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ORIGINAL ARTICLE

Greek indigenous streptomycetes as biocontrol agents against the soil-borne fungal plant pathogen *Rhizoctonia solani*

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Abstract

Aims: To examine the biocontrol potential of multiactive Greek indigenous *Streptomyces* isolates carrying antifungal activity against *Rhizoctonia solani* that causes damping-off symptoms on beans.

Methods and Results: A total of 605 *Streptomyces* isolates originated from 12 diverse Greek habitats were screened for antifungal activity against *R. solani* DSM843. Almost one-third of the isolates proved to be antagonistic against the fungus. From the above isolates, six were selected due to their higher antifungal activity, identified by analysing their 16S rRNA gene sequence and studied further. The obtained data showed the following: firstly, the isolates ACTA1383 and ACTA1557 exhibited the highest antagonistic activity, and therefore, they were selected for *in vivo* experiments using bean seeds as target; secondly, in solid and liquid culture experiments under optimum antagonistic conditions, the medium extracts from the isolates OL80, ACTA1523, ACTA1551 and ACTA1522 suppressed the growth of the fungal mycelium, while extracts from ACTA 1383 and ACTA1557 did not show any activity.

Conclusions: These results corresponded important indications for the utility of two Greek indigenous Streptomyces isolates (ACTA1557 and ACTA1383) for the protection of the bean crops from *R. solani* damping-off symptoms, while four of them (isolates OL80, ACTA1523, ACTA1551 and ACTA1522) seem to be promising producers of antifungal metabolites.

Significance and Impact of the Study: This is the first study on the biocontrol of *R. solani* using multiactive Streptomyces isolates originated from ecophysiologically special Greek habitats. Our study provides basic information to further explore managing strategies to control this critical disease.

Introduction

Among the most common phytopathogenic fungi, *Rhizoctonia solani* Kühn [teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk; basidiomycetes] is an important soil-borne pathogen responsible for the 'damping-off' of many widely cultured plants, such as potato and tomato plant (De Curtis *et al.* 2010; Lahlali and Hijri 2010; Montealegre *et al.* 2010), bean plant (Balali and Kowsari 2004; Godoy-Lutz *et al.* 2008; Nerey *et al.* 2010) and cotton (Abd-Elsalam *et al.* 2010) thus constituting a financial threat for farmers. The 'damping-off' symptom is characterized by the disability of seeds to shoot or by the mortification of seedlings either before or after their emergence. Especially for bean plants, 'damping-off' means the sudden decay of the young seedlings of the plant, a few days after their emergence (Balali and Kowsari 2004).

The pathogen is characterized by significant ecological advantages such as an extremely broad host range and a high survival rate of sclerotia, under various environmental conditions, and therefore, its control is difficult to accomplish. Currently, *Rhizoctonia* diseases are not adequately controlled and their severity can only be limited

through a combination of cultural and crop protection strategies. For instance, planting seeds in warm soils and covering them with as little soil as possible speeds the sprouting and development of the stem while reducing the risk of stem canker. Farmers also use chemical control and several products like azoxystrobin (Amistar; Syngenta), chlorothalonil (Daconil 2787; Aventis), cymoxanil (Curzate 50; Dupont), flutolanil (Monarch; Aventis), pencycuron (Monceren; Bayer) and propamocarb (Previcur N; Aventis) (van den Boogert and Luttikholt 2004), which have been developed for this purpose. They concern both seed treatment and soil application, although they resulted in poor *Rhizoctonia* control (Wharton *et al.* 2007).

In Greece, *Rhizoctonia solani* harms tobacco plants (northern Greece), tomato plants (northern and central Greece) and bean plants (central and southern Greece). The control of the soil-borne plant pathogens, including *R. solani*, is based mainly on cultural practices like decrease in soil moisture, soil coverage and the use of phytopathogen-resistant hybrids. Greek farmers also use chemical disinfectants with no significant effect, for example, metham sodium (Vapam), quintozene + etridiazole as Terrachlor Super-X and methyl bromide either prior to or after the infection, but their use is limited because of their high cost and their strong toxicity (Marouli and Tzavella-Klonari 2002). Also, due to the lack of coordination between the Greek Ministry of Agriculture and the agricultural cooperatives, the flow of information about treatment procedures is obscure and inadequate.

The increasing concern for environmental protection and demand for organic farming drives research towards alternative control measures, such as the use of natural antagonists to biologically control plant pathogens (De Curtis *et al.* 2010; Hernández-Suárez *et al.* 2011).

Actinobacteria and particularly members of the genus *Streptomyces* are characterized by their complex morphological differentiation and the ability to produce a wide variety of secondary metabolites (Challis and Hopwood 2003). These micro-organisms can be found in the rhizosphere of several plant species (Crawford *et al.* 1993; Kortemaa *et al.* 1994; Tokala *et al.* 2002; Ramakrishnan *et al.* 2009) behaving as endophytes that occur within the roots of barley (Sadeghi *et al.* 2009; Kluth *et al.* 2010) or the stems of potato (Sessitsch *et al.* 2002). Plant root exudates stimulate rhizosphere growth of streptomycetes that are strongly antagonistic to fungal pathogens (Yuan and Crawford 1995).

Several *Streptomyces* species such as *S. lydicus, S. lividans, S. olivaceoviridis, S. scabies, S. plicatus, S. hydroscopicus, S. violaceusniger, S. humidus, S. avermitilis, S. aurofaciens* and *S. roseoflavus* are well-known producers of important compounds that are active against a wide variety of fungal pathogens (Taechowisan *et al.* 2009). These include a wide range of antibiotics as well as a variety of enzymes (i.e., chitinases), which degrade the fungal cell wall (Chamberlain and Crawford 1999; Gomes *et al.* 2000; Hwang *et al.* 2001; Getha and Vikineswary 2002; Taechowisan *et al.* 2003; De Souza *et al.* 2008). Metabolites from streptomycetes have been used in agriculture as growth promoters (Igarashi *et al.* 2000; El-Tarabily 2008; Ichinose *et al.* 2008; Schrey and Tarkka 2008) and selected strains of the genus also have been used as direct biocontrol agents for other plant diseases (Yuan and Crawford 1995; Neeno-Eckwall *et al.* 2001; Shekhar *et al.* 2006; Godoy-Lutz *et al.* 2008; Bakker *et al.* 2010).

The Greek territory, due to its geographical position that is characterized by the Mediterranean climate conditions, has been proved to be a rich habitat for streptomycete populations with biotechnological interest (Katsifas *et al.* 1999, 2000; Baur *et al.* 2006; Paululat *et al.* 2008, 2010).

In this study, we aimed to select Greek *Streptomyces* isolates from the Athens University Microbiology Laboratory Culture Collection for their antifungal activity against *Rhizoctonia solani* DSM843. Two of them were used for *in vivo* studies to control the phytopathogenic fungus *R. solani* DSM843 using the plant *Phaseolus vulgaris* L. (Fabaceae) as a model fungal target. In addition, medium extracts from solid and liquid cultures of selected isolates were investigated for their antifungal activity. Gel filtration fractions of the above extracts were also used for *in vitro* antifungal assays to provide initial information on the molecular features of the possible bioactive compounds.

Materials and methods

Microbial strains

A total of 605 bacterial isolates assigned to the genus *Streptomyces* on the basis of their phenotypic characteristics (Herron and Wellington 1990) were screened *in vitro* for antifungal activity against the phytopathogenic fungus *R. solani* DSM843 (Table 1). These strains were derived from the Athens University Microbiology Laboratory Culture Collection and have been isolated from 12 different Greek habitats using selective media (Katsifas *et al.* 1999). According to the 12 selected habitats, the samples are grouped into soil samples from the rhizospheres of indigenous plants (Table 1A) and nonrhizosphere samples (Table 1B). All isolates were maintained as spore suspensions in 30% (w/v) glycerol at −20°C (Herron and Wellington 1990).

Table 1 *Streptomyces* strains and their antifungal activity from each of the 12 studied Greek habitats

Sampling area	Number of isolates tested	Number (percentage) of isolates with antifungal activity against *Rhizoctonia solani* DSM843 {highest – lowest – average}*
(A) Rhizosphere samples		
1. Rhizosphere of *Ebenus sibthorpii*	39	10 (25·6%) {7·4; 1·3; 4·5}
2. Rhizosphere of *Ceratonia silicva*	47	9 (19·1%) {5·4;1·3; 2·8}
3. Rhizosphere of *Olea europea*	75	25 (33·3%) {9·0; 1·3; 4·1}
4. Rhizosphere of *Abies cefalonica*	20	0 (0·0%)
5. Rhizosphere of *Pinus brutia* from Crete	24	12 (50·0%) {9·7; 1·9; 4·5}
6. Rhizosphere of evergreen woody shrubs from an island of the Aegean Sea	22	13 (59·1%) {11·5; 1·3; 5·0}
7. Rhizosphere of evergreen woody shrubs from an island of the Ionian Sea	30	0 (0·0%)
8. Rhizosphere of coniferous trees (Arcadian forest)	100	26 (26·0%) {7·6; 1·4; 3·7}
Rhizosphere subtotals	357	95 (26·6%)
(B) Nonrhizosphere samples		
9. Hot spring water of thermopiles thermal springs (Viotia District)	5	5 (100·0%) {7·0; 2·2; 5·1}
10. Sediment from a volcanic area (Santorini Island – Aegean Sea)	30	1 (3·3%) {5·6; 5·6; 5·6}
11. Soil derived from cultivated area (Marathon, Attica District)	186	100 (53·8%) {9·2; 1·3; 3·9}
12. Soil from protected natural forest area (Kessariani, Attica District)	27	12 (44·4%) {9·6; 2·5; 5·4}
Nonrhizosphere subtotals	248	118 (47·6%)
Total	605	213 (35·2%)

*Antagonistic activity levels as expressed by the quotient of the inhibition zone area over streptomycete colony area (See *In vitro* antagonism bioassays).

Rhizoctonia solani DSM843 that was used as target fungus for the antagonism bioassays belonged to the anastomosis group 1 (AG-1) and was maintained on potato dextrose agar (PDA) suggested by DSMZ, Germany, at 4°C.

Preparation of inoculum of biocontrol agents and fungi

Streptomycete aliquots (30 μl) from a spore suspension in 30% (w/v) glycerol were used as inoculum for all *in vitro* antagonism bioassays. For the same test, we used two full loops of *R. solani* mycelium from a 5-day-old culture on PDA.

For *in vivo* antagonism tests, a suspension of streptomycetes spores in Ringer ¼ salt solution (NaCl 2·15 g l^{-1}, KCl 0·15 g l^{-1}, $CaCl_2$ 0·075 g l^{-1}, K_2HPO_4 0·5 g l^{-1} according to Wellington *et al.* 1990) (10^9 spores per ml) was prepared from a 5-day-old culture on arginine–glycerol–salt agar (AGS), as described by Herron and Wellington (1990), and used for the bean seed treatments. *Rhizoctonia solani* was cultured in nutrient broth (NB, Biokar Diagnostics, Beauvais, France) for 5 days at 28°C and 180 rpm. The mycelium was aseptically collected on filter paper and washed with three culture volumes of deionized sterile water. Three grams of wet mycelium (dry weight, 15–18% w/w) was resuspended in 1000 ml deionized sterile water and was thoroughly mixed with 1 kg of either sterile or nonsterile soil (Lu *et al.* 2004).

In vitro antagonism bioassays

Antifungal antagonism was determined using a modified agar plate antagonism bioassay (Crawford *et al.* 1993). All streptomycetes were spot inoculated in the centre of NA agar plates (triplicate plates). Plates were incubated at 28°C for 2 days prior to fungal inoculation. The phytopathogenic fungus was inoculated in two antidiametrical positions, 1 cm from the plate edge. Following fungal inoculation, the plates were incubated at 28°C for 5 days. Antagonistic activity of the streptomycetes was determined by measuring the inhibition zone, the presence of which characterized the strain as positive.

Antagonism strength was determined by averaging (triplicate plates per strain x three independent experimental sets) the quotient of the area of the inhibition zone, which was formed around the streptomycetes colony, over the area of the streptomycetes colony itself [Antifungal activity = $\pi R_z^2/\pi R_{str}^2$ (R_z = radius of

inhibition zone and R_{str} = radius of streptomycetes colony, modified from Seeley *et al.* 1990)].

Taxonomy of streptomycetes

The 22-mer BOX A1R oligonucleotide (5′-CTACGGCAA GGCGACGCTGACG-3′) was used to generate BOX-PCR profiles (Versalovic *et al.* 1991; Martin *et al.*, 1992). Amplification reactions were performed in volumes of 25 μl, containing 2 μmol l^{-1} of the single BOX primer, 200 μmol l^{-1} each of dATP, dCTP, dGTP and dTTP (Bioprobe Systems/Quantum, Paris, France), PCR buffer [10 mmol l^{-1} Tris–HCl (pH 9·0), 50 mmol l^{-1} KCl, 1·5 mmol l^{-1} $MgCl_2$, 0·1% Triton X-100 and 0·2 mg ml^{-1} bovine serum albumin], 1·5 units of Taq DNA polymerase (Biotools, Surrey, UK) and 40 ng template DNA. After initial denaturation for 7 min at 95°C, samples were cycled for 35 cycles using the following profile: denaturation for 1 min at 94°C, primer annealing for 1 min at 53°C and primer extension for 8 min at 65°C, with a final elongation step of 16 min at 65°C. The BOX-PCR was repeated twice and yielded consisting results. We analysed the BOX-PCR profile of the isolates that showed the highest *in vitro* antifungal activity and selected for further studies. The same isolates were further characterized through the amplification of their 16S rRNA gene. The 16S rDNA fragment was amplified by PCR using two universal primers (Edwards *et al.* 1989; Lane 1991): pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and R1492 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′). Amplification reactions were performed in volumes of 50 μl containing 40 ng template DNA, 0·4 μmol l^{-1} of each primer, 1X buffer with Mg^{2+}, 1 unit of Taq DNA Polymerase (Biotools) and 0·2 mmol l^{-1} dNTPs. Nucleases-free water was used to bring the reaction volume to 50 μl. After initial denaturation at 95°C for 2 min, samples were cycled for 30 PCR cycles using the following cycle profile: 95°C denaturation for 30 s, primer annealing at 53°C for 30 s and primer extension at 72°C for 2 min, plus a final 2-min elongation step at 72°C. Amplified PCR products were separated by gel electrophoresis on 1·2% (w/v) agarose gel and then purified using Nucleospin® Extract PCR kit (Macherey-Nagel, Düren, Germany). The 16S rDNA fragment (>1400 bp) was fully sequenced (Macrogen, Seoul, Korea), and the results were used for strain identification following comparison with existing sequences of *Streptomyces* type strains (Altschul *et al.* 1997).

In vivo antagonism bioassays

Two *Streptomyces* isolates (ACTA1383 and ACTA1557) were selected for *in vivo* experiments due to the strong suppression they caused to *R. solani* DSM843 growth *in vitro*. A sandy silt loam soil (ASTM classification) with a pH of 7·9 taken from an area under intense agricultural exploitation in the Marathon area (42 km NE from the centre of Athens) was used. Prior to its use, the soil was air-dried in the dark at 22°C for at least 3 months, passed through a 2-mm sieve and autoclaved twice (121°C, 60 min) on two separate days.

Bean seeds were sterilized for 30 min in a 20% (w/v) chlorine suspension and then dried under sterile conditions. A number of sterile seeds were immersed into a suspension of streptomycetes spores in Ringer ¼ salt solution (Wellington *et al.* 1990) (10^9 spores per ml) for 30 min and then dried under sterile conditions. Untreated sterile bean seeds and sterile bean seeds treated with the selected streptomycetes were planted in pots containing sterile soil amended with *Rhizoctonia solani* (3 g of wet washed mycelium per kg of soil) or not (Lu *et al.* 2004). For every treatment, 24 seeds were planted in each pot (three replicates for each pot were prepared). Each full experiment was conducted in four different occasions, over a time period of 8 months. The pots were incubated at 28°C under fluorescent light, and moisture was controlled daily at the level of 40% (w/w) for 25 days. The number of seeds that survived and/or germinated was evaluated to estimate the ability of the examined streptomycetes to control the fungi *in vivo*. In addition, the height and weight of the emerged plants were measured for the estimation of the *in vivo* antagonism strength. The same set of experiments was carried out using nonsterile soil of the same origin.

Extraction and fractionation of streptomycetes metabolites from solid and liquid cultures

In parallel, the *Streptomyces* isolates that showed the highest antifungal activity *in vitro* were grown on SAB [Streptomyces antibiotic broth (Atlas 1993)] because it was selected as optimum medium for high antifungal activity expression by the *Streptomyces* isolates. The cultures were incubated at 28°C for 7 days in 1000-ml Erlenmeyer flasks containing 500 ml of liquid medium on orbital shaker S03, at 180 rpm. 500 μl of 10^8 spores ml^{-1} suspension was used as inoculum. Cultures were centrifuged (Biofuge 28RS; Heraeus, Hanau, Germany) at 9000 ***g*** for 20 min. Supernatant was collected, concentrated by lyophilization (1 : 100) and filtered (0·45 μm). For the determination of antifungal activity, 200 μl from the concentrated culture supernatant was placed into wells on SAA (Streptomyces antibiotic agar) plates (formed using a cork borer – diameter 1 cm, depth 1 cm) that were inoculated with the fungus.

Additionally, the inhibition zones on SAA plates were removed and blended for 3 min. The slurry was centrifuged at 4000×***g*** for 60 min and the supernatant was collected. After filtration, 200 μl was placed in a similar manner into wells on SAA agar plates inoculated with the fungus for the determination of the antifungal activity.

The extract from the solid culture of the four *Streptomyces* isolates (OL80, ACTA1523, ACTA1551 and ACTA1522) was fractionated into a high molecular weight (protein) and a low molecular weight (nonprotein) component on a PD-10 gel filtration column (GE Healthcare, Athens, Greece) using de-ionized water for elution, according to the manufacturer's recommendation. Each fraction was concentrated by lyophilization and examined for antifungal activity.

Data analysis

Statistical analysis of the various data sets was conducted through one-way ANOVA (with *post hoc* pairwise multiple comparisons by the Holm–Sidak method) and unpaired *t* tests using SigmaStat/Plot software program (ver. 12.0; Systat Software Inc., Chicago, IL, USA). In all runs, a significance level of <0·05 was used.

Results

In vitro antifungal activity of the streptomycetes

A total of 213 strains of 605 (Athens University Microbiology Laboratory Culture Collection) showed *in vitro* antagonistic activity against *R. solani* DSM843 (Table 1). None of the isolates from *Abies cefalonica* rhizosphere (sampling area 4) or from the rhizosphere of evergreen shrubs of Ionian Sea Island (sampling area 7) were able to suppress the phytopathogenic fungus, while only one isolate from the area of Santorini Island (sampling area 10) showed antifungal activity.

Analysing the level of antifungal activity of the antagonistic isolates, they ranged from the minimum detectable level of 1·3 to the maximum of 11·5. Comparing these results (Table 1), it was found that *Streptomyces* isolates with very high antagonistic activity against *R. solani* DSM843 originated from the rhizosphere of the indigenous plants *Olea europea*, *Pinus brutia* and evergreen shrubs spontaneous of the Aegean Sea Island (sampling areas 3, 5 and 6).

Elaboration of the above findings and taking into account the results from previous studies by our group (Katsifas *et al.* 1999, 2000; Paululat *et al.* 2008; Baur *et al.* 2006; Paululat *et al.* 2010; ACTAPHARM-Project, Final Report, 2005, http://cordis.europa. eu/library) led to the selection of six isolates, for further studies. One of them, encoded ACTA1383, was one among the 39 strains that were isolated from the rhizosphere of *Ebenus sibthorpii* (*Fabaceae*), an endangered endemic plant found in low numbers in the Kaisariani area, a preserved forest site, 4 km SE from the centre of Athens (Katsifas *et al.* 1999). Two of the selected isolates, encoded ACTA1557 and ACTA1551, were among the 24 isolates, found in large numbers, in the rhizosphere of *Pinus brutia,* from a forest with coniferous trees on Crete Island (Katsifas *et al.* 1999). The remaining three isolates, encoded OL80, ACTA1522 and ACTA1523, derived from the rhizosphere of *Olea europa*. All six isolates were among those that revealed the highest *in vitro* antifungal activity (>7) against *R. solani* DSM843 (Table 3). The antagonistic activities among the six isolates were statistically different as determined by one-way ANOVA ($F_{(5,12)} = 8{\cdot}2887$, $P = 0{\cdot}0014$). *Post hoc* paired comparisons (Holm–Sidak method) revealed statistically different antagonistic levels for all combinations of two among the six isolates ($P < 0{\cdot}05$), except for pairs that included any two among the ACTA1551, ACTA1523 and OL80 ($P > 0{\cdot}05$).

Considering the BOX-PCR fingerprints of the six selected micro-organisms, it was possible to group into four different profiles according to their bar code; three of these groups had only one representative (Fig. 1).

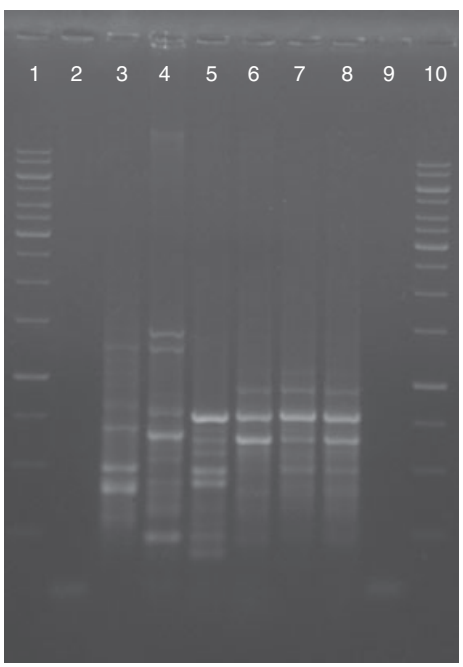

Figure 1 BOX-PCR-based fingerprinting analysis of the selected *Streptomyces* isolates. Lane 1: 1000-bp ladder, Lane 2: water (negative control), Lane 3: ACTA1383, Lane 4: ACTA1557, Lane 5: ACTA1551, Lane 6: ACTA1522, Lane 7: ACTA1523, Lane 8: OL80, Lane 9: water (negative control), Lane 10: 1000-bp ladder.

Three of the six isolates (ACTA1522, ACTA1523 and OL80) shared the identical BOX-PCR bar code and exhibited similar antifungal activity (Fig. 1). The rest of the isolates showed very high antifungal activity and revealed different BOX-PCR profiles, independently of their habitat of origin. 16S rRNA gene sequence data grouped the selected isolates to type strains of streptomycetes as shown in Table 2.

Table 3 Antagonistic activity levels of the selected isolates as expressed by the quotient of the inhibition zone area over streptomycete colony area (*In vitro* antagonism bioassays)

Streptomyces isolates	Antagonistic level
Streptomyces pseudovenezuelae ACTA1383	7·41 ± 0·05
Streptomyces fulvissimus ACTA1557	9·70 ± 0·40
Streptomyces rochei ACTA1551	9·01 ± 0·26
Streptomyces longisporoflavus ACTA1522	8·68 ± 0·09
Streptomyces longisporoflavus ACTA1523	9·08 ± 0·89
Streptomyces longisporoflavus OL80	9·01 ± 0·47

In vivo antifungal activity

Knowing that the culture medium is a crucial factor that can affect the antagonistic character that microbes express, we used the results from the *in vitro* antagonistic assay to lead us to the selection of ACTA1383 (*Streptomyces pseudovenezuelae*) and ACTA1557 (*Streptomyces fulvissimus*) so as to use them for *in vivo* studies. This selection was based on their very high antifungal activity expressed *in vitro* (Table 3) and the observation from previous work (Katsifas *et al.* 1999, 2000; Baur *et al.* 2006; Paululat *et al.* 2008, 2010) that they are multiproducers of bioactive substances. Thus, they characterized as promising biocontrol agents *in vivo*.

Analysis of particle size of the soil that used for this purpose indicated the presence (%, dry weight) of sand, 50; silt, 36; clay, 14. Mineralogy analysis showed the presence of (%, dry weight) illite, 65; chlorite, 7; kaolinite, 10; smectite, 12; talc, 6 and calcite <1. Phosphorus content was 124 mg l^{-1} dry soil and organic carbon was 1·23% (dry weight).

Macroscopic observation from these experiments indicated that, although the *R. solani* DSM843 mycelium was developed in all cases, the extent of mycelial growth was strongly dependent on the presence of the streptomycetes.

In sterile soil experiments, germination of the streptomycete-treated seeds was markedly increased when compared to the untreated seeds (Table 4A), because untreated seeds planted in sterile soil and infected with the fungus (negative control A) showed very low levels of germination (only the 2·7% of the planted seeds germinated) and very poor plant growth. Almost 40% of the streptomycete-treated seeds, planted in the same *R. solani* DSM843-infected sterile soil, were able to germinate (Table 4A). Comparison of germination results between the two streptomycete treatments did not yield any statistically significant differences. The mean weights of plants were very similar among the positive control and the two streptomyces-treated seeds. In fact, the plant weights between the positive control and ACTA1557-treated seeds did not significantly differ, while those of the ACTA1383-treated seeds were only 10% lower than the positive control. On the contrary, the plant heights of the streptomycete-treated seeds reached on average only the 15% the positive controls heights. This weight/height pattern of plants in the sterile soil experiment reflects the difference in the morphology of the treated seed plants that were generally 'shorter' and 'thicker' (Fig. 2).

The *in vivo* results for the nonsterile soil were similar to the sterile soil experiments as far as seed germination is concerned (Table 4B). The only statistically significant difference involved the reduced germination of the ACTA1557-treated seeds in nonsterile soil (as compared both against the same treatment in sterile soil and against ACTA1383 in nonsterile soil). This was also reflected in the plant weights where those of ACTA1557-treated seeds were significantly lower than those of the ACTA1383-treated seeds. Nonsterile soil environment in general, though, had a significant positive effect on plant weight and height of the positive controls and the streptomycete-treated seeds, when

Table 2 Taxonomy of the six selected streptomycetes by 16S rRNA gene sequence data

Streptomyces isolates	Closest phylogenetic relative (GeneBank Accession number)	% Identity*	GeneBank Accession number
ACTA1383	*Streptomyces pseudovenezuelae* (FR682807.1)	99·0	JN167527
ACTA1557	*Streptomyces fulvissimus* (AB184787.1)	99·0	JN167524
ACTA1551	*Streptomyces rochei* (HQ909756.1)	99·0	JN167525
ACTA1522	*Streptomyces longisporoflavus* (EF178687.1)	99·0	JN167526
ACTA1523	*Streptomyces longisporoflavus* (EF178687.1)	99·0	JN167528
OL80	*Streptomyces longisporoflavus* (EF178687.1)	99·0	JN167529

*The percentage identity with the 16 rDNA sequence of the closest phylogenetic relative.

Table 4 Germination data of bean seeds during *in vivo* antagonism bioassays. The total number of planted bean seeds was (24 per pot) × (three replicates for each pot) × (four independent experiments) = 288. Data from all experiments were combined for the analysis

Experimental set (abbreviation)	Total number (percentage of germinated seeds)	Average of germinated seeds per pot*	Mean weight of plants (g)†	Mean height of plants (cm)‡
(A) Sterile soil				
Positive control A (PcA)§	232 (80·6)	19·33 ± 0·58	1·41 ± 0·20	31·6 ± 4·32
Streptomyces fulvissimus ACTA1557 (1557A)	116 (40·3)	9·67 ± 0·58	1·30 ± 0·33	4·3 ± 0·75
S. pseudovenezuelae ACTA1383 (1383A)	100 (34·7)	8·33 ± 1·53	1·27 ± 0·22	3·95 ± 0·84
Negative control A (NcA)¶	8 (2·7)	0·67 ± 0·58	0·39 ± 0·27	0·92 ± 0·65
(B) Nonsterile soil				
Positive control B (PcB)§	252 (87·5)	21·00 ± 1·73	3·08 ± 0·43	36·4 ± 6·25
S. fulvissimus ACTA1557 (1557B)	76 (26·4)	6·33 ± 1·15	2·06 ± 0·52	33·5 ± 8·57
S. pseudovenezuelae ACTA1383 (1383B)	128 (44·4)	10·67 ± 1·53	2·75 ± 0·71	35·8 ± 9·90
Negative control B (NcB)¶	8 (2·7)	0·67 ± 0·58	0·32 ± 0·25	1·06 ± 0·91

*One-way ANOVA on the number of germinated seeds per pot: Sterile soil (A) groups: Differences in the mean values among the treatment groups [PcA, 1557A, 1383A and NcA] are statistically significant ($P = <0·001$), and the corresponding pairwise multiple comparisons (Holm–Sidak method) are all significantly different ($P = <0·001$) except 1557A *vs* 1383A, which was not statistically significant ($P = 0·155$). Nonsterile (B) soil groups: the differences in the mean values among the treatment groups [PcB, 1557B, 1383B and NcB] are statistically significant ($P = <0·001$) and the corresponding pairwise multiple comparisons (Holm–Sidak method) are all significantly different ($P = <0·001$). Pairwise comparisons among sterile (A) and nonsterile (B) groups: PcA *vs* PcB and 1383A *vs* 1383B, not significantly different ($P = 0·189$ and $0·135$, respectively). 1557B *vs* 1353B significantly different ($P = 0·011$).

†Unpaired *t*-test on plant weights: PcA *vs* 1557A and 1557A *vs* 1383A not significantly different ($P > 0·05$). PcA *vs* 1383A significantly different ($P < 0·001$). PcB *vs* 1557B or 1383B and 1557B *vs* 1383B, all significantly different ($P < 0·01$). PcA *vs* PcB, 1557A *vs* 1557B and 1383A *vs* 1383B all significantly different ($P < 0·001$).

‡Unpaired *t*-test on plant heights: PcA *vs* 1557A or 1383A significantly different ($P < 0·001$). 1557A *vs* 1383A, significantly different ($P < 0·02$). PcB *vs* 1557B or 1383B and 1557B *vs* 1383B all not significantly different ($P > 0·15$). PcA *vs* PcB, 1557A *vs* 1557B and 1383A *vs* 1383B all significantly different ($P < 0·001$).

§Positive control: Noninfected sterile (A) or nonsterile (B) soil planted with sterilized seeds.

¶Negative control: *Rhizoctonia solani*-infected sterile (A) or nonsterile (B) soil planted with sterilized seeds.

Figure 2 *In vivo* antifungal ability of the selected *Streptomyces* isolates in sterile soil experiments – Growth of the bean plants (from left to right). (a) Untreated seed planted in untreated sterile soil (positive control), (b) seed treated with *Streptomyces fulvisimus* ACTA1557 and planted in *Rhizoctonia solani* DSM843-infected soil, (c) seed treated with *Streptomyces pseudovenezuelae* ACTA1383 and planted in *R. solani* DSM843-infected soil and (d) untreated seed planted in *R. solani* DSM843-infected soil (negative control).

compared to sterile soil results. This was especially true for the heights of the plants that did not reveal any significant differences neither between the positive controls and the treated seeds nor between the two treatments (Table 4B and Fig. 3).

These results showed that *Streptomyces pseudovenezuelae* ACTA1383 proved more effective as a biocontrol agent against *Rhizoctonia solani* in nonsterile processes because seeds treated with its spore suspension promoted better growth of the bean plants resulting in plants with improved height and weight (Figs 2 and 3).

Streptomycetes metabolites from solid and liquid cultures; fractionation of the extracts

The concentrated extracts from the liquid cultures did not show any significant antifungal activity, while the extracts taken from the agar inhibition zones of four of the six isolates could strongly suppress the growth of *R. solani* DSM843 (Fig. 4).

Following fractionation of the bioactive extracts in a gel filtration column, the antifungal activity was observed in the low molecular weight fractions.

Discussion

Selected *Streptomyces* sp. has been used in several studies for the direct biocontrol of various plant diseases (Yuan

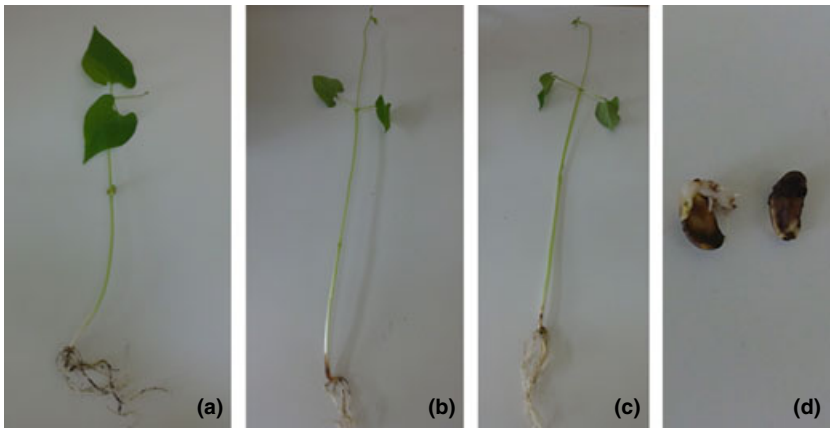

Figure 3 *In vivo* antifungal ability of the selected *Streptomyces* isolates in nonsterile soil experiments – Growth of the bean plants (from left to right). (a) Untreated seed planted in untreated sterile soil (positive control), (b) seed treated with *Streptomyces fulvisimus* ACTA1557 and planted in *Rhizoctonia solani* DSM843-infected soil, (c) seed treated with *Streptomyces pseudovenezuelae* ACTA1383 and planted in *R. solani* DSM843-infected soil and (d) Untreated seed planted in *R. solani* DSM843-infected soil (negative control).

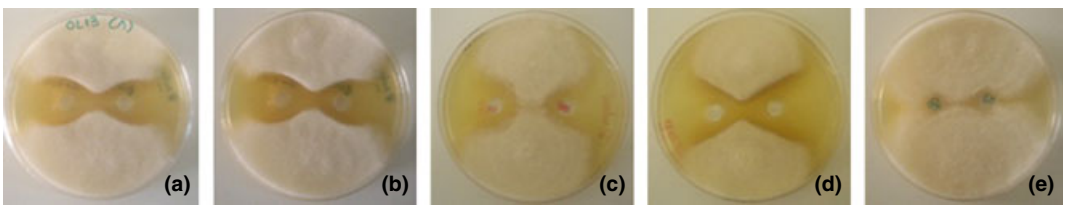

Figure 4 Antifungal activity of the concentrated medium extracts derived from the solid cultures of four *Streptomyces*. Inhibition zone caused from (a) *S. longisporoflavus* ACTA1522 extract, (b) *S. longisporoflavus* ACTA1523 extract, (c) *S. longisporoflavus* OL80 extract, (d) *S. rochei* ACTA1551 extract and negative control (no streptomycete extract added) (e).

and Crawford 1995; Abd-Allah 2001; Neeno-Eckwall *et al.* 2001; Getha and Vikineswary 2002; Shekhar *et al.* 2006). In addition, selected ecosystems such as tomato plant rhizosphere (Cao *et al.* 2004), banana roots (Cao *et al.* 2005) and wheat root tissues (Coombs and Franco 2003) have been recently used as a source for the isolation of streptomycetes with antifungal activity. However, studies involving the evaluation and comparison of indigenous streptomycete potential as biocontrol agents from a variety of different ecosystems are limited in the literature (Gomes *et al.* 2000; Neeno-Eckwall *et al.* 2001; Shekhar *et al.* 2006). The present work involved the screening of 605 *Streptomyces* isolates, originating from 12 different and important habitats within the Greek territory, against phytopathogenic fungus *R. solani* DSM843 and the evaluation of the *in vitro* and the *in vivo* antifungal activity of selected streptomycetes. Previous work on *Streptomyces* isolates of the Athens University Microbiology Laboratory Culture Collection used in this study (Katsifas *et al.* 1999) grouped them into 19 different clusters according to phenotypic identification with some species to be present in more than one habitat and some others to be unique in some sites.

The data of this work, additionally to the above studies, suggested that sampling areas 1, 3 and 5 (Table 1) were a rich pool of not only diverse species, but also active species with high antagonistic activity, a suggestion that may reflect the relation of their features with the habitat of origin.

Particularly, the results from the *in vitro* antifungal activity experiments showed that almost one-third of the examined isolates could be antagonistic to *R. solani* DSM843. Such a percentage (about 35%) justified that the genus of *Streptomyces* could be the most appropriate and promising target for biotechnological applications and especially for biocontrol uses. In addition, it could support a hypothesis that the Greek soil is a convenient substrate for hosting such microbial communities' biocontrol agents (Baur *et al.* 2006; Paululat *et al.* 2008, 2010). The latter is in agreement with the previous observations that the Greek *Streptomyces* isolates are multiactive compared to isolates from other countries (ACTAPHARM, Final Report, 2005, http://cordis.europa.eu/library).

The Aegean Sea climate, characterized by its high temperatures and low humidity, results in a soil poor in

nutrients. This fact creates conditions of environmental stress on the indigenous populations of streptomycetes and possibly leads to the prevalence of micro-organisms with antagonistic properties. Furthermore, combined with the high anthropogenic impact on the Aegean Sea Islands, this could probably explain the high percentages of isolates collected from the rhizosphere soil of the plant *Pinus brutia* (indigenous plant of Crete) and of the evergreen shrubs that were antagonistic to *R. solani in vitro*. On the contrary, both the rhizosphere of *Abies cefalonica* and evergreen shrubs in the Ionian Sea Islands, which were deliberately selected from areas less touristic and less anthropogenically disturbed, did not yield any isolates with antifungal activity obviously as a result of the very different climatic conditions of the soil of origin (areas of high humidity and low anthropogenic impact).

High percentages (53·8%) of isolates antagonistic to the phytopathogenic fungi were also collected from a nonrhizosphere sample of an agricultural area in Marathon (Attica District). This result again may be due to the fact that the soil of origin was characterized by medium–high temperatures and human activity influence (use of fertilizers and synthetic fungicides).

The results from the *in vivo* antagonism tests were encouraging as they supported the possible systematic use of the two selected *Streptomyces* strains for crop protection. It is known that the antagonistic profile of the micro-organisms is strongly depended on the growth conditions and there are differences between laboratory cultures and *in vivo* processes. In addition, *in vitro* assays are necessary for the screening of the potential antagonistic strains. Therefore, laboratory cultures were used as an original indication as it was the only way to choose the strains for further research. It was shown that the selected strains could express their antifungal activity *in vivo* quite well. Interestingly, these results are in agreement with those from previews studies on the potential of *Streptomyces* isolates to be used as biocontrol agents. For instance, Mahadevan and Crawford (1997) found that *Streptomyces lydicus* was identified as a broad spectrum biocontrol agent while the results from Farrag (2011) enhanced that finding. Moreover, Reddi and Rao (1971) reported that isolates of *Streptomyces ambofaciens* were able to control *Pythium* damping-off in tomato plants and *Fusarium* wilt in cotton plants, in an artificially infested soil. Rothrock and Gottlieb (1984) showed that *S. hygroscopicus* could effectively control *Rhizoctonia* root rot in pea plants in growth chambers, and Maldonado *et al.* (2010) proved that a *Streptomyces strain* originated from Argentina reduced damage caused by *P. digitatum* and *Geotrichum candidum* on citrus plants. These results correspond important indications for the utility of *Streptomyces fulvisimus* ACTA1557 and *Streptomyces pseudovenezuelae* ACTA1383 for the protection of the bean crops. Of additional importance is that through our experimental approach, the antifungal compounds excreted by the streptomycetes can provide their protective action to the plant just by simple coating of the seeds with spore suspension prior to sowing. This inoculation method has been proved more effective as the biocontrol agent can rapidly and extensively cover the surface of the seeds (Lu *et al.* 2004). Early colonization by a biocontrol agent often is required to fill the critical niches and to effectively compete against pathogenic fungi (Mitchell 1992). Thus, seed coating with bacterial and fungal biocontrol agents often is utilized or required to control aggressive, rapidly growing soil-borne pathogens, such as *R. solani* (Mitchell 1992; Crawford *et al.* 1993; Nerey *et al.* 2010). Additionally, this procedure is much easier to implement and more applicable for large scale cultivation, compared to the classic one that includes enrichment of the soil with the biocontrol agents, which is both time-consuming and difficult to apply in real farming conditions (Yuan and Crawford 1995; Whipps 2001). Moreover, the data from the nonsterile soil experiments enhanced the potential of the selected streptomycetes to be used as biocontrol agents in real farming conditions as it was shown that the coexistence of the examined micro-organisms, especially *Streptomyces pseudovenezuelae* ACTA1383, with the native microflora of the soil, made the former more effective. The type of soil that reflects on its microflora composition has been referred as an important factor that affects the results of *in vivo* biocontrol procedures. Suppressive soils contain microorganisms that are antagonistic to the pathogen or promote the growth of the target plant (Whipps 2001). The presence of these microbes, in combination with the high populations of the introduced biocontrol agents, can enhance the antimicrobial activity of the latter. Additionally, sterilization of soils by pasteurization, fumigation or autoclaving usually allows the pathogen to proliferate (Burgess *et al.* 1988), while in nonsterile soils, the native microflora slows up the growth of the pathogen giving the opportunity to the biocontrol agent to express its antimicrobial activity more effectively. This observation may lead to the conclusion that not only the germination of the seeds but also the growth of the plants could be enhanced *in situ* by planting bean seeds treated with *Streptomyces pseudovenezuelae* ACTA1383 in the crop. Some questions could be raised for the application of the selected *Streptomyces* isolates (*Streptomyces fulvisimus* ACTA1557 and *Streptomyces pseudovenezuelae* ACTA1383) as biocontrol agents against *Rhizoctonia solani* in real farming conditions, but the above results could be characterized as a promising first step against

the agricultural–commercial exploitation of the selected indigenous *Streptomyces* isolates, although the experiments were performed using only one type of soil and one plant type as fungal target.

Considering that the mechanism of the antifungal activity may vary among the production of antibiotic compounds, the promotion of plant growth, the induction of systemic resistant to plants and the mycoparasitism (Haas and Défago 2005), we examined, in parallel, the antifungal activity of both liquid culture concentrates and solid culture extracts, in an effort to evaluate the biochemical characteristics of the antifungal metabolites. The fact that antifungal activity was observed only in the solid culture extracts and not in the liquid culture supernatants may be attributed to the intrinsic differences between the two growth conditions that may very well result in different excretion phenotypes. For instance, in liquid cultures, the possible antifungal metabolites may be more susceptible to enzymatic breakdown, a phenomenon that is much less intense in solid media where the antifungal/microbial substances are continually diffusing into the agar (Buynitzky *et al.* 1979). The difference in oxygen availability, which is higher for micro-organisms growing in solid media, is another important factor that causes general changes in the metabolism that probably results in differences in the expression levels of the various metabolites by the streptomycete strains. In addition, the nutrient limitation in the microenvironment around a growing colony on solid agar media could induce the biosynthesis of secondary metabolites (Buynitzky *et al.* 1979; Doelle *et al.* 1992; Lahlali and Hijri 2010). Finally, the observation that the antifungal activity was present only in the solid media extracts and not in the concentrates of the streptomycete liquid cultures could also indicate a specific induction pattern that was caused by the simultaneous presence of a growing fungal mycelium on the agar plates.

The fact that the antifungal activity was detected at the low molecular weight fraction of the streptomycete solid medium extracts suggested that the active compound(s) was probably not a protein (hydrolytic enzyme) but rather a smaller organic compound, such as a secondary metabolite. Further biochemical analysis, using high-performance liquid chromatography fractionation coupled with mass spectrometry and nuclear magnetic resonance techniques, will allow us to refine its structure.

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