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# Selection of a *Streptomyces* Strain Able to Produce Cell Wall Degrading Enzymes and Active against *Sclerotinia sclerotiorum*

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**Control of plant pathogen *Sclerotinia sclerotiorum* is an ongoing challenge because of its wide host range and the persistence of its sclerotia in soil. Fungicides are the most commonly used method to control this fungus but these can have ecotoxicity impacts. Chitinolytic *Streptomyces* strains isolated from Brazilian tropical soils were capable of inhibiting *S. sclerotiorum* growth *in vitro*, offering new possibilities for integrated pest management and biocontrol, with a new approach to dealing with an old problem. Strain *Streptomyces* sp. 80 was capable of irreversibly inhibiting fungal growth. Compared to other strains, its crude enzymes had the highest chitinolytic levels when measured at 25°C and strongly inhibited sclerotia from *S. sclerotiorum*. It produced four hydrolytic enzymes involved in fungal cell wall degradation when cultured in presence of the fungal mycelium. The best production, obtained after three days, was 0.75 U/ml for exochitinase, 0.9 U/ml for endochitinase, 0.16 U/ml for glucanase, and 1.78 U/ml for peptidase. Zymogram analysis confirmed two hydrolytic bands of chitinolytic activity with apparent molecular masses of 45.8 and 206.8 kDa. One glucanase activity with an apparent molecular mass of 55 kDa was also recorded, as well as seven bands of peptidase activity with apparent molecular masses ranging from 15.5 to 108.4 kDa. Differential interference contrast microscopy also showed alterations of hyphal morphology after co-culture. *Streptomyces* sp. 80 seems to be promising as a biocontrol agent against *S. sclerotiorum*, contributing to the development of new methods for controlling plant diseases and reducing the negative impact of using fungicides.**

***Keywords***: biological control, chitinase, β-1,3-glucanase, peptidase, *Sclerotinia sclerotiorum*, *Streptomyces* sp.

## Introduction

*Sclerotinia sclerotiorum* (Lib.) de Bary is a cosmopolitan fungus and hugely destructive pathogen attacking many economically important crops including soybean, bean, pea, lettuce, tomato, sunflower, and canola (Hegedus and Rimmer, 2005). Control of this pathogen is by use of long crop rotations, soil fumigation and fungicide sprays. It has a wide host range of at least 408 plant species and its sclerotia can survive for up to 8 years in soil (Bae and Knudsen, 2007). Environmental problems caused by fungicides, their cost and difficulties in obtaining resistant cultivars make biological control a very interesting additional or alternative tool for the suppression of this particular fungus (Inbar and Chet, 1996). Several microorganisms have already been recorded as antagonists for *S. sclerotiorum* (Adams and Ayers, 1979); however, studies including actinomycetes are rare (Tahtamouni *et al.*, 2006). Filamentous bacteria, especially of the genus *Streptomyces*, produce a wide spectrum of antibiotics as secondary metabolites, as well as a variety of fungal cell wall-degrading enzymes including chitinases, glucanases, and peptidases (Yuan and Crawford, 1995). These enzymes are very important in biological control since chitin, glucan, and protein are the major components of various cell walls of phytopathogenic fungi, including *S. sclerotiorum* (Jones, 1970). El-Tarabily *et al.* (2006, 2009) and several others (Valois *et al.*, 1996; Prapagdee *et al.*, 2008; Gopalakrishnan *et al.*, 2011) have described the production of cell wall-degrading enzymes by *Streptomyces* spp. and highlighted their potential importance for biological control of fungi. In Brazil, losses in revenue from cash crops such as bean and soybean, caused by *S. sclerotiorum*, is of major concern for producers and a challenge for researchers (Junior and Abreu, 1994) to ameliorate. Currently there is no microbial product registered in Brazil for the biocontrol of this fungus. The development of new formulations is of great interest and importance to the global protein market and Brazil is the largest exporter of soybeans in the World. In this study the selection and molecular characterization of a chitinolytic *Streptomyces* strain able to interfere with *S. sclerotiorum* growth, *in vitro,* has been reported. Levels of endochitinases, exochitinases, β-1,3-glucanase and proteolytic enzyme production were quantified and the antifungal biocontrol potential of the selected *Streptomyces* strain is discussed.

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## Materials and Methods

### Microbial cultures

*S. sclerotiorum* was isolated from infected bean plants, provided by EMBRAPA Meio Ambiente (Brazil). The isolated strain was grown on potato-dextrose-agar medium (PDA) and plugs (3–5 mm diameter) of actively growing mycelium

were used as inoculum. The strain was maintained as plugs in sterile distilled water. Four *Streptomyces* strains (70, 80, Q11, and M08) previously isolated from a tropical Brazilian soil (Gomes *et al*., 1999) were characterized as chitinolytic and promising for biocontrol of various phytopathogenic fungi (Gomes *et al*., 2000). Strains were stored as spore suspensions in 20% glycerol at -20°C until use. Whenever not specified, growth of each culture was obtained on yeast extract-malt extract-agar (YMA) (Shirling and Gottlieb, 1966) after two weeks incubation.

**Antagonism assays by dual culture**

This test was performed with the four streptomycetes strains. Each one was previously inoculated onto PDA near the border of a Petri dish and incubated at 28°C. After two days incubation an agar plug from a five-day-old culture of *S. sclerotiorum* was center inoculated and the Petri dish incubated at 22°C, for five days. *Sclerotinia sclerotiorum* plugs were also placed on dishes without *Streptomyces* strains, as control. After five days incubation, inhibition was calculated by subtracting the distance (mm) of the fungal growth in the direction of the antagonistic colony ($\gamma$) from the fungal growth radius ($\gamma_0$) of the control culture, to give $\Delta\gamma = \gamma_0 - \gamma$, where $\Delta\gamma > 5$ mm = +; $\Delta\gamma > 10$ mm = ++; and $\Delta\gamma > 20$ mm = +++ (El-Tarabily *et al*., 2000). This same test was also performed growing the actinomycetes previously at 22°C (best temperature for *S. sclerotiorum* growth) instead of 28°C. Three replicates were prepared for each strain grown at each temperature.

**Fungal mycelium viability test**

This was performed after five days incubation of the four paired cultures described above. Plugs from inhibition zones still containing some hyphae were transferred to a fresh PDA medium. The plates were incubated for 10 days at 22°C and checked for fungal growth (El-Tarabily *et al*., 2000). Experiments were conducted with three replicates for each isolate.

**Enzyme production**

**Preparation of *S. sclerotiorum* mycelium:** Erlenmeyer flasks (250 ml) containing 100 ml of a potato broth (PB) (20 g potato infusion in 100 ml distilled water plus 0.4% yeast extract) were inoculated with plugs of PDA with actively growing mycelium of *S. sclerotiorum* and incubated at 22°C for 5 days. The mycelium was washed three times with sterile saline (0.85%), transferred to Erlenmeyer flasks (3 L) containing one liter of PB and further incubated at 22°C for 10 days. Mycelia were then collected by filtration using commercial filter paper, washed with distilled water three times and lyophilized. The lyophilized mycelium was homogenized in a blender (IKA, Germany), sieved (Granutest, 0.42 mm) and stored in a desiccator until use.

**Growth conditions:** *Streptomyces* strains were grown in triplicate for up to four days (28°C/200 rpm) in TLE medium (*Trichoderma* Liquid Enzyme, [Bara *et al*., 2003]) containing bactopeptone 0.1%, urea 0.03%, $KH_2PO_4$ 0.2%, $(NH_4)SO_4$ 1.4%, $MgSO_4{\cdot}7H_2O$ 0.03%, glucose 0.03%, 1 ml of trace elements solution ($FeSO_4{\cdot}7H_2O$ 0.11%, $ZnSO_4{\cdot}7H_2O$ 0.15%, $MnCl_2{\cdot}4H_2O$ 0.79%, $CuSO_4{\cdot}5H_20$ 0.64%) and 0.5% of *S. sclerotiorum* mycelium. Each Erlenmeyer flask (100 ml) containing 25 ml of the medium was inoculated with $10^7$ spores/ml and at the end of each day, the crude extract of a whole flask was obtained by filtration (Whatman N° 1) and centrifugation at 15300×g. The crude extracts were maintained at -20°C until use.

**Hydrolytic enzyme assays: chitinase, glucanase and peptidase**

Chitinase activity assays were carried out in 96-well plates with a reaction mixture containing 50 µl of crude extract, 100 µl of Tris-HCl 50 mM pH 7.4 and 50 µl of the substrate, 4-methylumbeliferil-*N*-acetyl-β-D-glucosaminide 50 mM/L for exochitinase or 4-metylumbeliferil-β-D-N,N′,N″-tetracetyl chitotetraose 50 mM/L, for endochitinase. After 1 h of incubation at 25°C or 50°C the amount of methylumbeliferil produced was determined using a Fluoroskan autoreader (Fluoroskan II version 6.3, excitation wavelength 366 nm and emission wavelength 450 nm). One unit of enzyme activity (U) corresponded to the amount of enzyme required to produce 1 µmol of metylumbeliferil in 1 min of reaction (Souza *et al*., 2003). Beta-1,3-glucanase activity was assayed using 100 µl of crude extract, 200 µl of sodium citrate - citric acid 50 mM/L pH 5.0 and 100 µl of laminarin (0.5%) (Bara *et al*., 2003). The reaction was conducted for 1 h at 50°C and the amount of reducing sugar liberated was determined according to Nelson-Somogyi methodology (Spiro, 1966). Glucose was used as the calibration standard. One unit of enzyme activity (U) corresponded to the amount of enzyme required to produce 1 µmol of reducing sugar in 60 min of reaction. Peptidase activity was measured spectrophotometrically using the gelatin as a substrate (Merck, Germany). The assay consisted of 100 µl of crude extract, 100 µl of Tris-HCl 50 mM/L pH 7.4 and 50 µl of gelatin (0.5%) and was conducted for 1 h at 30°C using a 96-well microplate (Buroker-Kilgore and Wang, 1993; Jones *et al*., 1998). Bovine serum albumin was used as standard. One unit of enzyme activity (U) corresponded to the amount of enzyme required to reduce 0.01 absorbance unit per min at 595 nm under standard assays conditions. All measurements were conducted using three replicates. Results represent the mean of these three assays.

**Sclerotia viability tests**

**Preliminary test in liquid medium:** Sclerotia from *S. sclerotiorum* were obtained according to Ferraz and Café Filho (1998). Two sclerotia (3–5 mm) were aseptically introduced into 125 ml Erlenmeyers flasks containing 25 ml of PDB (Potato Dextrose Broth) medium along with a loopful of spores for one of each of the four *Streptomyces* strains. After six days incubation at 28°C (120 rpm) sclerotia were collected and reintroduced into fresh PDA medium (22°C) for up to 10 days for a visual viability test. Experiments were performed separately for each actinomycete strain, and controls without the *Streptomyces* spores were also prepared. Assays were conducted with three replicates for each isolate.

***In vitro* test using concentrated *Streptomyces* sp. 80 crude extract:** After the third day of incubation, crude extract from *Streptomyces* sp. 80 (obtained according to *Growth conditions*

section) was concentrated 10 fold at 4°C using an ultra-filtration membrane (Amicon Diaflo), with a 10 kDa molecular mass (MM) cut off (Souza *et al.*, 2003). Both fractions, the concentrated one (MM>10 kDa), and the excluded one (MM<10 kDa) were used. The experiments were conducted in a 24-well microplate, in triplicate, under sterile conditions, each well containing 3 small sclerotia of about 3 mm each, and 2 ml of each of the following: the crude extract, the concentrated fraction and the excluded fraction. Three controls were also prepared, a negative control, using autoclaved sclerotia; and two positives controls, one using sclerotia without treatment and the other using sclerotia incubated for 2 days in TLE medium. All fractions were filtrated through a 0.22 µm membrane before the experiments. Microplates were incubated for 2 days at 37°C and then the sclerotia of each well were transferred, individually, to the wells of another microplate containing Neon-S medium (Napoleão *et al.*, 2006), in order to verify its viability. The microplates were incubated at 22°C for up to 7 days. Neon-S medium contains bromophenol blue, and the viability is detected by the production and release of oxalic acid from the viable sclerotia, turning the blue color of the dye into yellow (Gracia-Garza *et al.*, 1997).

***In vivo* test using *Streptomyces* sp. 80 suspension:** Eight gerboxes (11 cm × 11 cm × 3.5 cm) were filled in with 120 g of an autoclaved commercial substrate (West garden, www.westgarden.com.br). Substrates from 4 boxes were moistened with 12 ml of a spore suspension of *Streptomyces* sp. 80 (final concentration of $10^8$ spores/g of substrate), whereas the other 4 boxes, used as control, were moistened with 12 ml of sterile distilled water. Ten sclerotia were buried in each gerbox, equidistantly. The boxes were maintained at 25°C for 30 days and then all sclerotia still present were unearthed. After visual observation they were disinfected (by immersion in 50% alcohol for 3 min and then in a 1% sodium hydrochloride solution for 3 min), washed three times with distilled water and dried in a laminar flow hood. The dried sclerotia were placed individually into each well of a 24-well microplate containing Neon-S medium (Napoleão *et al.*, 2006) and incubated for 4–6 days at 22°C in order to evaluate its viability.

### DNA extraction, PCR amplification, and phylogenetic analysis of *Streptomyces* sp. 80

Extraction of genomic DNA was carried out as described previously (Kurtzman and Robnett, 1998) after growth in a liquid medium of yeast extract-malt extract-agar (YMA) (Shirling and Gottlieb, 1966) for three days at 28°C with agitation (200 rpm). PCR amplification of the 16S rRNA gene (*rrs*) gene was carried out using the GoTaq® Green Master Mix kit (Promega Corporation, USA), with primers 27F (Hayashi *et al.*, 2004) and 1541R (Loffler *et al.*, 2000), in a thermal cycler model Gene Amp® PCR System 9700 (Applied Biosystems, USA). Amplified fragments were purified using the Illustra™ GFX™ PCR DNA as well as Gel Band Purification kit (GE Healthcare) and were sequenced directly using an ABI Prism dye terminator cycle sequencing reaction kit (Applied Biosystems) in an automatic sequencer (ABI model 3730; Applied Biosystems). The 16S rRNA sequence obtained was compared with sequences online at the Ribosomal Database Project (RDP) release 10 (Cole *et al.*, 2009) and NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) by using the BLAST algorithm (Altschul *et al.*, 1990) available at NCBI. Sequences retrieved were aligned with the most similar type strains obtained using CLUSTAL W (Thompson *et al.*, 1994). Evolutionary distance matrices and trees were generated using the Kimura 2-parameter and neighbor-joining algorithms with a complete deletion option, as implemented in the MEGA version 5.0 software package (Tamura *et al.*, 2011). The resultant unrooted tree topologies were evaluated by bootstrap analyses based on 1000 replications. The sequence obtained was deposited in the GenBank database under accession number HM447593.

### Zymograms

After the third day of incubation, crude extract from *Streptomyces* sp. 80 (obtained according to *Growth conditions* section) was concentrated 10 fold at 4°C using an ultra-filtration membrane (Millipore Corp., USA), with a 10 kDa molecular mass (MM) cut off (Souza *et al.*, 2003). Chitinases and β-1,3-glucanases from the crude extract were analyzed by a 10% (w/v) polyacrylamide gel electrophoresis (PAGE) using the Hoefer minielectrophoresis system (Amersham Biosciences, Sweden), and carried out at a constant voltage of 120 V and 30 mA at 4°C (for 1 h). Chitinolytic enzymes were renatured with 1% Triton X-100 in water for 30 min at room temperature and transferred to a 7.5% polyacrylamide overlay gel containing glycol-chitin (1% w/v) as substrate. The overlay gel was incubated with Tris-HCl 50 mM pH 7.4 buffer for 1 h at 50°C and lytic zones were revealed by UV (365 nm) after staining with calcofluor white M2R (0.01% w/v) (Sigma) for 10 min and destaining with distilled water for about 4 h at 25°C, in the dark (Trudel and Asselin, 1989). The native gel for β-1,3-glucanase activity was equilibrated in sodium citrate – citric acid buffer 100 mM pH 5.0 for 15 min and then incubated in a laminarin solution (0.5%) using the same buffer described above for 3 h at 50°C. The gel was washed three times with distilled water and activity bands were revealed by boiling the gel in a solution containing 0.15% of 2,3,5-triphenyltetrazolium chloride dissolved in 1 M NaOH (Pan *et al.*, 1989; Monteiro and Ulhoa, 2006). Peptidase activity was detected in a 7.5% SDS-PAGE co-polymerized with gelatin (1%) gel under the same conditions as described for chitinase activity, except that incubation was carried out at 37°C for 18 h. Hydrolytic bands were detected after staining the gel with comassie blue R-250 (Heuseen and Dowdle, 1980).

### Microscopic observations of *Sclerotinia* hyphal morphology

*Sclerotinia sclerotiorum* was grown in PDB medium. After three days incubation at 22°C (120 rpm) the culture broth was removed and mycelium aseptically washed three times with sterile distilled water. Live mycelium was inoculated in PDB medium along with a loopful of a spore suspension of *Streptomyces* sp. 80 culture. After 4 days of co-cultivation at 22°C (120 rpm) mycelia were observed using differential interference contrast microscopy (400× magnifications). Control mycelia were observed from cultures in which the *Streptomyces* strain had not been inoculated with the fungus.

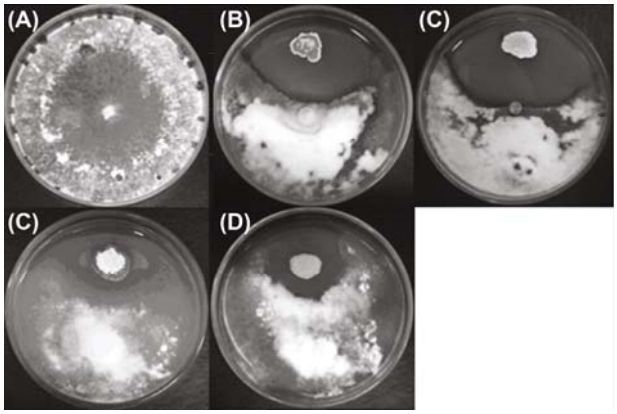


**Fig. 1. Dual culture antagonism assay.** (A) control, *S. sclerotiorum*; (B, C, D, E) *S. sclerotiorum* center inoculated and *Streptomyces* sp. 80 (B), *Streptomyces* sp. Q11 (C), *Streptomyces* sp. 70 (D), *Streptomyces* sp. M08 (E) inoculated at the border of the plate.

## Results

### Preliminary tests

In dual culture tests in Petri dishes, when streptomycete growth was at 28°C, the four *Streptomyces* strains presented good levels of inhibition against *S. sclerotiorum* (Fig. 1). However, when the actinomycete strains were cultivated previously at 22°C, *Streptomyces* sp. 80 was the only one capable of growing and inhibiting *S. sclerotiorum* (data not shown). *Streptomyces* sp. 80 was also the only strain capable of irreversibly inhibiting *S. sclerotiorum* growth, according to the mycelium viability test (Table 1). Concerning sclerotia from *S. sclerotiorum*, when the viability test was performed in liquid media in the presence of each of the four strains, an effective inhibition was observed in all cases. After 6 days of co-cultivation, sclerotia collected from each broth and re-introduced into fresh medium were not able to germinate, even after 10 days at 28°C (Table 1). As to the endochitinase and exochitinase activities produced by the four strains measured at 25°C (Fig. 2), the best results were obtained after 3 to 4 days of cultivation. Enzyme activities after the third day are presented in Table 1. High levels of endochitinase were detected, except for strain Q11 (Fig. 2A). *Streptomyces* sp. 80 was the only strain able to produce exochitinase in significant amounts (Fig. 2B). Considering the results so far obtained (Table 1) strain 80 was selected as the most promising for biocontrol, deserving further studies.

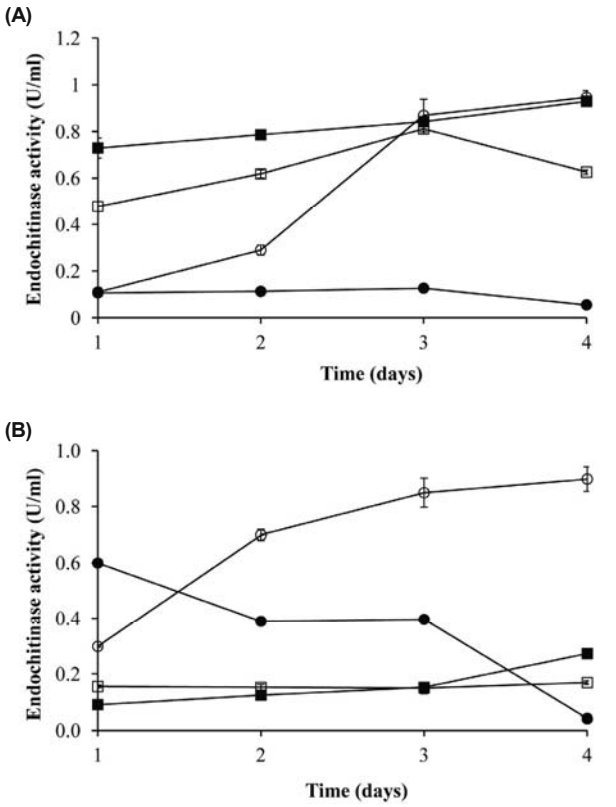


**Fig. 2. Kinetics of endochitinase (A) and exochitinase (B) production measured at 25°C.** *Streptomyces* sp. 80 (—○—), M08 (—■—), 70 (—□—), and Q11 (—●—), grown in TLE medium containing 0.5% of *S. sclerotiorum* mycelium. Bars indicate the standard deviation.

### Characterization of *Streptomyces* sp. 80

For molecular characterization, 1498 nucleotides of the 16S rRNA were sequenced and analyzed. According to the phylogenetic tree (Fig. 3) the most closely related strain observed was *Streptomyces lunalinharesii* RC1071 DSM 41876$^T$, sharing 99.2% similarity. Other close relatives included: *Streptomyces lydicus* ATCC 25470$^T$ (98.9%) and *Streptomyces albulus* IMC S-0802$^T$ (98.7%).

### Production of hydrolytic enzymes

Figure 4 shows that optimum production of each enzyme type occurred after different incubation periods. Based on this, and with the objective of testing a crude extract with

**Table 1. Preliminary tests for *Streptomyces* strain selection**

| Streptomycete strain | Dual culture | | Inhibition of sclerotia germination | Chitinolytic activity at 25°C (U/ml) (after three days cultivation) | |
|---|---|---|---|---|---|
| | Antagonism[a] | Viability[b] | | Exochitinase | Endochitinase |
| 80 | +++ | - | + | 0.85 | 0.87 |
| 70 | +++ | + | + | 0.15 | 0.81 |
| M08 | ++ | + | + | 0.15 | 0.84 |
| Q11 | +++ | + | + | 0.40 | 0.13 |

[a] Antagonism test: length of the inhibitory zone according to Δ γ: Δ γ ≥ 5 mm = +; Δ γ ≥ 10 mm = ++; Δ γ ≥ 20 mm = +++; (See text for details).
[b] Viability of fungal plugs obtained from the inhibitory zone of the dual culture, still containing some hyphae.

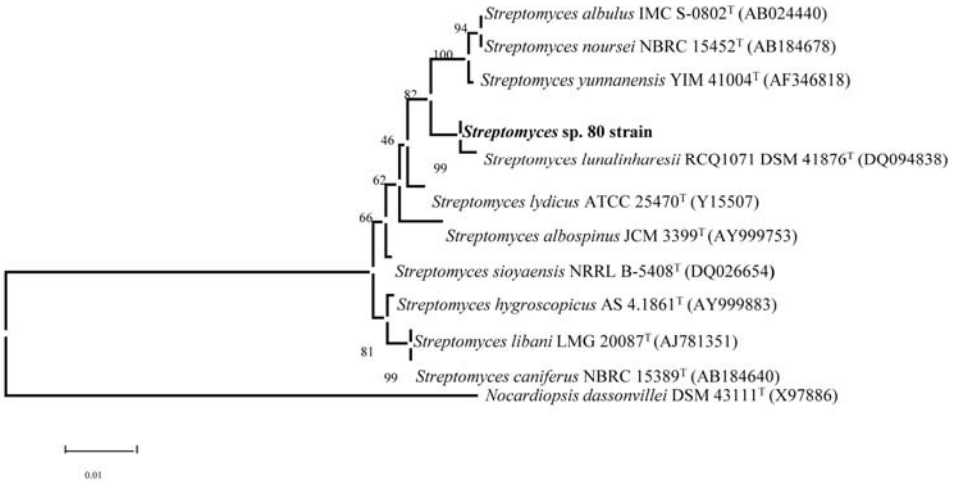


**Fig. 3. Phylogenetic placement of *Streptomyces* strain sp. 80.** Neighbor-joining tree based on 16S rRNA gene (*rrs*) sequences (>1300 bp) placing the strain studied in this work within the genus *Streptomyces*. Numbers at the nodes indicate levels of bootstrap support, the % based on an analysis of 1000 re-sampled datasets. The scale bar corresponds to 0.01 substitutions per nucleotide position.

all enzymes present, the third day was considered the most likely to obtain a crude extract efficient against the degradation of the cell wall of *S. sclerotiorum in vivo*. The enzymatic activities on the third day were 0.75 U/ml for exochitinase, 0.9 U/ml for endochitinase, 0.16 U/ml for β-1,3-glucanase and 1.78 U/ml for peptidase. Zymograms presented in Fig. 5A showed that *Streptomyces* sp. 80 produced two chitinases, one with apparent molecular mass of approximately 45.8 kDa, and another with a high apparent molecular mass of 206.8 kDa. A single β-1,3-glucanase was also detected, with an apparent molecular mass of approximately 55 kDa (Fig. 5B). Concerning the peptidases, zymograms revealed seven different hydrolysis zones, with apparent molecular masses varying from 108 to 15 kDa (Fig. 5C).

### Sclerotia viability tests using *Streptomyces* sp. 80

Sclerotia treated with crude enzymatic extract from *Streptomyces* sp. 80 were not viable even after 7 days cultivation in Neon-S medium. The two other fractions tested (ten fold concentrated and the excluded ones) were also both active against sclerotia, indicating the presence of bioactive molecules in both ranges, with MM > 10 kDa or <10 kDa. All the controls ran correctly, indicating that sclerotia were originally viable and TLE medium did not interfere with the viability. Each triplicate gave the same result. When sclerotia were buried in an artificial substrate for 30 days in the presence of *Streptomyces* sp. 80 spores, 67% were completely degraded or, when recovered, were not viable, their morphology being visibly altered. In the control experiment, only 25% of the sclerotia were not viable and none were completely degraded.

### Microscopic observations

Microscopic observations of *S. sclerotiorum* hyphae co-cultured with *Streptomyces* sp. 80 (Fig. 6) showed morphological alterations, with hyphal deformation, and a lytic effect with visible loss of hyphal content.

## Discussion

Four chitinolytic streptomycete strains were tested for antagonism against *Sclerotinia sclerotiorum*. During the pre-

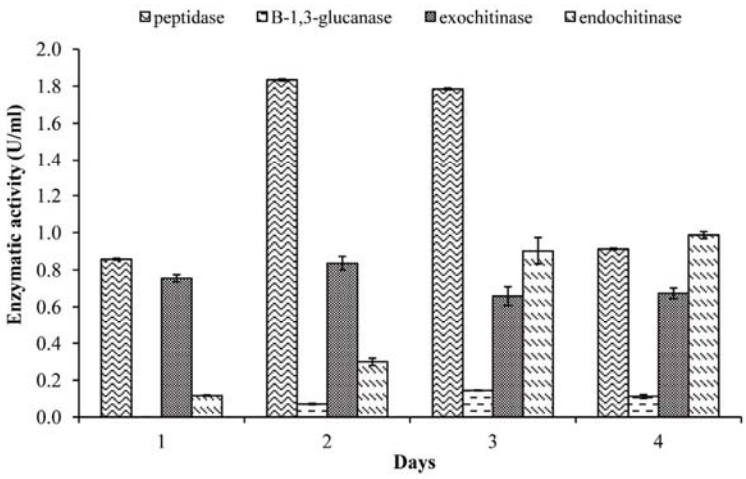


**Fig. 4. Kinetics of peptidase, β-1,3-glucanase, exochitinase, and endochitinase production by *Streptomyces* sp. 80 (indicated in the legend above).** The strain was grown, in triplicate, in TLE medium containing 0.5% dried mycelium of *S. sclerotiorum* during 4 days at 28°C/200 rpm. Error bars indicate one standard deviation.

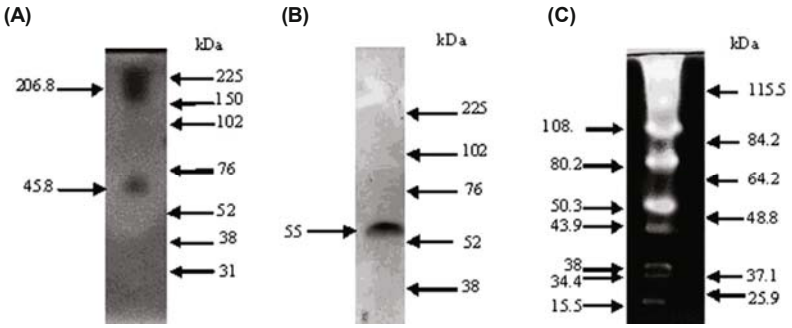


**Fig. 5. Zymogram analysis of chitinase (A), β-1,3-glucanase (B), and peptidase (C) activity in the concentrated crude extract of *Streptomyces* sp. 80;** Numbers and arrows on the right of the gel strips indicate relative molecular mass (in kDa). Numbers and arrows on the left indicate the enzymes detected and their relative molecular mass.

liminary tests, *Streptomyces* sp. 80 presented the best antagonistic effects over the different conditions investigated. In the dual culture in Petri dishes, it was the only one capable of growing and inhibiting *S. sclerotiorum* at 22°C. This temperature is lower than optimal for streptomycete growth, in general, but within the optimal growth range for *S. sclerotiorum* (20–25°C) (Young *et al.*, 2004). It was also the only strain capable of irreversible inhibition of *S. sclerotiorum* growth, according to the mycelium viability test, thereby confirming a fungicidal effect (El-Tarabily *et al.*, 2000). Although the four strains were effective in inhibiting *S. sclerotiorum* sclerotia, and three of them were good endochitinase producers, *Streptomyces* sp. 80 was the only one able to produce exochitinase in significant amounts. Molecular characterization of *Streptomyces* sp. 80 has shown that *Streptomyces lunalinharesii* RC1071 DSM 41876$^T$ was its most closely related strain, sharing 99.2% similarity. It is interesting to comment that *Streptomyces lunalinharesii* RC1071 was first isolated from a Brazilian soil by our group and characterized as a new species (Souza *et al.*, 2008). The strain was able to produce a thermophilic endochitinase, already purified, and its antagonism against other phytopathogenic fungi such as *Aspergillus parasiticus* has already been reported (Gomes *et al.*, 2001). However, it should be stressed that similarities as high as 99.4% have been observed between strains considered to be different species (Dastager *et al.*, 2008), and a DNA-DNA hybridization will be necessary to confirm whether or not *Streptomyces* sp. 80 is a strain belonging to the type strain *Streptomyces lunalinharesii* RC1071. In this study, antibiotic production was not evaluated, however, the excluded fraction (MM<10 kDa) taken after ultrafiltration of the crude extract of *Streptomyces* sp. 80 was active against *S. sclerotiorum* sclerotia, indicating the possibility of an antibiotic mechanism of action. In addition, *Streptomyces* sp. 80 was able to produce many hydrolytic enzymes, when grown in TLE medium containing 0.5% of *S. sclerotiorum* mycelium. Biological control agents are capable of reducing diseases by several mechanisms. These include antibiotics, toxic metabolites and siderophore production, hyperparasitism, induction of resistance, as well as nutrient competition and secretion of cell wall-degrading enzymes (El-Tarabily, 2006; Prapagdee *et al.*, 2008). For several authors the success of various phytopathogenic antagonists has been attributed to their ability to produce chitinases and glucanases (El-Tarabily, 2006) and also, in some cases, peptidases (De Marco and Felix, 2007). In this study, although antibiotic production was not evaluated, it was observed that the excluded fraction (MM<10 kDa) from the ultra-filtration of the crude extract of *Streptomyces* sp. 80 was active against *S. sclerotiorum* sclerotia, indicating the possibility of antibiotic activity. In addition, *Streptomyces* sp. 80 was able to produce several hydrolytic enzymes, when grown in TLE medium containing 0.5% of *S. sclerotiorum* mycelium. This could be considered a rich medium, containing mineral salts plus bactopeptone, urea and glucose, however the amount of the fungal mycelium present is far more significant and, apparently, these rich ingredients have permitted the actinomycete to grow and didn't interfere with the induction of the cell wall-degrading enzymes. The third day was considered optimum for the production of a crude extract with all enzymes present. The values of endochitinase and exochitinase thus obtained were higher than those obtained by other authors using crude extracts of actinomycetes, but they were cultivated in other substrates, and different methods for detection of enzymatic activity were used (El-Tarabily *et al.*, 2000; De Marco *et al.*, 2003; Souza *et al.*, 2003). Earlier, our group detected endochitinase activity in the crude extract of *Streptomyces lunalinharesii* (strain RC1071) (Gomes *et al.*, 2000), which is the most closely related type strain to *Streptomyces* sp. 80. Values were about 10 times lower than those now obtained, using the same methodology, but colloidal-chitin was the carbon

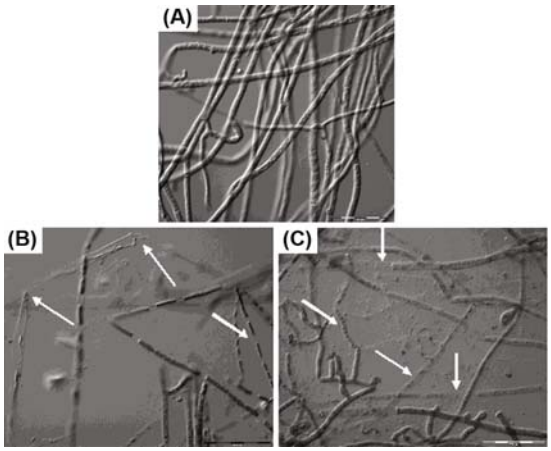


**Fig. 6. Morphology of *S. sclerotiorum* hyphae in an actively growing co-culture with *Streptomyces* sp. 80 after 4 days at 22°C (120 rpm).** (A) healthy mycelium of *S. sclerotiorum* (control); (B and C) loss of hyphal content and deformation (indicated by white arrows). Bar=50 µm.

source used for growth. According to the zymograms *Streptomyces* sp. 80 produces two chitinases. One with molecular mass of approximately 45.8 kDa, different from the purified endochitinase (70 kDa) produced by *S. lunalinharesii* strain RC1071 (Souza *et al.*, 2008) but within the range currently described for *Streptomyces* (Han *et al.*, 2009). The other with a high molecular mass, 206.8 kDa, which is not common among chitinases, but could correspond to monomeric associations, as suggested for other hydrolytic enzymes (Sunna and Antranikian, 1997). Several glucanolytic actinomycetes have recently been described in studies focusing on biological control. El-Tarabily detected β-1-3 glucanase production in non-streptomycetes isolated from the cucumber rizosphere (El-Tarabily, 2006), and from several endophytic actinomycetes antagonistic to *Pythium aphanidermatum* (El-Tarabily *et al.*, 2009). Their strains were cultivated in minimal medium containing laminarin and the maximum enzyme activity values obtained were around 6.0 U/ml. Earlier, Valois *et al.* (1996) using the same medium, detected β-1,3-glucanase production varying between 0.015 and 2.21 U/ml from actinomycete antagonists to *Phytophora fragariae*. Our results were within 0.07 and 0.14 U/ml range; however, fungal mycelium was used as substrate instead, and assays were performed using different substrates. In our studies, a single β-1,3-glucanase was detected in the zymogram, with a molecular mass of approximately 55 kDa, different from that described by Monteiro and Ulhoa (2006), who observed a 75 kDa β-1,3-glucanase produced by *Trichoderma koningii,* grown in *Rhizoctonia solani* cell wall medium. It is also different from that described by Leelasuphakul *et al.* (2006), produced by *Bacillus subtilis* NSRS 89-24, having a molecular mass of 95.5 kDa. Indeed, there are no reports in the literature about zymograms or molecular masses of glucanase activity produced by actinomycetes. Reports describing the action of peptidases on inhibition of phytopathogenic fungi and/or their utilization in biocontrol are scarce. Dunne *et al.* (1997) have demonstrated that the biocontrol of *Pythium ultimum* in the rhizosphere of sugar beet by *Stenotrophomonas maltophila* was due to the production of extracellular peptidases. De Marco *et al.* (2003) have described peptidase production (2.0 U/ml) by *Trichoderma harzianum* 1051 with antagonistic activity against *Crinipelis perniciosa*. *Streptomyces* sp. 80 was able to produce 7 different peptidases detected as hydrolysis zones, having molecular masses varying from 108 to 15 kDa. Although biological control of *S. sclerotiorum* has been proposed by some authors (Kamensky *et al.*, 2003; Gupta *et al.*, 2006; Hou *et al.*, 2006) none of them have dealt with the role of peptidases in this process. The results now obtained indicate that fungal mycelium may have an important inducer role for cell wall hydrolytic enzymes, as has been suggested earlier by Monteiro and Ulhoa (2006). Sclerotia are resistant structures produced by some fungal pathogens, remaining viable in soil for years, which makes disease control more difficult (Hou *et al.*, 2006). Thus, it would be interesting to select biocontrol agents capable of acting with efficiency on this kind of resistant structure. Both experiments performed for testing the ability of *Streptomyces* sp. 80 to inhibit sclerotia were favorable. *In vitro*, using the crude extract from the microbial growth, or *in vivo*, when the sclerotia were buried in soil along with the *Streptomyces* spores. These preliminary results suggest that *Streptomyces* sp. 80 can be effective in degrading *S. sclerotiorum* sclerotia, however a more detailed study, subject to statistical analysis, must be performed. It is well known that *Streptomyces* species are capable of mycoparasitizing fungal spores and hyphal structures (Sutherland and Papavizas, 1991). Microscopic observations of *S. sclerotiorum* hyphae co-cultured with *Streptomyces* sp. 80 suggest some morphological alterations. The *S. sclerotiorum* cell wall is largely composed of chitin, β-glucans, and also proteins (Jones, 1970) so it is supposed that chitinases, β-1,3-glucanases and peptidases produced by the *Streptomyces* sp. 80 may play a crucial role in the degradation of the fungal cell wall and could be involved in the observed antagonism. Although several studies involving biological control of phytopathogenic fungi are described in the literature, research on the use of actinomycetes acting against *S. sclerotiorum* is scarce (Tahtamouni *et al.*, 2006) and there seems to be a gap in our knowledge in this area. In conclusion, we have selected a streptomycete strain, *Streptomyces* sp. 80, which was able to inhibit *S. sclerotiorum* growth in its mycelial form, and also sclerotia germination. When grown in TLE medium containing the fungal mycelium for three days at 28°C/200 rpm, the strain produced the four main fungal cell wall-degrading hydrolases (exochitinase, endochitinase, β-1,3-glucanase and peptidases) after three days cultivation in the presence of the fungal mycelium. *Streptomyces* sp. strain 80 seems to be a promising strain for biological control against *S. sclerotiorum,* however, further *in vivo* experiments will be necessary to evaluate its real potential.

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