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Trichoderma harzianum strain SQR-T37 and its bio-organic fertilizer could control *Rhizoctonia solani* damping-off disease in cucumber seedlings mainly by the mycoparasitism

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Abstract Damping-off disease is caused by *Rhizoctonia solani* and leads to serious loss in many crops. Biological control is an efficient and environmentally friendly way to prevent damping-off disease. Optical micrographs, scanning electron micrographs, and the determination of hydrolytic enzymes were used to investigate the antagonism of *Trichoderma harzianum* SQR-T37 (SQR-T37) against *R. solani*. Experiments were performed in pots to assess the in vivo disease-control efficiency of SQR-T37 and bio-organic fertilizer. The results indicate that the mycoparasitism was the main mechanism accounting for the antagonistic activity of SQR-T37. In one experiment, the population of *R. solani* was decreased from 10^6 internal transcribed spacer (ITS) copies per gram soil to 10^4 ITS copies per gram soil by the presence of the antagonist. In this experiment, 45% of the control efficiency was obtained when 8 g of SQR-T37 hyphae per gram soil was applied. In a second experiment, as much as 81.82% of the control efficiency was obtained when bio-organic fertilizer (SQR-T37 fermented organic fertilizer, BIO) was applied compared to only 27.27% of the control efficiency when only 4 g of SQR-T37 hyphae per gram soil was applied. Twenty days after incubation, the population of *T. harzianum* was 4.12×10^7 ITS copies per gram soil in the BIO treatment, which was much higher than that in the previous treatment (8.77×10^5 ITS copies per gram soil), where only SQR-T37 was applied. The results indicated that SQR-T37 was a potent antagonist against *R. solani* in a mycoparasitic way that decreased the population of the pathogen. Applying BIO was more efficient than SQR-T37 application alone because it stabilized the population of the antagonist.

Keywords *Trichoderma harzianum* SQR-T37 · *Rhizoctonia solani* · Mycoparasitism · Bio-organic fertilizer (BIO) · Quantification real-time PCR · DGGE

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Introduction

The widespread soil-borne pathogen *Rhizoctonia solani* Kühn is responsible for serious damages to many agricultural and horticultural crops and trees worldwide (Baker 1970; Anderson 1982; Li 1995). *R. solani* causes damping-off and stem rot in young plants and a disease of the lower stem and root called sore shin in older plants (Lucas 1975; Sneh et al. 1996). Furthermore, sclerotia-dormant forms of the fungi are resistant to unfavorable environmental conditions. The importance of this pathogen has increased dramatically in Europe in recent years (Grosch et al. 2005). However, even though the pathogen causes serious economic loss through reduced yield in many crops, no effective strategy to control the pathogen is currently available (Kai et al. 2007).

Agricultural production has increased dramatically over the past few decades, and farmers rely on chemical pesticides as a relatively dependable method of protecting plants from soil-borne pathogens (Compant et al. 2005). However, the increased use of chemical pesticides can have several negative effects on both the environment and on human health (Gerhardson 2002). As a result, restrictions have increased on a variety of chemical pesticides (Adesina et al. 2007). Furthermore, growing concerns regarding food safety and environmental pollution have generated interest in compost and biological control agents to help prevent and control soil-borne diseases (Trillas et al. 2006).

Many species of *Trichoderma* spp. have been used as potent biocontrol agents for a variety of soil-borne phytopathogenic fungi (Papavizas 1985). The response of *Trichoderma* to the presence of a potential host includes production of antibiotic compounds, formation of specialized structures, and degradation of the host's cell wall followed by the assimilation of its cellular content, mainly the mycoparasitism (Benitez et al. 2004). This last response has been proposed as the central mechanism accounting for the antagonistic activity of the *Trichoderma* species (Mendoza-Mendoza et al. 2003).

Using composts to minimize organic waste pollution and to reduce the addition of chemical fertilizers and fungicides in crop production is a promising strategy (Ling et al. 2010). However, only approximately 20% of natural composts are suppressive to *Rhizoctonia* damping off (Hoitink and Boehm 1999). The capability of certain composts to repress *R. solani* may be due to the presence and activity of specific antagonists (Kuter et al. 1983; Tuitert et al. 1998; Scheuerell et al. 2005). Furthermore, this ability depends on the degree of compost decomposition (Hoitink and Boehm 1999) because mature composts can sustain biological control agents (Litterick et al. 2004).

Rapid and sensitive quantification methods are necessary to detect invading pathogens even in the absence of visible host plant symptoms, when investigating early plant response reactions and disease control (Leisova et al. 2006). The high sensitivity, specificity, and reproducibility provided by real-time PCR are the main reasons justifying this application for pathogen detection and comparison (Ruiz-Ruiz et al. 2007). The amount of template can be determined as absolute or relative quantification. When using absolute quantification, the actual number of nucleic acid targets present in a sample is determined using a standard curve, constructed by amplifying known amounts of the target under conditions identical to those of the sample (Bustin 2000; Freeman et al. 1999; Mackay et al. 2002; Rutledge and Côté 2003).

Determining the structure of soil microbial communities is important for understanding the biological processes that occur in soil–plant systems (Dong and Reddy 2010). However, research conducted in the last two decades have revealed that more than 99% of the bacteria present in many environmental samples cannot be cultivated in the laboratory (Sharma et al. 2005). The use of molecular biological methods makes it possible to study microbial diversity in environmental samples without cultivation (Niemi et al. 2001). Denaturing gradient gel electrophoresis (DGGE) is a high-throughput technique, and it has been more widely applied in bacteria and fungi community analyses than any other method (Li et al. 2008; Luo et al. 2010). DGGE is an electrophoretic method which is capable of detecting differences between DNA fragments of the same size but with different composition. This is because these fragments can be separated in a denaturing gradient gel based on their differential denaturation (melting) profile (Ercolini 2004). The theoretical aspects of this separation were first described by Fisher and Lerman (1983).

The objective of this study was to investigate the antagonism of the *Trichoderma harzianum* SQR-T37 (SQR-T37) strain against *R. solani* Q1 and to assess its control efficiency in pot experiments. Molecular biological methods (real-time PCR and PCR-DGGE) were used for the quantification of the two species in soil samples and for comparison of structures of the soil microbial communities in different treatments in pot experiments.

Materials and methods

Strains

A *T. harzianum* strain SQR-T37 (CGMCC accession no. 3308, China General Microbiology Culture Collection Center) was used throughout this study. The strain was screened and identified by our laboratory (Chen et al. 2010) and was stored on a potato dextrose agar (PDA) medium. An *R. solani* Q1 (ATCC accession no. 76168, American Type Culture Collection) was used as the pathogen and was obtained from the institute of vegetables and flowers, Chinese Academy of Agricultural Sciences (Beijing, China).

Dual cultures

SQR-T37 was evaluated for its effectiveness against *R. solani* Q1 in dual culture tests. One 5-mm mycelial disk of a 2-day-old culture of *R. solani* Q1 was placed in a Petri dish containing PDA. Another 5-mm mycelial disk of SQR-T37 (4-day-old culture) was placed at the opposite side of the Petri dish. The distance between the two mycelial disks was 5 cm. Inoculated plates were kept at 28 °C in an incubator. The radial growth of SQR-T37 and *R. solani* Q1 were observed 2 and 5 days after inoculation. Controls inoculated with *R. solani* Q1 or SQR-T37 individually were also maintained, and the assay was repeated four times.

Hyphal interaction between *R. solani* Q1 and SQR-T37

The hyphae of *R. solani* Q1 and SQR-T37 were kept in touch with each other in dual cultures. These cultures were chosen for the observation of the interaction between the two fungi. An optical microscope (OLYMPUS CX21, Tokyo, Japan) and a scanning electron microscope (HITACHI S-3000N, Tokyo, Japan) were used for the observations.

Determination of chitinase and β-1,3-glucanase of SQR-T37

The *R. solani* Q1 cell walls were prepared according to El-Katatny et al. (2000). Erlenmeyer flasks (250 ml) containing 100 ml of potato dextrose broth (PDB) were incubated with 1-cm^2 discs of the actively growing mycelium of *R. solani* Q1. The inoculated flasks were incubated at 28 °C for 4 days. The mycelium was then collected by filtration through a gauze, washed with sterile distilled water, and homogenized in distilled water using a laboratory homogenizer. The suspension was then centrifuged three times (6,000 g for 10 min). The mycelia were stored at 4 °C or oven-dried for 24 h and were used as a C-source. The moisture of the fresh mycelia was calculated.

Erlenmeyer flasks (250 ml) each containing 50 ml of minimal synthetic medium (MSM) (glucose as C-source) were seeded with 1 ml of a suspension (10^6 conidia of SQR-T37 per milliliter) and incubated in a rotary shaker at 180 rpm for 4 days at 28 °C. The cultures were centrifuged aseptically at 6,000 g for 10 min at 4 °C. The mycelia were washed with sterile distilled water. Equal amounts of SQR-T37 mycelia were transferred to flasks containing fresh MSM of various C-source compositions (colloidal chitin, laminarin, fresh and dried *R. solani* Q1 cell wall) and incubated at 28 °C for 4 days. The culture filtrates were centrifuged for 10 min at 6,000 g at 4 °C and the clear supernatants were either immediately used as crude enzyme or stored at −20 °C until assayed. The formula of the MSN was as follows: 5 g/L C-source, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.9 g/L K_2HPO_4, 0.2 g/L KCl, 1.0 g/L NH_4NO_3, 0.002 g/L $FeSO_4 \cdot 7H_2O$, 0.002 g/L $MnSO_4$, and 0.002 g/L $ZnSO_4$. The colloidal chitin 1% (*w*/*v*) was prepared according to the methods of Lee et al. (2009).

The activity of β-1,3-glucanase was determined by a dinitrosalicylic acid (DNS) colorimetric method using laminarin as the substrate (Noronha and Ulhoa 2000), and the reaction, carried out for 2 h at 45 °C, was stopped by immersing the tubes in a boiling water bath for 15 min. Chitinase activity was determined by the DNS method using colloidal chitin as the substrate (Han et al. 2009). The reaction was carried out for 4 h at 40 °C and was stopped by the method described above. The amount of reducing sugars released was calculated from standard curves for *N*-acetyl-glucosamine and glucose. One unit of enzyme activity (nanokatals) was expressed as the amount of enzyme that released 1 nmol of reducing sugar per minute. The assay was repeated three times.

Pot experiments

Cucumber seedling, soil, and fertilizer for pot experiments

Cucumber (*Cucumis sativus* L.) seeds of the cultivar JinChun 5, were surface-sterilized in 2% NaClO for 3 min, rinsed three times in sterile water, and then germinated on 9-cm plates covered with sterile wet filter paper at 25 °C for 36 h (Ling et al. 2010).

The soil used in the pot experiments came from a rice paddy field and was solarized to minimize the influence of other microorganisms and air-dried prior to the experiment. The dried soil had the following properties: pH 6.7, 0.9% organic matter, 0.1% nitrogen, and 4.4% H_2O. The organic fertilizer (OF) used in the pot experiment was an amino acid fertilizer. The amino acid fertilizer was made from oil rapeseed cakes that were enzymatically hydrolyzed by aerobic microbial fermentation at <50 °C for 7 days (Zhang et al. 2008). The amino acid fertilizer contained 44.2% organic matter and 12.9% amino acids, small molecular peptides, and oligo peptides. The nutrient content was 4.4% N, 2.3% P_2O_5, and 0.7% K_2O.

Pathogen, SQR-T37, and SQR-T37-fermented organic fertilizer (BIO) preparation

The mycelia of *R. solani* Q1 and SQR-T37 were prepared as described above with a few modifications. Erlenmeyer flasks (250 ml) containing 100 ml of PDB were incubated with 1-cm^2 discs of the actively growing mycelia. The inoculated flasks were incubated at 28 °C for 4 days without shaking. The mycelia were then collected by filtration through a gauze and were washed with distilled water. Finally, the mycelia were physically dried, but not killed.

The BIO product used in the experiments was obtained by aerobically fermenting a mixture of amino acid fertilizer with SQR-T37 (5:2, *w*/*w*) for 5 days at <45 °C. The mycelia of SQR-T37 was prepared as above and homogenized in distilled water. The mixture was thoroughly mixed and maintained at 40–45% moisture during the fermentation stage. At the end of the fermentation stage, the density of *T. harzianum* was 2.57×10^9 internal transcribed spacer (ITS) copies per gram of dry weight BIO. The BIO product was stored at less than 25 °C, and it was used in experiments only if the density of *T. harzianum* was kept at the level of 10^9 ITS copies per gram dry weight BIO.

Description of pot experiments

Six treatments were applied in pot experiment 1 (Exp. 1). The treatments were as follows: (1) $control_1$ (CK_1) (soil not inoculated with any microbe), (2) CK_2 (soil inoculated with 1.5 g fungal biomass of *R. solani* Q1 per kilogram soil), (3) T1 (soil inoculated with 1.5 g fungal biomass per kilogram soil of *R. solani* Q1 and 0.5 g fungal biomass per kilogram soil of SQR-T37), (4) T2 (soil inoculated with 1.5 g fungal biomass per kilogram soil of *R. solani* Q1 and 2 g fungal biomass per kilogram soil of SQR-T37), (5) T3 (soil

inoculated with 1.5 g fungal biomass per kilogram soil of *R. solani* Q1 and 4 g fungal biomass per kilogram soil of SQR-T37), and (6) T4 (soil inoculated with 1.5 g fungal biomass per kilogram soil of *R. solani* Q1 and 8 g fungal biomass per kilogram soil of SQR-T37). Experiment 1 contained two growth cycles of 20 days each. Four and 24 days after inoculation, pre-germinated cucumber seeds were planted in the soils. Twenty and 40 days after inoculation, the cucumber seedlings were harvested. At this time, the diseased plants, shoot dry weights, and shoot lengths of the surviving plants were evaluated. The diseased plants were detected by visual observations of disease symptoms.

In pot experiment 2 (Exp. 2) the following five treatments were applied: (1) CK_1 (soil not inoculated with any microbe), (2) CK_2 (soil inoculated with 1.5 g fungal biomass per kilogram soil of *R. solani* Q1), (3) OF (soil inoculated with 1.5 g fungal biomass per kilogram soil of *R. solani* Q1 and amended with 1% OF), (4) T (soil inoculated with 1.5 g fungal biomass per kilogram soil of *R. solani* Q1 and 4 g fungal biomass per kilogram soil of SQR-T37), and (5) BIO (soil inoculated with 1.5 g fungal biomass per kilogram soil of *R. solani* Q1 and 1% BIO). Four days after inoculation, pre-germinated cucumber seeds were planted in the soil. Twenty days after the inoculation, the cucumber seedlings were harvested. The diseased plants, shoot dry weights, and shoot lengths of the surviving plants were evaluated.

The pot experiments were carried out in a greenhouse located at Yixing, China from May to June 2010. The temperature ranged from 23 to 30 °C and the relative humidity from 60% to 85%. In both pot experiments, each pot was filled with 150 g of infested soil, and one seed was planted per pot. Each treatment contained three replications, and each replication contained ten pots. Exp. 1 and Exp. 2 were both repeated twice.

Soil DNA extraction

In the pot experiments, three plant samples from each treatment were randomly collected. The rhizosphere soil was collected as described by Hervás et al. (1998). The cucumber plants were carefully removed from the pots and gently shaken to remove the soils. The soils remaining within the root systems were defined as the rhizosphere soil. The soil DNA was extracted with the E.Z.N.A.™ Soil DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions (Dineen et al. 2011), and the DNA of 1 g soil was dissolved in 100 μl of elution buffer.

Quantification of *R. solani* and *T. harzianum*

The standard curves were developed according to López-Mondéjar et al. (2010). The 209-bp fragment from the selected ITS region of the SQR-T37 and the 192-bp fragment of *R. solani* Q1, were cloned into a PMD-18 vector (TaKaRa Bio, Inc., Dalian, China). The plasmids were used to transform *Escherichia coli* DH5α (Invitrogen, Shanghai, China) cells and were purified using an AxyPrep™ Plasmid Miniprep Kit (Axygen Scientific, Inc., Union City, CA, USA). The presence of the inserts were verified by restriction fragment length polymorphism using the two restriction enzymes, *Eco*RI and *Hin*dIII (Fermentas, Shenzhen, China). The DNA concentrations of the plasmid standard solution were measured using a spectrophotometer (GeneQuant 100, General Electric Company, Shanghai, China) and were compared to the known molecular weight of a single plasmid molecule. The concentrations were then adjusted to 10^{11} ITS copies and the standards were diluted in tenfold steps to obtain the standard curves. The slopes of the linear regression curve analyses indicated very efficient amplification rates in the TaqMan (−3.315) and SYBR Green (−3.191) real-time PCR systems. The soil DNA solution was also tested for the inhibitory effect of coextracted substances by determining the ITS copy number in tenfold dilutions of soil DNA and by adding 10^5 copies of the target gene in the lowest dilution of soil DNA, and the results showed that there were no inhibitors in the DNA solutions.

The number of *R. solani* ITS copies was determined by SYBR Green real-time PCR. Real-time PCR amplifications were performed using eight-well tubes with a total volume of 50 μl in each using the SYBR® Premix Ex Taq™ (TaKaRa) on a PRISM® thermocycler (Applied Biosystems 7500 Real-Time PCR, Applied Biosystems Inc., Foster City, CA, USA). Each PCR reaction contained 2 μl of the target DNA extract, 25 μl of SYBR Green premix EX Taq (2×), 1 μl of primer ST-RS1 and ITS4 (10 μM, Table 1), 1 μl RoxII and 20 μl of sterile distilled water. Thermal cycling conditions lasted 1 min at 95 °C followed by 40 amplification cycles of 10 s at 95 °C, 34 s at 60 °C. Fluorescence was detected at the second stage of each cycle. To evaluate amplification specificity, a melt curve analysis was performed at the end of each PCR run. A melt curve profile was obtained by heating the mixture to 95 °C, cooling to 60 °C (15 s) and slowly heating to 95 °C at 0.1 °C s^{-1} with a continuous measurement of fluorescence.

The number of *T. harzianum* ITS copies was determined by TaqMan real-time PCR. Real-time PCR amplifications were performed in a total volume of 20 μl using Premix Ex Taq™ (TaKaRa) on a 7500 Real-time PCR system. The reaction mixtures contained 0.4 μl of primer RT37_ITS1S and RT37_ITS1 (10 μM, Table 1), 0.8 μl TaqMan probe RT37_ITS1 (10 μM, Table 1), 10 μl Premix Ex Taq™, 2 μl template DNA, 0.4 μl RoxII, and 6 μl sterile distilled water. The thermal cycling conditions for amplification were the same as described above. The amplification results were

Table 1 Primers and probe used in the experiments

Primers and probe	Sequence (5′–3′)[a]	Product size (bp)	Reference
ST-RS1(F) ITS4(R)	AGTGTTATGCTTGGTTCCACT TCCTCCGCTTATTGATATGC	192	Lievens et al. (2006)
RT37_ITS1S(F)	TACAACCTCCA -AACCCAATGTGA	209	López-Mondéjar et al. (2010)
RT37_ITS1(R)	CCGTTGTTGAAAGT -TTTGATTCATTT		
RT37_ITS1(P)[b]	AACTCTTTTTGTATA -CCCCCTCGCGGGT		
GC-U968(F) L1401(R)	GC.-AACGCGAAGAACCTTAC GCGTGTGTACAAGACCC	490[c]	Ercolini (2004)
NS1(F) GC-fungi(R)	GTAGTCATATGCTTGTCTC GC.-ATTCCCCGTTACCCGTTG	388	Das et al. (2007)
GC(bacteria)	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG		
GC(fungi)	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC		

F forward primer, *R* reverse primer, *P* TaqMan probe

[a] A GC-rich sequence (GC.-) attached to the 5′ end of sequence is indicated

[b] The fluorescent dye at the 5′ end of the probe is FAM (6-carboxyfluorescein), and the quencher dye at the 3′ end is TAMRA (6-carboxy-tetramethylrhodamine)

[c] Expected amplicon sizes in *E. coli* for bacteria and size in *S. cerevisiae* for fungi

analyzed with the Sequence Detection Software version 1.4 (Applied Biosystems).

PCR-DGGE conditions

The microbial diversity of the sample was determined by PCR–DGGE. The bacterial DNA was amplified using the bacterial primers U968+GC (Table 1) and L1401 (Table 1) (Ercolini 2004; Das et al. 2007), which amplified a 490-bp fragment, including a 40-bp GC clamp (Table 1). A PCR was performed using 2.5 μl 10× Ex Taq buffer (20 mM Mg^{2+}, TaKaRa), 2 μl 2.5 mM dNTP mixture, 0.25 μl of 5 U per microliter Ex Taq polymerase (TaKaRa), 1 μl of each primer (10 μM), 1 μl diluted template (1:2), and H_2O with a total of 25 μl. A bacterial PCR was performed using the following cycle conditions: an initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and elongation at 72 °C for 45 s, and then a final elongation step at 72 °C for 10 min. For the fungi, the primer pair NS1 (Table 1) and the fungus-specific primer Fungi+GC (Table 1) (Das et al. 2007) were used to amplify a 370-bp fragment, including a 40-bp GC clamp (Table 1). The cycle conditions in the fungal PCR were the following: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and elongation at 72 °C for 15 s, and a final elongation step at 72 °C for 10 min. The products from the bacterial and fungal PCR reactions were verified by agarose gel electrophoresis.

DGGE was performed using the D-GENE System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) DGGE equipment. The PCR products were loaded onto 8% (*w*/*v*) polyacrylamide gels (40% acrylamide/bis-solution, 37.5:1, Bio-Rad) with denaturing gradients ranging from 45–60% for the bacterial DNA and 30–45% for the fungal DNA. The gels with bacterial and fungal DNA were run for 16 h at 60 °C and 80 V. The digital images of the gels were obtained and analyzed using an image analysis (Quantity One 4.6.3, Bio-Rad).

DNA sequencing

The DGGE bands were excised with a sterile scalpel, and the DNA from each band was eluted overnight at 4 °C in 20 μl of sterile water. The eluted DNA (2 μl) was re-amplified using the conditions described above (Hu et al. 2009). The resulting PCR products were analyzed by DGGE to confirm that the expected products had been isolated. The samples yielding a single band comigrating with that of the original sample were then excised and amplified with the primers without the GC clamp, purified, and sent for sequencing at the Genscript Company (Nanjing, China). The sequences recovered were aligned to the bacteria and fungi gene fragments available from the National Center for Biotechnology Information databases. Searches in BLAST from GenBank were used to find the closest known relatives to the partial bacteria and fungus sequences.

Statistical analysis

The means and standard deviation of the data were calculated and statistically analyzed using analysis of variance and Duncan's multiple range tests ($P \leq 0.05$). The SPSS (version 13.0) was used for statistical analysis (SPSS, Inc., Chicago, IL, USA). A cluster analysis was performed with the UPGMA algorithm to study the general patterns of community similarity in the Quantity One (Quantity One 4.6.3, Bio-Rad).

Results

Dual cultures

Figure 1 showed the growth situations of the two microbes in the three treatments inoculated with only SQR-T37 (Fig. 1a), with both SQR-T37 and *R. solani* Q1, and with only *R. solani* Q1. SQR-T37 and *R. solani* Q1 were grown towards each other on 9-mm Petri dishes containing PDA. The firstly observed contact between the hyphae of both fungi occurred within 2 days after inoculation. After stopping the growth of the mycelium of *R. solani* Q1, the mycelium of SQR-T37 continued to grow on the mycelium of the pathogen. Five days after the inoculation, the mycelium of *R. solani* Q1 was completely covered by the mycelium of SQR-T37 (Fig. 1b). Only a few sclerotia on the edge of the Petri dish were formed by *R. solani* Q1 (Fig. 1b). In contrast, the control Petri dishes inoculated with *R. solani* Q1 alone were filled with the sclerotia of *R. solani* Q1 (Fig. 1c).

Hyphal interaction between *R. solani* Q1 and SQR-T37

Two days after the inoculation, dual cultures of the hyphae that had made contact with each other were observed under an optical microscope and scanning electron microscope. The optical micrographs showed that SQR-T37 was closely twisted onto *R. solani* Q1 hyphae via coiling, hooks, and appressorium-like bodies (Fig. 2). Scanning electron micrographs were taken for an intensive investigation of the interaction. Similarly, the SQR-T37 hyphae established close contact with the *R. solani* Q1 hyphae by coiling around the hyphae. The coils were usually dense (Fig. 3a) and appeared to tightly encircle the hyphae of *R. solani* Q1 (Fig. 3b). However, at this stage of interaction, the integrity of the *R. solani* Q1 cell surface was well preserved (Fig. 3b). At the next stage, the hyphae of the antagonist inserted into the hyphae of the host, causing cell wall breakdown and loss of the cytoplasm of the host (Fig. 3c). Early signs of collapse, including a wrinkled appearance of the host cell surface, were often observed (Fig. 3c, d). These features of cell alteration were not seen with the unparasitized hyphae of *R. solani* Q1. The SEM observations indicated that coiling of the antagonist around the pathogen was an early event preceding hyphal damage of the host.

Chitinase and β-1,3-glucanase activity of SQR-T37

When grown on laminarin- or colloidal-chitin-supplemented mineral medium, SQR-T37 produced an extracellular chitinase or β-1,3-glucanase that degraded these polymers, respectively. The chitinase activity was much lower than the activity of β-1,3-glucanase. Both β-1,3-glucanase and chitinase were produced by SQR-T37 in the culture media amended with dried or fresh mycelium of *R. solani* Q1; however, the levels of enzyme activities in the presence of the mycelium of the pathogen were much lower than those in the presence of colloidal chitin or laminarin (Table 2). A dried mycelium of *R. solani* Q1 was more effective than fresh mycelium on inducing the production of the enzymes, but the differences were not significant. These results indicated that laminarin, colloidal chitin, and *R. solani* Q1 cell walls were capable of inducing SQR-T37 to produce these enzymes.

Pot experiment 1

The effect of SQR-T37 on disease intensity

In this experiment, two growth cycles (20 days each) of cucumber seedlings were carried out in the same soil. The

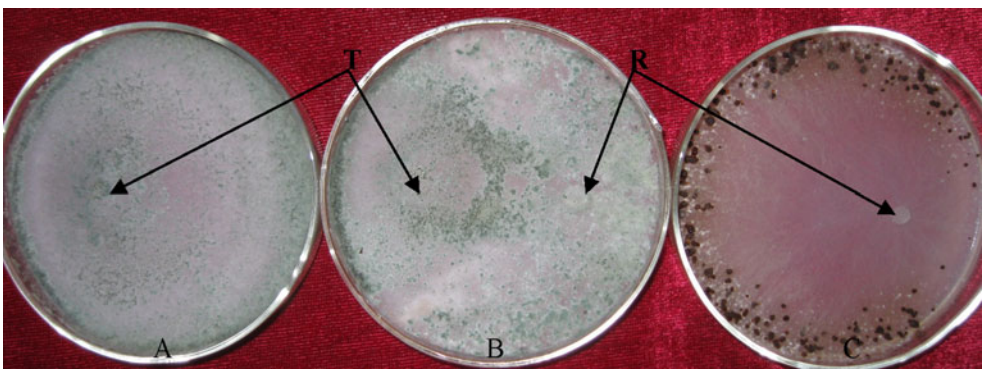

Fig. 1 Dual cultures (5 days after inoculation; (*R*) *R. solani* Q1; (*T*) SQR-T37)

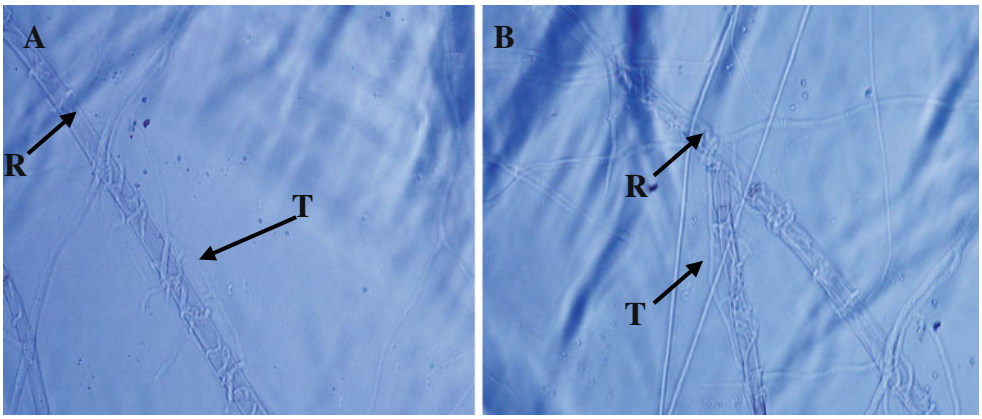

Fig. 2 Light micrographs of SQR-T37 (*T*) hyphae interacting with cells of *R. solani* Q1 (*R*) in dual cultures (2 days after inoculation; ×400)

treatment T4 (8 g SQR-T37 hyphae per kilogram soil) had the highest control efficiency in both growth cycles. Furthermore, the SQR-T37 had a long-term effect, and its efficiency increased during the second growth cycle (Table 3). No diseased plant was found in the treatment of CK_1, no matter in what growth cycle (data not shown). These results showed that the *R. solani* Q1 used in this experiment was the pathogen causing the damping-off disease in the cucumber, and SQR-T37 could prevent the disease.

The effect of SQR-T37 on the growth tendency of surviving plants

SQR-T37 not only suppressed damping-off disease but also significantly promoted cucumber growth and increased shoot dry weights and shoot lengths. In the first growth season, the lowest shoot dry weight and shoot length were found in the CK_2 treatment plants while the highest shoot dry weight and shoot length were found in plants in treatment T4. The shoot dry weights and shoot lengths of plants in all SQR-T37-added treatments were higher than those in the CK_1 and CK_2 plants (Table 4). Similarly, in the second growth season, the lowest shoot dry weights and shoot lengths were found in the CK_2 treatment plants while the highest shoot dry weight was found in plants in the T2 treatment, and the highest shoot length was found in plants in the T4 treatment. However, the shoot dry weights and shoot lengths of the seedlings in the same soil had no significant differences between the two growth seasons (Table 4).

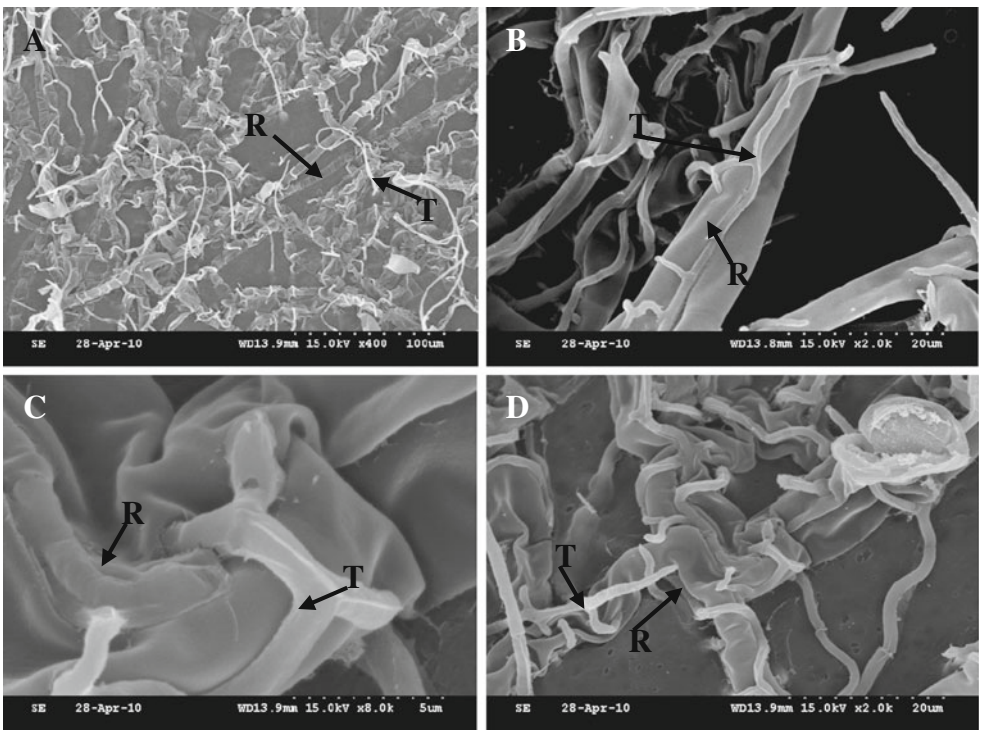

Fig. 3 Scanning electron micrographs of SQR-T37 (*T*) hyphae interacting with cells of *R. solani* Q1 (*R*) in dual cultures (2 days after inoculation)

Table 2 The effect of various carbon sources on the activity of extracellular chitinase and β-1,3-glucanase produced by SQR-T37 grown on a mineral medium

C-source	Substrate	Chitinase (nkat ml^{-1} of crude enzyme)	β-1,3-glucanase (nkat ml^{-1} of crude enzyme)
Colloidal chitin	Colloidal chitin	14.73±0.91a	
Laminarin	Laminarin		66.88±3.37a
Fresh *R. solani* Q1 cell wall	Colloidal chitin	9.49±1.45b	
	Laminarin		37.57±2.68b
Dried *R. solani* Q1 cell wall	Colloidal chitin	10.80±2.17b	
	Laminarin		43.18±3.84b

Values with the different letter within the same column are significantly different at $P<0.05$ according to Duncan's test. Numbers followed by "±" are standard errors (SE)

Quantification of pathogen and antagonist in experiment 1

There were significant differences in ITS copies of *R. solani* in the soil between the treatments with or without application of SQR-T37 in both day 20 and day 40 soil samples (Table 5). The highest population of *R. solani* was found in the CK_2 treatment in the day 20 soil sample and the day 40 soil sample. In the soil treated with SQR-T37, the ITS copies of *R. solani* were inhibited and remained within the level of 10^4 ITS copies per gram soil in the day 20 and day 40 samples, which was significantly lower than those in CK_2. The population of *R. solani* in the treatments applied with SQR-T37 was almost invariable between the day 20 soil sample and the day 40 soil sample. These results indicated that the population of *R. solani* in the soil was significantly reduced by the presence of SQR-T37. In the soils treated with SQR-T37, the ITS copies of *T. harzianum* were decreased from day 0 soil samples to day 20 soil samples regardless of how many SQR-T37 hyphae were inoculated in the soil (Table 6). However, similar to *R. solani*, the variation of the population of *T. harzianum* between the day 20 soil samples and the day 40 soil samples was very small.

Pot experiment 2

Disease intensity and growth tendency of surviving plants in different treatments

SQR-T37 significantly inhibited damping-off disease in cucumber during the greenhouse experiments. The incidence of damping-off disease in cucumber plants (Table 7) planted in soil treated with SQR-T37 was significantly lower than in the CK_2 treatment. A similar difference was observed between soil treated with BIO and OF. Furthermore, the disease incidence of the BIO treatment was significantly lower than that of the T treatment. However, no significant difference was found between the CK_2 treatment and the OF treatment. These results indicated that the application of BIO could significantly increase the

Table 3 The effect of SQR-T37 on the incidence of damping-off disease caused by *R. solani* Q1 in cucumber seedlings grown in two successive periods in the same soil

Treatments	First growth season		Second growth season	
	Diseased plants (%)	Control efficiency (%)[a]	Diseased plants (%)	Control efficiency (%)
CK_2	66.67±11.55a	–[b]	53.33±5.77a	–[b]
T1	66.67±5.77a	0.00	43.33±5.77ab	18.74
T2	53.33±5.77ab	20.00	36.67±15.28bc	31.25
T3	40.00±10.00b	40.00	26.67±5.77cd	50.00
T4	36.67±11.55b	45.00	20.00±0.00d	62.50

Values with the different letter within the same column are significantly different at $P<0.05$ according to Duncan's test. Numbers followed by "±" are SE

DP diseased plants, *X* one of the treatments

[a] The calculation of control efficiency (CE) was according to the formula: $CE_x(\%)=(DP_{CK2}-DP_x)/DP_{CK2}\times 100\%$

[b] The value is incalculable

Table 4 The effect of SQR-T37 on the growth tendency of surviving cucumber seedlings grown during two successive times in the same soil

Treatments	First growth season		Second growth season	
	Shoot dry weight (mg)	Shoot length (cm)	Shoot dry weight (mg)	Shoot length (cm)
CK_1	94.57±14.04ab	8.00±0.28b	107.10±8.36b	8.32±0.79b
CK_2	84.80±7.33a	6.98±0.23a	83.00±15.32a	6.63±0.38a
T1	119.73±11.67c	8.52±0.67bc	124.23±7.23bc	9.00±0.46bc
T2	114.00±7.04bc	8.27±0.55b	138.20±5.21c	9.57±0.32cd
T3	113.87±10.52bc	9.07±0.13 cd	130.60±19.45c	9.50±0.40cd
T4	128.17±20.81c	9.65±0.23 d	122.73±5.41bc	10.23±0.38d

Values with the different letter within the same column are significantly different at $P<0.05$ according to Duncan's test. Numbers followed by "±" are SE

efficiency of controlling damping-off disease compared to the application of SQR-T37 alone.

SQR-T37 not only suppressed damping-off disease but also significantly promoted cucumber growth, which was similar with the results in Exp. 1. The lowest shoot dry weight and shoot length were found in the CK_2 treatment plants, whereas the highest shoot dry weight and shoot length were found in plants treated with BIO. Even for the lower soil fertility, the SQR-T37-treated plants had a higher shoot dry weight and a higher shoot length than those in the OF treatment (Table 7). These data confirmed that the addition of SQR-T37 could significantly control damping-off disease and increase the shoot dry weight and shoot length of the cucumber seedlings in a protected cultivation system.

Quantification of pathogen and antagonist in experiment 2

In the day 20 soil sample, the ITS copies of *R. solani* in the treatments with SQR-T37 application (treatments T and BIO) were significantly lower than in treatments without SQR-T37 application (treatments CK_2 and OF) (Fig. 4a). With the exception of the CK_1 treatment (without application of *R. solani* Q1), the lowest population of *R. solani* was obtained in the BIO treatment. In contrast, the highest amount of *R. solani* was found in the CK_2 treatment (Fig. 4a). The *R. solani* ITS copies in the SQR-T37 and BIO treatments from day 20 soil samples decreased to 10^4 g^{-1}soil compared to 10^6 g^{-1} soil in day 0 soil samples. However, the *R. solani* ITS copies in the CK_2 and OF treatments in day 20 soil samples increased to 10^7 g^{-1} soil compared with 10^6 g^{-1} soil in day 0 soil samples. The results indicated that the population of *R. solani* in the soil was significantly reduced by the presence of SQR-T37.

In the day 0 soil sample, the population of *T. harzianum* in the BIO treatment was higher than in the T treatment, which was attributed to the additional nutrition supplied by the organic fertilizer. In the day 20 soil sample, the ITS copies of *T. harzianum* declined from 1.18×10^7 g^{-1}soil to 8.77×10^5 g^{-1}soil in the T treatment. In contrast, the ITS copies of *T. harzianum* in the BIO treatment maintained a level of 10^7 g^{-1} soil (Fig. 4b). The *T. harzianum* ITS copies in the treatments without application of SQR-T37 (treatment CK_1, CK_2, and OF) had little change. These results suggested that the application of organic fertilizer helps the SQR-T37 to survive in the soil.

Changes in fungal and bacterial diversity

The PCR–DGGE analysis of the fungal communities from cucumber rhizosphere soils showed that the fungal communities had significant dissimilarities in species composition (position of bands) and in species diversity (number of occurring bands). The number of fungal species from the T

Table 5 SYBR Green real-time PCR quantification of *R. solani* ITS copies in different soil samples

Treatments	$\log_{10}$ *R. solani* ITS copies per gram soil		
	Day 0 soil sample	Day 20 soil sample	Day 40 soil sample
CK_1	3.59±0.08a	2.84±0.13a	2.88±0.13a
CK_2	6.54±0.12b	7.59±0.20d	6.15±0.18c
T1	6.55±0.14b	4.91±0.10c	4.51±0.15b
T2	6.45±0.09b	4.67±0.14bc	4.27±0.11b
T3	6.42±0.07b	4.33±0.16b	4.35±0.10b
T4	6.38±0.12b	4.70±0.18bc	4.17±0.20b

Values with the different letter within the same column are significantly different at $P<0.05$ according to Duncan's test. Numbers followed by "±" are SE

Table 6 TaqMan real-time PCR quantification of *T. harzianum* ITS copies in different soil samples

Treatments	$\log_{10}$ *T. harzianum* ITS copies per gram soil		
	Day 0 soil sample	Day 20 soil sample	Day 40 soil sample
CK_1	4.47±0.13a	3.91±0.14a	3.44±0.14a
CK_2	4.59±0.08a	3.95±0.11a	3.24±0.17a
T1	6.07±0.18b	4.14±0.21b	4.00±0.12b
T2	6.80±0.12c	4.12±0.09b	4.54±0.15c
T3	7.09±0.22cd	5.49±0.19c	5.65±0.10d
T4	7.26±0.20d	5.93±0.14d	5.45±0.14d

Values with the different letter within the same column are significantly different at $P<0.05$ according to Duncan's test. Numbers follow by "±" are SE

and BIO treatments was lower than from the CK_2 and OF treatments (Fig. 5a). For further information, we sequenced six bands on the gel of Fig. 5a. The bands on the gel were classified as shown in Table 8.

In contrast to fungal diversity, the DGGE fingerprinting of bacteria showed that bacterial communities in the T and BIO treatments were more prolific than those in the CK_2 and OF treatments (Fig. 6a). The BIO treatment had the largest bacterial diversity among the four treatments. The bands on the gel were classified as described in Table 9.

Cluster analyses were performed with the UPGMA algorithm to study the general patterns of community similarity of fungal and bacteria communities among the different treatments (Fig. 5b and Fig. 6b). The T and BIO treatments clustered together, and the CK_2 and OF treatments clustered together.

Discussion

The *T. harzianum* SQR-T37 strain, which was separated by our lab, could prevent cucumber from *Fusarium* wilt disease (Chen et al. 2010). Based on our results, the SQR-T37 also showed an effective inhibition against *R. solani* Q1. In dual culture, 5 days after inoculation, the hyphae of *R. solani* Q1 were completely covered by the hyphae of SQR-T37. This phenomenon has also been observed in other studies (Benhamou and Chet 1993; Reithner et al. 2007), indicating that *Trichoderma* sp. could effectively inhibit the growth of *R. solani*.

As previously mentioned, mycoparasitism has been proposed as the central mechanism accounting for the antagonistic activity of *Trichoderma* species. The complex process of mycoparasitism involves several steps, including recognition of the host, attacking and subsequent penetrating, and killing the host (López-Mondéjar et al. 2011). Parts of the mycoparasitism processes, such as coiling, were observed in previous studies (Benhamou and Chet 1993; Almeida et al. 2007). In this study, the coiling, penetration, leakage of cytoplasm, and crimpling of the pathogen cell walls, were distinctly observed. During the mycoparasitism process, *Trichoderma* spp. secretes hydrolytic enzymes that destroy the cell wall of the host fungus (Verma et al. 2007). These enzymes mainly consist of chitinase and β-glucanase. Thus, the main mechanism of antagonism of *Trichoderma* spp. against pathogens is the extracellular secretion of chitinase and β-1,3-glucanase (Elad et al. 1982). As a result, we evaluated the activities of chitinase and β-1,3-glucanase produced by SQR-T37. Both chitinase and glucanase were detected in the SQR-T37 culture supernatant using colloidal chitin and laminarin as C-source. Furthermore, both the fresh and dried cell walls of *R. solani* Q1 also induced the two

Table 7 The diseased plants, control efficiency, shoot dry weight, and shoot length of the cucumber seedlings in different treatments (16 days after planting)

Treatments	Diseased plants (%)	Control efficiency (%)	Shoot dry weight (mg)	Shoot length (cm)
CK_1	0.00±0.00a	–[a]	94.93±7.13c	8.63±0.25bc
CK_2	73.33±5.77d	–	76.03±0.81a	6.02±0.30a
OF	66.67±5.77cd	9.09	86.40±4.27b	7.73±0.39ab
T	53.33±11.55c	27.27	104.30±12.54c	8.97±0.33c
BIO	13.33±11.55b	81.82	132.40±2.04d	13.11±0.46d

Values with the different letters within the same column are significantly different at $P<0.05$ according to Duncan's test. Numbers followed by "±" are SE

CK_1 not inoculated with any microbe or fertilizer, *CK_2* only inoculated with *R. solani* Q1, *OF* inoculated with *R. solani* Q1 and organic fertilizer, *T* inoculated with *R. solani* Q1 and SQR-T37, *BIO* inoculated with *R. solani* Q1 and BIO

[a] The value is incalculable

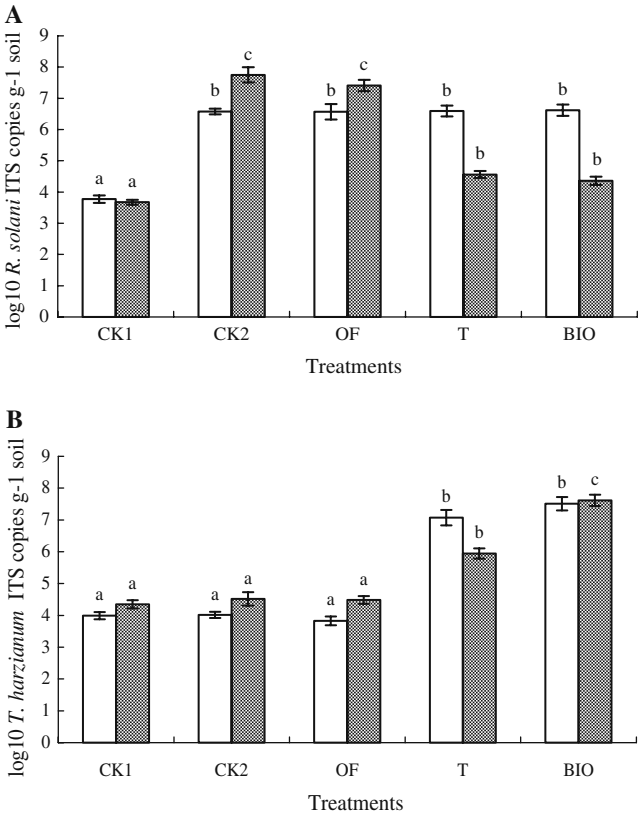

Fig. 4 Real-time PCR quantification of *R. solani* ITS copies (**a**) and *T. harzianum* ITS copies (**b**) in pot experiment 2 (*empty bars*, day 0 soil samples; *filled bars*, day 20 soil samples). CK_1 (not inoculated with any microbe or fertilizer), CK_2 (only inoculated with *R. solani* Q1), *OF* (inoculated with *R. solani* Q1 and organic fertilizer), *T* (inoculated with *R. solani* Q1 and SQR-T37), *BIO* (inoculated with *R. solani* Q1 and BIO). *Bars with the same letters* are not statistically different among the five treatments following Duncan's test ($P<0.05$)

types of hydrolytic enzymes of SQR-T37. Based on these results, we assume that the SQR-T37 strain is capable of the entire process of mycoparasitism against *R. solani* Q1. Besides, El-Katatny et al. (2000) reported that the dried cell wall was more effective in inducing the production of chitinase and glucose than the fresh cell wall. However, our results showed no significant difference between the fresh and the dried cell wall.

The pot experiments were designed to test the in vivo biocontrol abilities of SQR-T37. In Exp. 1, SQR-T37 showed an inhibition effect on damping-off disease caused by *R. solani* Q1. Furthermore, SQR-T37 had a long-term effect, and the efficiency increased during the second growth season. In Exp. 2, the disease incidence of the cucumber seedlings receiving the BIO treatment was much lower than in the seedlings receiving the T treatment. Because the individual application of organic fertilizer could not significantly inhibit the occurrence of the damping-off disease, the decline of the disease incidence in the BIO treatment compared with the T treatment was attributed to the combination of organic fertilizer and biocontrol agent SQR-T37. This is in agreement to the previous reports that a combination of antagonistic microbes with organic substrates may be more efficient in inhibiting disease than using single antagonistic microbial strains alone (Kay and Stewart 1994; Trillas et al. 2006; Luo et al. 2010). This was mainly because after fermenting in OF, SQR-T37 was more effective on the biocontrol against *R. solani* Q1 in the soil. Generally, a biocontrol efficiency of 50% is acceptable and can be extended in field (Minuto et al. 2006). Thus, these results indicated that SQR-T37 is a promising strain for the control of damping-

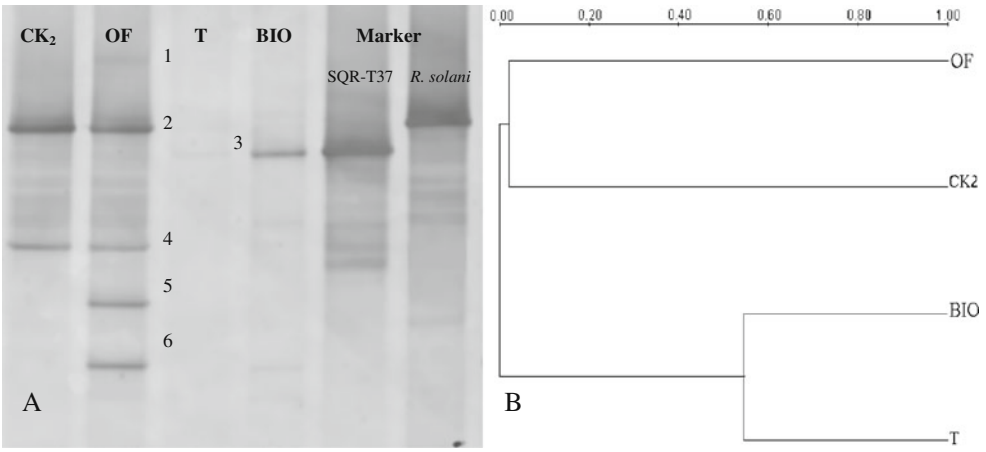

Fig. 5 The DGGE profiles of the fungal community in the soil rhizosphere. **a** The original DGGE pattern. Bands indicated by numbers *1–6* were excised, re-amplified, and subjected to sequencing. **b** Dendrogram of the DGGE profiles. The dendrogram was obtained by UPGMA clustering of a matrix containing Dice coefficients (Quantity One 4.6.3, Bio-Rad). The markers (SQR-T37 and *R. solani*) were the PCR products which carried the genomic DNA of pure cultures as the templates. CK_2 was only inoculated with *R. solani* Q1, *OF* was inoculated with *R. solani* Q1 and organic fertilizer, *T* was inoculated with *R. solani* Q1 and SQR-T37, and *BIO* was inoculated with *R. solani* Q1 and BIO

Table 8 Fungal 18S ribosomal RNA gene sequences obtained from the rhizosphere of cucumber

Representative sequence (GenBank accession number)	Best match database (GenBank accession number)	Similarity (%)
Band 1 (HQ424849)	*Actinomucor elegans* (AF157119)	99
Band 2 (JF499071)	*R. solani* (DQ917659)	100
Band 3 (JF499072)	*Trichoderma* sp. (FJ026619)	99
Band 4 (HQ424850)	*Setosphaeria* sp. (GU190183)	99
Band 5 (HQ424851)	Uncultured *Ascobolus* (EU120947)	98
Band 6 (HQ424852)	Uncultured *Lecythophora* (FJ748585)	100

off disease, and the application of BIO is more effective in controlling the disease than the individual application of SQR-T37.

Chen et al. (2010) reported that *T. harzianum* SQR-T37 can increase the dry weights of cucumber plants in the soil infested by *Fusarium oxysporum*. In Exp. 1, SQR-T37 showed the effect on promoting the growth of the cucumber seedlings. This is consistent with the previous reports (Chen et al. 2010). Furthermore, in Exp. 2, the BIO treatment had a greater positive effect on the shoot dry weights and shoot lengths of the surviving seedlings than SQR-T37 or OF individually.

The *Trichoderma* species have been used as biological agents against *R. solani* in many studies (Hadar et al. 1979; Lewis and Lumsden 2001; Trillas et al. 2006). However, little is known about the populations of the *R. solani* in the process of biological control. Li (1995) quantified the population of *R. solani* in the soil with a selective culture medium. However, the results from this study turned out to be inaccurate, especially in quantifying the population of the fungi which had no conidia. Real-time quantification PCR, as a high sensitivity, specificity, and reproducibility method, was used in this study to investigate the changes of the populations of *R. solani* in the soil. The PCR results showed that the population of *R. solani* in the soil was decreased by the presence of SQR-T37 in both Exp. 1 and Exp. 2. In contrast, the population of *R. solani* in the soil that did not receive the application of SQR-T37 was invariable. This indicated that SQR-T37 could kill the pathogen not only in the culture medium but also in the soil. The results are in accordance with the fact that the population of pathogens in the soil could be decreased by the presence of biocontrol agents (Ling et al. 2010; Zhao et al. 2011). Besides, the *R. solani* detected in the CK_1 (not inoculated with *R. solani* Q1) was the native isolates in the soil. In Exp. 2, the *R. solani* populations of CK_2 and OF in the day 20 soil samples were much higher than in the day 0 soil samples. The possible reason was that *R. solani* Q1 invaded the cucumber seedlings and assimilated the nutrient from the host.

One of the main problems of microbial introduction in practice is that the applied microorganisms cannot survive well in soils and thus cannot execute their specific functions (Jagnow 1987; Jagnow et al. 1991; van Veen et al. 1997). Several studies have indicated that organic substrates could provide functional microbes with good nutrients that could

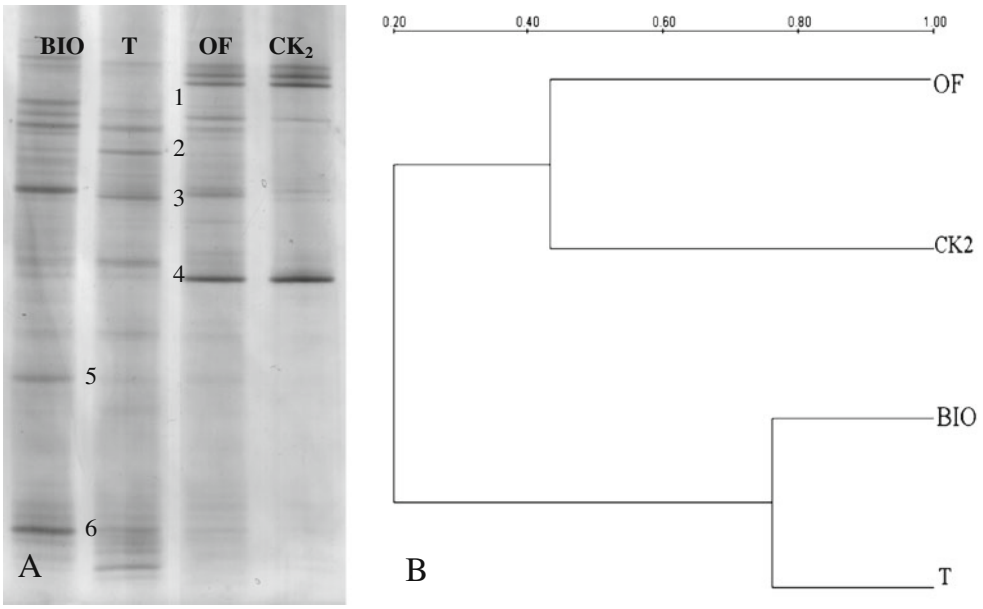

Fig. 6 The DGGE profiles of the bacterial community in the soil rhizosphere. **a** The original DGGE pattern. **b** Dendrogram of the DGGE profile that was obtained by UPGMA clustering of a matrix containing Dice coefficient (Quantity One 4.6.3, Bio-Rad). *CK_2* was only inoculated with *R. solani* Q1, *OF* was inoculated with *R. solani* Q1 and organic fertilizer, *T* was inoculated with *R. solani* Q1 and SQR-T37, and *BIO* was inoculated with *R. solani* Q1 and BIO

Table 9 Bacterial 16S ribosomal RNA gene sequences obtained from the rhizosphere of cucumber

Representative sequence (GenBank accession number)	Best match database (GenBank accession number)	Similarity (%)
Band 1 (HQ424853)	*Staphylococcus sciuri* (HQ154580)	99
Band 2 (HQ424854)	Uncultured bacterium (HM559212)	99
Band 3 (HQ424855)	Uncultured *Pseudomonas* sp. (FN994920)	98
Band 4 (HQ424856)	*Comamonas* sp. (GQ246691)	99
Band 5 (HQ424857)	Uncultured bacterium (GQ129977)	96
Band 6 (HQ424858)	Uncultured bacterium (HM332250)	96

make them more competitive in the soil (Uyanoz 2007; Wu et al. 2009; Ling et al. 2010; Zhao et al. 2011). However, there were no direct evidences in their reports to demonstrate that the combination of organic substrates and antagonists could maintain the population of antagonists in the soil. In this work, the *T. harzianum* population in the BIO treatment remained constant at the same level from day 0 to day 20 after inoculation, while that in the treatment applied with individual SQR-T37 was declined in both Exp. 1 and Exp. 2 over the same period. These results showed that the combination of SQR-T37 and organic fertilizer in the BIO treatment maintained a high population of the antagonist in the soil. After fermenting, the antagonists established itself in the OF and utilized the nutrients provided by OF (Trillas et al. 2006; Postma et al. 2003), which increased the viability of the antagonists and made them more competitive in the soil. It was the possible reason why BIO was more effective in preventing the disease than SQR-T37 alone. The conclusion is consistent with previous reports (El-Hassan and Gowen 2006) that the combination of *Bacillus subtilis* and organic substrates was more effective at suppressing *F. oxysporum* and promoting the growth of lentil plants compared with individual *B. subtilis* because organic substrates helped the antagonist to survive in the environment.

Soil microbial diversity is critical to the maintenance of soil health and quality (Garbeva et al. 2006). Luo et al. (2010) found that the application of bio-organic fertilizer decreased the diversity of fungi in cotton rhizosphere. However, there were very few reports on the detailed community of microorganisms after the application of organic fertilizers and antagonists in cucumber cropping soils infested by *R. solani*. In this study, we detected the community of fungi and bacteria using the DGGE technique. The number of fungal species from the T and BIO treatments was lower than from the CK_2 and OF treatments, which was caused by SQR-T37 inhibition of the other fungi in the soil. The number of bands in the OF treatment was greater than that in the CK_2 treatment because the applied organic fertilizer intensified the growth of fungal species. The applications of SQR-T37 and BIO both decreased the fungal diversity and increased the bacterial diversity in the cucumber rhizosphere. However, the largest effect was observed for the application of BIO. Furthermore, parts of the fungi species decreased by BIO were potentially pathogenic to plants or animals, such as *R. solani*, *Setosphaeria* sp., and *Lecythophora* sp. (Garbeva et al. 2006; Simcox and Bennetzen 1993; Scott et al. 2004). Many strains of bacteria and actinomycota can cause plant disease, but more damages are caused by fungi (Brussaard et al. 2007). Some bacteria have the potential for pathogen suppression (Boulter et al. 2002). Many bacterial species, including *Bacillus* spp.; *Erwinia* spp.; *Pseudomonas* spp., have been reported that they could promote the growth of the plants (Nelson 1998; Mayak et al. 1999; Ramos et al. 2003). These results indicated that BIO is effective in both amending and keeping the balance of the plant rhizosphere microbial community. In the DGGE profiles of the fungal community, the bands of *R. solani* in T and BIO treatments were almost invisible while those in CK_2 and OF were very distinct. Furthermore, the *T. harzianum* band in the BIO treatment was more distinct than in the T treatment. These results further verified that SQR-T37 could decrease the population of *R. solani*, and organic fertilizer could assist in better growth and reproduction of *T. harzianum* in soil conditions.

In summary, SQR-T37 is a powerful antagonist against *R. solani* Q1. This is attributed to the mycoparasitism, in which SQR-T37 coiled the mycelium of *R. solani* Q1, excreted the hydrolytic enzymes and destroyed the cell wall of the pathogen, penetrated the *R. solani* Q1 cell wall, and made the leakage of its cytoplasm. SQR-T37 protected the cucumber seedlings from damping-off diseases and decreased the population of *R. solani* in the soil. Furthermore, SQR-T37 significantly promoted the growth of the cucumber seedlings. A bio-organic fertilizer product which was produced by the combination of organic fertilizer and SQR-T37 was necessary to make the biocontrol more effective. The product was able to provide the antagonists with nutrients and stabilize the population of them, which then increased the antagonists' viability and made them more competitive in the soil, rhizosphere, and on the plant roots. These results together with other reports (Zhang et al. 2008; Ling et al. 2010; Luo et al. 2010) provide a breakthrough in the research work of the biological control of soil-borne pathogen diseases.

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References

Adesina MF, Lembke A, Costa R, Speksnijder A, Smalla K (2007) Screening of bacterial isolates from various European soils for in vitro antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: site-dependent composition and diversity revealed. Soil Biol Biochem 39:2818–2828

Almeida FBdR, Cerqueira FM, Silva RdN, Ulhoa CJ, Lima AL (2007) Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: evaluation of coiling and hydrolytic enzyme production. Biotechnol Lett 29:1189–1193

Anderson NA (1982) The genetics and pathology of *Rhizoctonia solani*. Annu Rev Phytopathol 20:329–374

Baker KF (1970) Types of *Rhizoctonia* diseases and their occurrence. In: Parmeter JR Jr (ed) *Rhizoctonia solani*, biology and pathology. University of California Press, California, pp 125–148

Benhamou N, Chet I (1993) Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold cytochemistry of mycoparasitic process. Phytopathology 83:1062–1071

Benitez T, Rincon AM, Limon MC, Codon AC (2004) Biocontrol mechanisms of *Trichoderma* strains. Int Microbiol 7:249–260

Boulter JI, Trevors JT, Boland GJ (2002) Microbial studies of compost: bacterial identification, and their potential for turfgrass pathogen suppression. World J Microb Biot 18:661–671

Brussaard L, Ruiter PC, Brown GG (2007) Soil biodiversity for agricultural sustainability. Agr Ecosyst Environ 121:233–244

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193

Chen LH, Yang XM, Raza W, Li JH, Liu YX, Qiu MH, Zhang FG, Shen QR (2010) *Trichoderma harzianum* SQR-T037 rapidly degrades allelochemicals in rhizospheres of continuously cropped cucumbers. Appl Microbiol Biot. doi:10.1007/s00253-010-2948-x

Compant S, Duffy B, Nowak J, Clement C, Barka E (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action and future prospects. Appl Environ Microb 71:4951–4959

Das M, Royer TV, Leff LG (2007) Diversity of fungi, bacteria, and actinomycetes on leaves decomposing in a stream. Appl Environ Microb 73:756–767

Dineen SM, Aranda R IV, Anders DL, Robertson JM (2011) An evaluation of commercial DNA extraction kits for the isolation of bacterial spore DNA from soil. J Appl Microbiol. doi:10.1111/j.1365-2672.2010.04816.x

Dong XL, Reddy GB (2010) Soil bacterial communities in constructed wetlands treated with swine wastewater using PCR-DGGE technique. Bioresource Technol 101:1175–1182

Elad Y, Chet I, Henis Y (1982) Degradacion of plant pathogenic fungi by *Trichoderma harzianum*. Can J Microbiol 28:719–725

El-Hassan SA, Gowen SR (2006) Formulation and delivery of the bacterial antagonist *Bacillus subtilis* for management of lentil vascular wilt caused by *Fusarium oxysporum* f. sp. *lentis*. J Phytopathol 154:148–155

El-Katatny MH, Somitsch W, Robra KH, El-Katatny MS, Gübitz GM (2000) Production of chitinase and β-1,3-glucanase by *Trichoderma harzianum* for control of the phytopathogenic fungus *Sclerotium rolfsii*. Food Technol Biotech 38:173–180

Ercolini D (2004) PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. J Microbiol Meth 56:297–314

Fisher SG, Lerman LS (1983) DNA fragments differing by single base pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. Proc Natl Acad Sci USA 80:1579–1583

Freeman WM, Walker SJ, Vrana KE (1999) Quantitative RT-PCR: pitfalls and potential. Biotechniques 26:112–125

Garbeva P, Postma J, Veen JA, Elsas JD (2006) Effect of above-ground plant species on soil microbial community structure and its impact on suppression of *Rhizoctonia solani* AG3. Environ Microbiol 8:233–246

Gerhardson B (2002) Biological substitutes for pesticides. Trends Biotechnol 20:338–343

Grosch R, Faltin F, Lottmann J, Kofoet A, Berg G (2005) Effectiveness of three antagonistic bacterial isolates to suppress *Rhizoctonia solani* Kühn on lettuce and potato. Can J Microbiol 51:345–353

Hadar Y, Chet I, Henis Y (1979) Biological control of *Rhizoctonia solani* damping-off with wheat bran culture of *Trichoderma harzianum*. Phytopathology 69:64–68

Han Y, Yang BJ, Zhang FL, Miao XL, Li ZY (2009) Characterization of antifungal chitinase from marine *Streptomyces* sp. DA11 associated with south China sea sponge *Craniella australiensis*. Mar Biotechnol 1:132–140

Hervás A, Landa B, Datnoff LE (1998) Effects of commercial and indigenous microorganisms on *Fusarium* wilt development in chickpea. Bio Control 13:166–176

Hoitink HAJ, Boehm MJ (1999) Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. Annu Rev Phytopathology 37:427–446

Hu P, Zhou GH, Xu XL, Li CB, Han YQ (2009) Characterization of the predominant spoilage bacteria in sliced vacuum-packed cooked ham based on 16 S rDNA-DGGE. Food Control 20:99–104

Jagnow G (1987) Inoculation of cereal crops and forage grasses with nitrogen-fixing rhizosphere bacteria: possible causes of success and failure with regard to yield response-a review. Z Pflanzenernahr Bodenkd 150:361–368

Jagnow G, Höflich G, Hoffmann KH (1991) Inoculation of non-symbiotic rhizosphere bacteria-possibilities of increasing and stabilizing yields. Angew Bot 65:97–126

Kai M, Effmert U, Berg G, Piechulla B (2007) Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. Arch Microbiol 187:351–360

Kay SL, Stewart A (1994) Evaluation of fungal antagonists for control of white rot in soil box trials. Plant Pathol 43:371–377

Kuter GA, Nelson EB, Hoitink HAJ, Madden LV (1983) Fungal populations in container media amended with composted hardwood bark suppressive and conducive to *Rhizoctonia* damping-off. Phytopathology 73:1450–1456

Lee YG, Chung KC, Wi SG, Lee JC, Bae HJ (2009) Purification and properties of a chitinase from *Penicillium* sp. LYG 0704. Protein Expres Purif 65:244–250

Leisova L, Minarikova V, Kucera L, Ovesna J (2006) Quantification of *Pyrenophora teres* in infected barley leaves using real-time PCR. J Microbiol Meth 67:446–455

Lewis JA, Lumsden RD (2001) Biocontrol of damping-off of greenhouse-grown crops caused by *Rhizoctonia solani* with a formulation of *Trichoderma* spp. Crop Prot 20:49–56

Li SD (1995) Quantitative assay of *Rhizoctonia solani* Kühn AG-1 in soil. Soil Biol Biochem 27:251–256

Li XY, Zhang HW, Wu MN, Zhang Y, Zhang CG (2008) Effect of methamidophos on soil fungi community in microcosms by plate count, DGGE and clone library analysis. J Environ Sci 20:619–625

Lievens B, Brouwer M, Vanachter ACRC, Cammue BPA, Thomma BPHJ (2006) Real-time PCR for detection and quantification of

fungal and oomycete tomato pathogens in plant and soil samples. Plant Sci 171:155–165

Ling N, Xue C, Huang QW, Yang XM, Xu YC, Shen QR (2010) Development of a mode of application of bioorganic fertilizer for improving the biocontrol efficacy to *Fusarium* wilt. Biocontrol 55:673–683

Litterick A, Harrier L, Wallace P, Watson C, Wood M (2004) The role of uncomposted materials, composts, manures and compost extracts in reducing pest and disease incidence and severity in sustainable temperate agricultural and horticultural crop production-a review. Crit Rev Plant Sci 23:453–479

López-Mondéjar R, Antón A, Raidl S, Ros M, Pascual JA (2010) Quantification of the biocontrol agent *Trichoderma harzianum* with real-time TaqMan PCR and its potential extrapolation to the hyphal biomass. Bioresource Technol 101:2888–2891

López-Mondéjar R, Ros M, Pascual JA (2011) Mycoparasitism-related genes expression of *Trichoderma harzianum* isolates to evaluate their efficacy as biological control agent. Biol Control 56:59–66

Lucas GB (1975) Diseases of tobacco, 3rd edn. Biological Consulting Associates, North California

Luo J, Ran W, Hu J, Yang XM, Xu YC, Shen QR (2010) Application of bio-organic fertilizer significantly affected fungal diversity of soils. Soil Sci Soc Am J 74:2039–2048

Mackay IM, Arden KE, Nitsche A (2002) Real time PCR in virology. Nucleic Acids Res 30:1292–1305

Mayak S, Tirosh T, Glick BR (1999) Effect of wild-type and mutant plant growth-promoting rhizobacteria on the rooting of mung bean cuttings. J Plant Growth Regul 18:49–53

Mendoza-Mendoza A, Pozo MJ, Grzegorski D, Martínez P, García JM, Olmedo-Monfil V, Cortés C, Kenerley C, Herrera-Estrella A (2003) Enhanced biocontrol activity of *Trichoderma* through inactivation of a mitogen-activated protein kinase. Proc Natl Acad Sci USA 100:15965–15970

Minuto A, Spadaro D, Garibaldi A, Gullino LM (2006) Control of soil borne pathogens of tomato using a commercial formulation of *Streptomyces griseoviridis* and solarization. Crop Prot 15:735–742

Nelson EB (1998) Biological control of *Pythium* seed rot and preemergence damping-off of cotton with *Enterobacter cloacae* and *Ervinis herbicola* applied as seed treatments. Plant Dis 72:140–142

Niemi RM, Heiskanen I, Wallenius K, Lindström K (2001) Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. J Microbiol Meth 45:155–165

Noronha EF, Ulhoa CJ (2000) Characterization of a 29-KDa β-1,3-glucanase from *Trichoderma harzianum*. FEMS Microbiol Lett 183:119–123

Papavizas GC (1985) *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. Annu Rev Phytopathol 23:23–54

Postma J, Montanari M, van den Boogert PHJF (2003) Microbial enrichment to enhance the disease suppressive activity of compost. Eur J Soil Biol 39:157–163

Ramos B, García JAL, Probanza A, Barrientos ML, Mañero FJG (2003) Alterations in the rhizobacterial community associated with European alder growth when inoculated with PGPR strain *Bacillus licheniformis*. Environ Exp Bot 49:61–68

Reithner B, Schuhmacher R, Stoppacher N, Pucher M, Brunner K, Zeilinger S (2007) Signaling via the *Trichoderma atroviride* mitogen-activated protein kinase Tmk1 differentially affects mycoparasitism and plant protection. Fungal Genet Biol 44:1123–1133

Ruiz-Ruiz S, Moreno P, Guerri J, Ambrós S (2007) A real-time RT-PCR assay for detection and absolute quantitation of *Citrus tristeza* virus in different plant tissues. J Virol Methods 145:96–105

Rutledge RG, Côté C (2003) Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res 31:93

Scheuerell SJ, Sullivan DM, Mahafee WF (2005) Suppression of seedling damping-off caused by *Pythium ultimum*, *P. irregulare*, and *Rhizoctonia solani* in contained media amended with a diverse range of Pacific Northwest compost sources. Phytopathology 95:306–315

Scott IU, Cruz-Villegas V, Flynn HW, Miller D (2004) Delayed-onset, bleb-associated endophthalmitis caused by *Lecythophora mutabilis*. Am J Ophthalmol 137:583–585

Sharma R, Ranjan R, Kapardar RK, Grover A (2005) 'Unculturable" bacterial diversity: an untapped resource. Curr Sci 89:72–77

Simcox KD, Bennetzen JL (1993) The use of molecular markers to study *Setosphaeria turcica* resistance in maize. Phytopathology 83:1326–1330

Sneh B, Jabaji-Hare S, Neate S, Dijst G (1996) *Rhizoctonia* species: taxonomy, molecular biology, ecology, pathology, and disease control, 5th edn. Kluwer Academic Publishers, The Netherlands

Trillas MI, Casanova E, Cotxarrera L, Ordovas J, Borrero C, Aviles M (2006) Composts from agricultural waste and the *Trichoderma asperellum* strain T-34 suppress *Rhizoctonia solani* in cucumber seedlings. Biol Control 39:32–38

Tuitert G, Szczech M, Bollen GJ (1998) Suppression of *Rhizoctonia solani* in potting mixtures amended with compost made from organic household waste. Phytopathology 88:764–773

Uyanoz R (2007) The effects of different bio-organic, chemical fertilizers and their combination on yield, macro and micro nutrition content of dry bean (*Phaseolus vulgaris* L.). Int J Agr Res 2:115–125

Van Veen JA, Van Overbeek LS, Van Elsas JD (1997) Fate and activity of microorganisms introduced into soil. Microbiol Mol Biol R 61:121–135

Verma M, Brar SK, Tyagi RD, Surampalli RY, Valéro JR (2007) Antagonistic fungi, *Trichoderma* spp.: panoply of biological control. Biochem Eng J 37:1–20

Wu HS, Yang XN, Fan JQ, Miao WG, Ling N, Xu YC, Huang QW, Shen QR (2009) Suppression of *Fusarium* wilt of watermelon by a bio-organic fertilizer containing combinations of antagonistic microorganisms. Biocontrol 54:287–300

Zhang SS, Raza W, Yang XM, Hu J, Huang QW, Xu YC, Liu XH, Ran W, Shen QR (2008) Control of *Fusarium* wilt disease of cucumber plants with the application of a bio-organic fertilizer. Biol Fert Soils 44:1073–1080

Zhao QY, Dong CX, Yang XM, Mei XL, Ran W, Shen QR, Xu YC (2011) Biocontrol of *Fusarium* wilt disease for *Cucumis melo* melon using bio-organic fertilizer. Appl Soil Ecol 47:67–75