological control agent.

The findings from our *in vitro* dual culture assays, an established method for initial evaluation of a microbial antagonist (Gromadzka et al., 2009; Whipps, 1987), provide strong evidence in support of *C. rosea* f. *rosea* BAFC1646 as a biocontrol agent. Although other strains of *C. rosea* have been shown to inhibit phytopathogens (Mejía et al., 2008; Whipps, 1987; Zizzerini & Tosi, 1985), it is noteworthy that our BAFC1646 strain showed an unusually high %RGI in all the conditions evaluated and against all strains of *S. sclerotiorum* evaluated. Interestingly, the power of inhibition exerted varied among the three *S. sclerotiorum* strains tested.

Inhibition in sclerotia formation and alteration of mycelial morphology were observed in *S. sclerotiorum* colonies interacting with *C. rosea* f. *rosea* BAFC1646. Similar modifications of hyphae morphology have been described previously when phytopathogenic fungi were confronted with a range of microorganisms. Their effects have been related to antifungal metabolites (Aryantha & Guest 2006; Rodríguez et al., 2011; Zizzerini & Tosi, 1985). The antifungal activity of *C. rosea* f. *rosea* BAFC1646 was confirmed in diffusion assays with metabolite extracts associated with the stationary growth phase. Metabolite extracts changed the morphology of the mycelium of the pathogen in dual cultures. These changes corresponded with melanization of pathogen structures in diffusion assays. The purification and complete structural elucidation of the metabolite(s) involved is being undertaken currently and the results will be published elsewhere.

In our greenhouse assays, *C. rosea* f. *rosea* reduced *S. sclerotiorum* disease in soybean plants. In addition, in the absence of the pathogen, it significantly increased the shoot lengths of soybean plants. Using similar conditions, Harman, Petzoldt, Comis, and Chen (2004) and

Rodríguez et al. (2011) showed growth promotion of *Zea mays* plants and *Lactuca sativa* plants with *T. harzianum* and *C. rosea* inoculation, respectively. Thus, *C. rosea* f. *rosea* could be used as both a biocontrol agent and a plant growth-promoting agent. Multi-faceted mechanisms can be at work in microbial-plant interactions (Whipps, 2001). Here, our *C. rosea* f. *rosea* strain showed growth promotion and various hydrolytic enzyme activities in addition to its antifungal activity. These multiple effects could have implications for improving plant growth, facilitating access to nutrient sources otherwise not available to the plant (Whipps, 2001).

The observed production of IAA by *C. rosea* f. *rosea* could be involved directly or indirectly in the promotion of soybean plant growth. IAA production by saprotrophs and endophytes has been reported to be involved in promoting growth in various plants and thus could be responsible, at least in part, for the presently observed plant growth promotion (Yuan, Zhang, & Lin, 2010; Zhang et al., 2012). Saprophytic ability and IAA production have been described as common attributes of fungal biocontrol agents (Altomare et al., 1999; Shores, Harman, & Mastouri, 2010).

A few consistent patterns relating plant and soil community composition have been detected; however, they have not been studied in detail (Singh et al., 2013). Soil microbial populations are immersed in a system of interactions that affect plant fitness and soil quality (Barea, Pozo, Azcon, & Azcon-Aguilar, 2005). Here, we uncovered characteristic differences between soils that are or are not suppressive against *S. sclerotiorum* through a two-pronged approach. On the one hand, we isolated active fungi, identified the isolated fungi, and determined their frequencies of occurrence. On the other hand, we evaluated the antagonistic activity of the isolates against *S. sclerotiorum*, and identified *C. rosea* f. *rosea* strain BAFC1646 as a promising biocontrol agent. Our characterizations of fungal diversity structures allowed us to elucidate the differential behavior of suppressive versus non-suppressive soils. Those findings led us to select suppression-associated fungal species for further analysis. The present results indicate that *C. rosea* f. *rosea* has a

bioprotective activity that protects soybean plants from *S. sclerotiorum* diseases and further suggest that this activity may be mediated through antifungal metabolite production.

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Table 1. Types of interaction considered in dual-culture confrontation assays (modified from Whipps, 1987).

Type of interaction	Summary of types of interaction between colonies in confrontation
TI1	Colonies meet and form a straight line in center of the Petri dish, with both ceasing growth and a mixing zone of mycelia in the interaction zone (Iz), with $Iz \leq 2$ mm.
TI1+	Like TI1, but $Iz > 2$ mm.
TI2a	Antagonist colony growth surrounds pathogen colony with $Iz \leq 2$ mm.
TI2b	Pathogen colony growth surrounds antagonist colony with contact between the hyphae, $Iz \leq 2$ mm.
TI2a+	Antagonist colony growth surrounds pathogen with subsequent growth over the pathogen with $Iz > 2$ mm.
TI2b+	Pathogen colony growth surrounds antagonist colony with subsequent growth over the pathogen with $Iz > 2$ mm.
TI3a	Antagonist colony growth surrounds pathogen colony without contact between the hyphae and with a growth inhibition halo (Ih) ≤ 2 mm.
TI3b	Pathogen colony growth surrounds fungal antagonist without contact between the hyphae and with $Ih \leq 2$ mm.
TI4	Mutual inhibition with $Ih < 2$ mm, straight-line interaction between the colonies.
TI5	Mutual inhibition with $Ih > 2$ mm.

Table 2. Indices of antagonism determined in *in vitro* assays and considered in the Principal Components Analysis (PCA) of antagonism of suppressive and non-suppressive soils.

Index	Antagonist quantification
Inhibition of radial growth in dual cultures assay (IGId)	$IGId = (r1 - r2)/r1$ where: r2 = radius of the pathogen in the line of confrontation and r1 = radius of the control colony
Inhibition of sclerotia formation in zone 1 in dual culture assay (ISF) (Fig. 1)	$ISF = (n1 - n2)/n1$ where: n1 = number of sclerotia in confrontation control n2 = number of sclerotia in confrontation with the antagonist
Inhibition of formation and position of immature sclerotia (Fig. 1) at day 6 in dual-culture assay	1: no formation of sclerotia in any of the zones. 0.75: sclerotia only in zone 4. 0.50: sclerotia in zones 3 and 4. 0.25: sclerotia in zones 2, 3 and 4. 0: sclerotia in all areas.
Inhibition of formation and position of mature sclerotia (Fig. 1) at day 13 in dual culture assay	1: no formation of sclerotia in any of the zones. 0.75: sclerotia only in zone 4. 0.50: sclerotia in zones 3 and 4. 0.25: sclerotia in zones 2, 3 and 4. 0: sclerotia in all areas.
Type of interaction (Table 1)	1: TI1+, TI2a+, TI2b+ and TI5 (most inhibitory reactions). 0.75: TI4, TI3a. 0.50: TI2a, TI3b, TI1. 0.25: TI2b (less inhibitory reactions).
Growth inhibition in cellophane membrane assay (IGIc)	$IGIc = (d1 - d2)/d1$ where: d2 = diameter of the pathogen colony on medium with exudates of the antagonist and d1 = diameter of the control colony
Growth inhibition in volatile metabolites assay (IGIv)	$IGIv = (d1 - d2)/d1$ where: d2 = diameter of pathogen colony in volatile metabolites assay, and d1 = diameter of control colony

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Table 3. Frequency of occurrence (Fr), relative abundance (Ar), and percentage of antagonistic isolates (% Ant) of each morpho-species present in suppressive and non-suppressive soil.

Fungal morpho-species	Suppressive soil			Non-suppressive soil		
	Fr	Ar	% Ant	Fr	Ar	% Ant
<i>Actinomucor microsporus</i>	2.67	2.22	0	-	-	-
<i>Aspergillus fumigatus</i>	-	-	-	4.00	3.51	100
<i>Clonostachys rosea</i> f. <i>rosea</i>	8.00	6.68	100	-	-	-
<i>Clonostachys rosea</i> f. <i>catenulata</i>	2.67	2.22	100	8.00	7.02	100
<i>Drechslera biseptata</i>	-	-	-	2.00	1.75	0
<i>Fusarium dimerum</i>	6.67	5.57	20	-	-	-
<i>Fusarium equiseti</i>	2.67	2.22	100	-	-	-
<i>Fusarium oxysporum</i>	21.33	28.92	100	14.00	12.28	71
<i>Fusarium semitectum</i>	1.33	1.11	100	-	-	-
<i>Fusarium solani</i>	5.33	4.44	100	-	-	-
<i>Humicola fucoastrata</i>	-	-	-	2.00	1.75	0
<i>Humicola grises</i>	18.67	15.58	0	12.00	10.53	0
<i>Idriella lunata</i>	-	-	-	4.00	3.51	0
Moniliaceous sterile mycelium 1	1.33	1.11	0	-	-	-
Pigmented sterile mycelium 1	2.67	2.22	100	-	-	-
Moniliaceous sterile mycelium 5	2.67	2.22	0	-	-	-
Moniliaceous sterile mycelium 2	-	-	-	2.00	1.75	0
Moniliaceous sterile mycelium 3	-	-	-	6.00	5.26	100
Moniliaceous sterile mycelium 4	-	-	-	4.00	3.51	0
Indeterminate mycelium	-	-	-	2.00	1.75	100
<i>Penicillium</i> sp1	-	-	-	2.00	1.75	0
<i>Penicillium</i> sp2	-	-	-	2.00	1.75	0
<i>Phoma exigua</i>	-	-	-	10.00	8.77	0
<i>Phoma glomerata</i>	2.67	2.22	0	-	-	-
<i>Phoma medigaginis</i>	-	-	-	2.00	1.75	0
<i>Phoma putaminum</i>	1.33	1.11	0	-	-	-
<i>Talaromyces flavus</i> var. <i>flavus</i>	1.33	1.11	100	-	-	-
<i>Talaromyces helicus</i> var. <i>helicus</i>	4.00	3.33	100	2.00	1.75	100
<i>Trichoderma harzianum</i>	10.67	8.90	100	4.00	3.51	100
<i>Trichoderma koningii</i>	10.67	8.90	75	26.00	22.81	69
<i>Trichoderma viride</i>	-	-	-	6.00	5.26	67

Fr = number of occurrences of a species x 100/total No. of inoculated soil particles.

Ar = number of isolates of a species x 100/total No. of isolates obtained.

% Ant = number of antagonistic isolates of a species x 100/total isolates evaluated.

Table 4. Percentage of radial growth inhibition (%RGI) and width of inhibition halo (Ih) for each tested strain of *S. sclerotiorum* (BAFC217, BAFC225 and BAFC2232) in dual cultures with *Clonostachys rosea* f. *rosea* BAFC1646 on MEA (malt extract agar) and PDA (potato dextrose agar).

<i>S. sclerotiorum</i> strain	MEA		PDA	
	%RGI	Ih	%RGI	Ih
BAFC 217	43.9 ± 1.1 b	2.8 ± 0.5 a	47.2 ± 2.1 b	0.0 ± 0.0
BAFC225	50.0 ± 1.4 a	1.5 ± 0.7 a	52.8 ± 2.8 a	2.3 ± 0.3
BAFC2232	44.4 ± 0.9 b	2.5 ± 0.3 a	53.9 ± 2.1 a	0.0 ± 0.0

Values are means for each treatment with standard errors. Different letters indicate significant differences between treatments (ANOVA Tukey test $p < .05$). Ih on PDA data were not been analysed due to 0 counts for both BAFC217 and BAFC2232.

Figure 1. Dual cultures of some fungal isolates from soybean soils and *Sclerotinia sclerotiorum* BAFC225, 13 days after confrontation. a) Illustration of areas assessed to determine antagonist effects on sclerotial production in dual cultures. X corresponds to the location of inoculum of each strain evaluated (modified from Jackson, Whipps, and Lynch, 1991). Some of the observed patterns of sclerotia formation are shown in panels b-f as follows: b) sclerotia in zones 1, 2, 3, and 4 (in dual culture with *Fusarium semitectum*); c) sclerotia in zones 2, 3, and 4 (*F. oxysporum*); d) sclerotia in zones 3 and 4 (*F. equiseti*); e) sclerotia only in zone 4 (vs. moniliaceous sterile mycelium 5); and f) absence of sclerotia formation (*Trichoderma koningii*).

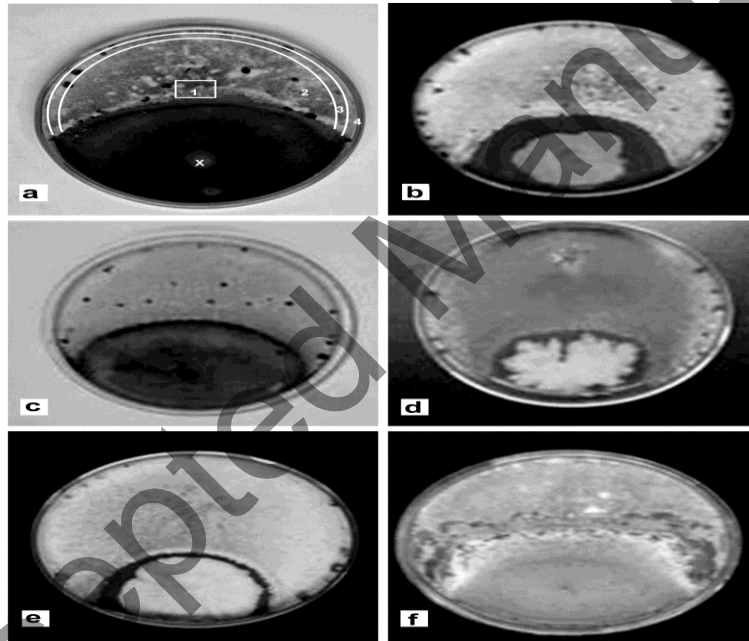


Figure 2. Principal components analysis (PCA). (a) Variables were colonization (i.e. frequency of occurrence) of suppressive and non-suppressive soils by the isolated fungal species (components or factors 1 and 5). (b, c) Variables were antagonism of the fungal species isolated (indices from Table 2), represented by components or factors 3 and 4 in panel b and 3 and 5 in panel c. The analysis included the first 5 components or factors as a combination of the original variables. S1, S2, S3: suppressive soil samples. N1, N2: non-suppressive soil samples.

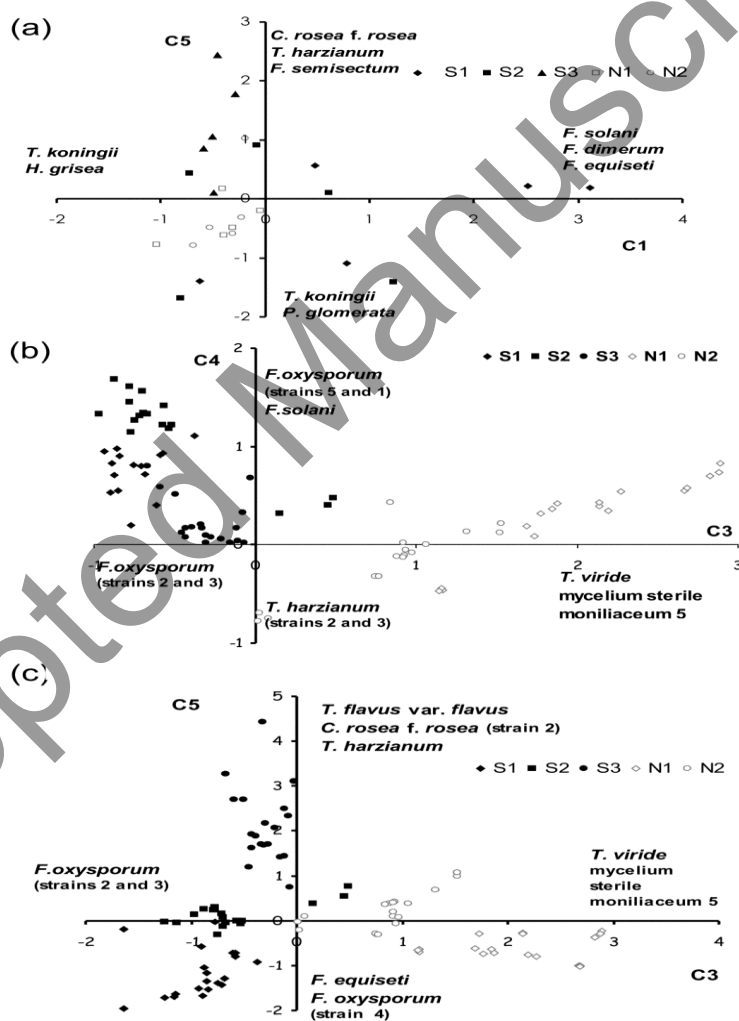


Figure 3. (a, b) Dual cultures of *C. rosea* BAFC1646 with *S. sclerotiorum* strains (from left to right BAFC225, BAFC2232 and BAFC217) on MEA (a) and PDA (b). (c, d) Antimicrobial assays based on diffusion of collected fractions (100 μ g) after extraction and vacuum chromatography against *S. sclerotiorum* BAFC225 colony. Note the presence of melanization in the pathogen mycelium in contact with the inhibition zone (arrowheads).

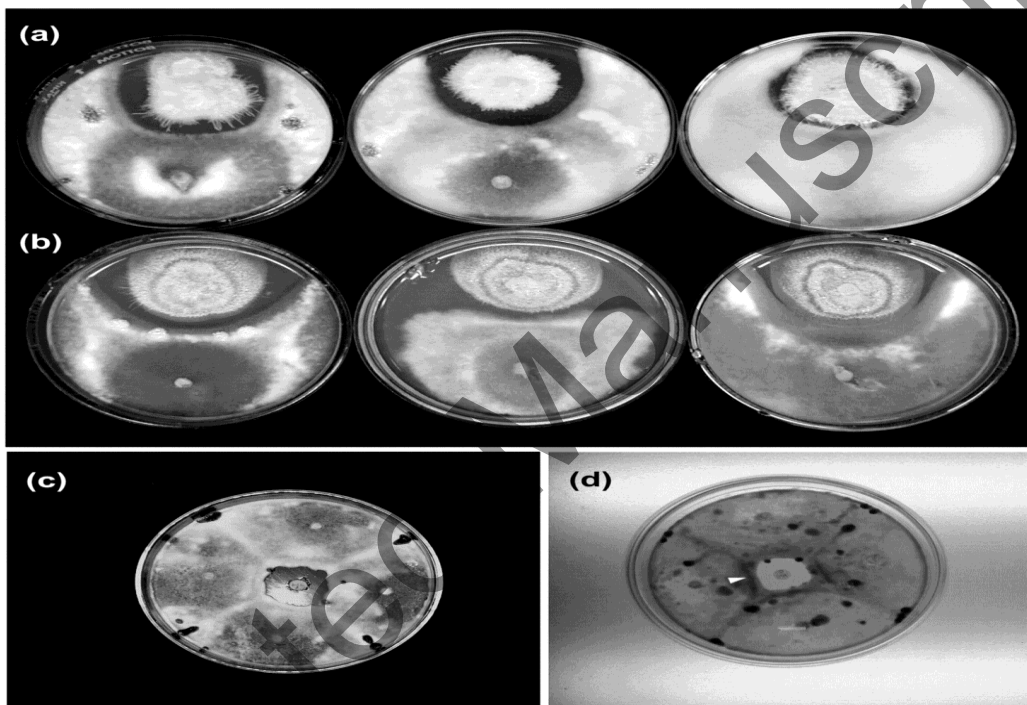


Figure 4. Microscopic observations of *Sclerotinia sclerotiorum* mycelium. (a) Observations at colony edge in untreated control on MEA. (b) Observations at edge of colony in contact with *Clonostachys rosea* f. *rosea* BAFC1646; note collapsed cytoplasm, increased branching, and shortened hyphal branches. Scale bar: 10 μ m.

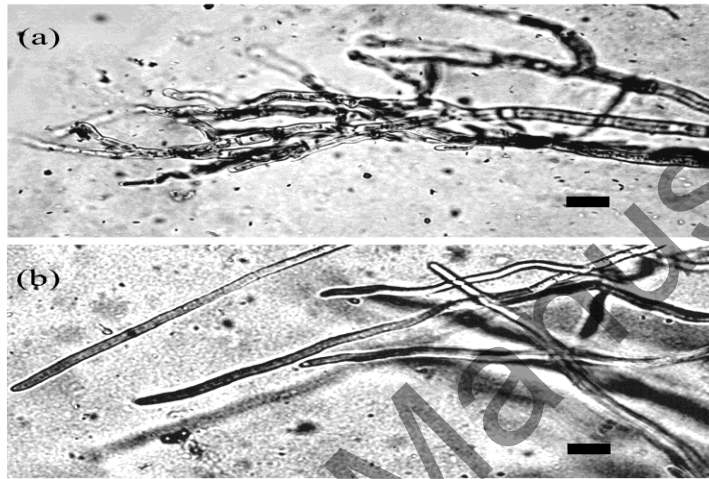
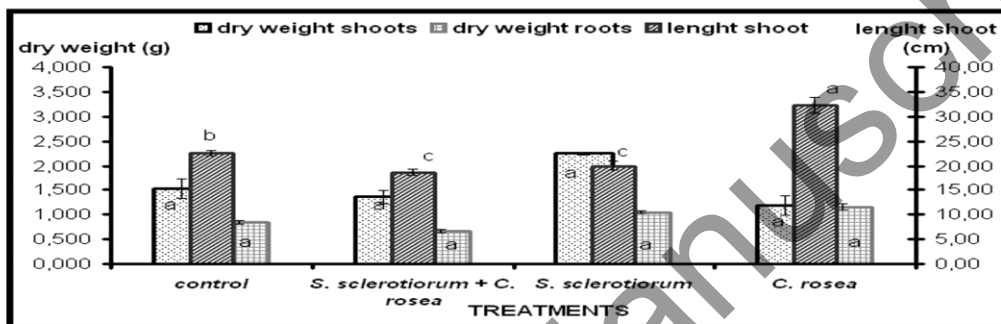


Figure 5. Growth parameters in greenhouse experiments (combined data from duplicate assays). Length and dry weight of shoots and dry weight of roots of surviving soybean plants in the biocontrol experiments with *C. rosea* BAFC1646 and *S. sclerotiorum* BAFC225 in the following four treatments: Control (without *C. rosea* or *S. sclerotiorum* inoculation); *C. rosea* co-inoculated with *S. sclerotiorum*; *S. sclerotiorum* only; and *C. rosea* only. Different letters indicate significant differences between treatments (ANOVA Tukey test $p < .05$). Bars indicate standard errors.



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