Biocontrol Potential of *Streptomyces griseus* H7602 Against Root Rot Disease (*Phytophthora capsici*) in Pepper

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The root rot of pepper (*Capsicum annuum* L.) caused by *Phytophthora capsici* is one of the most important diseases affecting this crop worldwide. This work presents the evaluation of the capacity of *Streptomyces griseus* H7602 to protect pepper plants against *Phytophthora capsici* and establishes its role as a biocontrol agent. In this study, we isolated an actinomycete strain H7602 from rhizosphere soil, identified it as *Streptomyces griseus*, and established its antifungal activity against various plant pathogens including *P. capsici*. H7602 produced lytic enzymes such as chitinase, β-1,3-glucanase, lipase and protease. In addition, crude extract from H7602 also exhibited destructive activity toward *P. capsici* hyphae. In the pot trial, results showed the protective effect of H7602 against pepper from *P. capsici*. Application of H7602 culture suspension reduced 47.35% of root mortality and enhanced growth of pepper plants for 56.37% in fresh root and 17.56% g in 47.35% of root mortality and enhanced growth of pepper plants for 56.37% in fresh root and 17.56% g in fresh shoot as compared to control, resulting in greater protection to pepper plants against *P. capsici* infestation. Additionally, the enzymatic activities, chitinase and β-1,3-glucanase, were higher in rhizosphere soil and roots of pepper plants treated with H7602 than other treated plants. Therefore, our results indicated a clear potential of *S. griseus* H7602 to be used for biocontrol of root rot disease caused by *P. capsici* in pepper.

**Keywords**: antifungal activity, biocontrol, *Phytophthora capsici*, *Streptomyces griseus*

Based on increasing public concern about residues of fungicides in food products, as well as about their soil-degrading effects, availability of a sustainable and environmentally friendly method for disease control in pepper is highly desirable. Technical, economical and environmental factors are forcing to adopt new sustainable methods, such as use of microbial antagonists for the control of soilborne pathogens. Recently, the interest in biological control by beneficial microorganisms has increased consistently as an alternative disease control to substitute for various chemical controls against airborne or soilborne plant pathogens (An et al., 2010; Mukherjee and Sen, 2006; Sang et al., 2011), and antagonistic microorganisms have been shown to inhibit the growth and proliferation of various phytopathogens with little or no side effects (Arthurs et al., 2009).

*Phytophthora* root rot (PRR), caused by *Phytophthora capsici*, is one of the most devastating soilborne diseases in the world. Due to a lack of resistant cultivars, control of soilborne pathogens of pepper is mainly aimed at *P. capsici* and involves numerous applications of fungicides both before and after transplanting in the field (Hwan and Kim, 1995). To combat wilt caused by *P. capsici* in pepper, cultural practices based on the biology and ecology of the pathogen have been suggested (Ristaino and Johnston, 1999). One of the strategies is use of biocontrol agents in order to reduce the number of applications of fungicides and the pathogen population resistant to the fungicides (Sang et al., 2008).

There have been considerable efforts to find biological control agents against PRR of pepper and several potential candidates have been reported including: *Penicillium striatiaporum* (Ma et al., 2008), *Pseudomonas fluorescens* (Paul and Sarma, 2006), and *Streptomyces rochei* (Ezziyyani et al., 2007). The mechanisms of disease suppression by antagonistic bacteria have been extensively studied and they involved the production of antibiotics, extracellular enzymes, biosurfactant or cyanide production and/or the...
Isolation and identification of antagonistic microorganism. H7602 was cultured in gelatin chitin (GC) medium containing [gelatin 0.5 g; chitin powder 0.5 g; NaCl 0.05%; NH₄Cl 0.1%; MgSO₄·7H₂O 0.05%; CaCl₂·2H₂O 0.05%; yeast extract 0.025%; Agar 2%; and potato dextrose broth 0.5%]. One isolate having the strongest enzyme activities was selected and stored in glycerol solution 25% at −70°C for further experiments. This isolate was later identified by 16S rRNA gene sequence analysis and matching sequences using BLAST search at gene bank database of NCBI (Bethesda, MD).

Materials and Methods

Isolation and identification of antagonistic microorganism. Rhizosphere soils were collected from crop fields (rice, bean or vegetables) in Gunsan, Korea in March, 2011. Soil samples were serially diluted with sterile distilled water and inoculated on chitin agar plates containing [colloidal chitin 0.5%; Na₃HPO₄ 0.2%; KH₂PO₄ 0.1%; NaCl 0.5%; NH₄Cl 0.1%; MgSO₄·7H₂O 0.05%; CaCl₂·2H₂O 0.05%; yeast extract 0.05%; Agar 2%; and pH 7.0]. The plates were incubated at 30°C for 3 days, after that several colonies possessing strong chitin clearance zones were selected and sub-cultured on the same medium for more purification. All isolates were tested by a dual culture assay against P. capsici on chitin potato dextrose (CP) agar medium containing [colloidal chitin 0.25%; Na₃HPO₄ 0.1%; KH₂PO₄ 0.05%; NaCl 0.25%; NH₄Cl 0.05%; MgSO₄·7H₂O 0.025%; CaCl₂·2H₂O 0.025%; yeast extract 0.025%; Agar 2%; and potato dextrose broth 0.5%]. One isolate having the strongest activity was selected and stored in glycerol solution 25% at −70°C for further experiments. This isolate was later identified by 16S rRNA gene sequence analysis and matching sequences using BLAST search at gene bank database of NCBI (Bethesda, MD).

Antagonism of H7602 to various plant pathogenic fungi. H7602 was tested for antagonism against several fungal pathogens namely: Phytophthora capsici KACC 40483, Fusarium oxysporum f. sp. lycopersici KACC 40032 and Rhizoctonia solani AG-2-2 (IV) KACC 40132 purchased from KACC (Korea Agriculture Culture Collection, 225 Seodun-dong, Suwon, Gyunggi Province, Korea). H7602 was inoculated on CP agar medium one day before inoculation of the fungal pathogens. The antagonist and the test pathogen were placed 4 cm apart on the same CP plate, and all cultures were incubated at 26°C. All tests were carried out in five replications. Growth of fungal pathogens was evaluated at 7 days after inoculation by measuring colony radius from the original point of inoculation in the direction of the antagonist using the following formula:

\[
\frac{(R - r)}{R} \times 100
\]

where, R is the distance of fungal growth from the point of inoculation to the colony margin

Effect of crude extract from H7602 on P. capsici hyphae morphology. H7602 was cultured in gelatin chitin (GC) medium containing [gelatin 0.5 g; chitin powder 0.5 g; complex fertilizer 3 g (21-17-17; N 0.63 g; P₂O₅ 0.51 g; K₂O 0.51 g; Dongbu Hitek company, Korea); dry grass powder 6.0 g; rice bran 0.5 g; yeast extract 0.03 g; water 1 L; and pH 7.0] at 30°C on a rotary shaker at 170 rpm for 5 days. The supernatant was acidified with concentrated HCl to pH 3.0 and extracted with an equal volume of n-Hexane (4 L, two times). The n-Hexane soluble organic fraction was concentrated by a rotary evaporator (Büchi,

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where, R is the distance of fungal growth from the point of inoculation to the colony margin

Antifungal activity was defined in the terms of size of the inhibition zone and denoted as follows: − no inhibition zone; + (very weak), 0-5 mm; ++ (weak), > 5-10 mm; +++ (moderate), > 10-15 mm; ++++ (strong), > 15-20 mm; +++++ (very strong), > 20 mm as the distance between the fungal pathogen and the area of the antagonist growth after 7 days.

Lytic enzyme assay. To examine chitinase and β-1,3-glucanase activities, H7602 was cultured on medium containing [Na₃HPO₄ 0.2%; KH₂PO₄ 0.1%; NaCl 0.05%; NH₄Cl 0.1%; MgSO₄·7H₂O 0.05%; CaCl₂·2H₂O 0.05%; and yeast extract 0.01%] supplemented with 1.0% of P. capsici hyphae powder [made from the fungal mycelium grown in PDB at 30°C for 15 days, filtered through Whatman no. 1 filter paper, thoroughly washed with distilled water, autoclaved at 121°C for 15min, dried at 70°C for 3 days, and then ground using mixer (SMB-S20HSI, SPN Corporation, Korea)] at 30°C on a rotary shaker at 170 rpm for 7 days. The supernatant was daily collected and assayed by method of Tabatabai (1982) and Yedidia et al. (2000), separately. Also, protease and lipase activities were determined by using skim milk agar (MA) plate and Luria-Bertani (LB) plate supplemented with 1% Tween 80, respectively (Folman et al., 2003).
Switzerland) to obtain crude extract. To examine the effect of this crude extract on hyphae of *P. capsici*, one ml of *P. capsici* culture grown on potato dextrose broth (PDB) medium at 30°C for 5 days was put in test tube, and the crude extract dissolved in methanol was added to the test tubes to a final concentration of 500 and 1,000 ppm, respectively. The same volume of methanol was used as control. The mixtures of crude extract and *P. capsici* were incubated at 30°C for 72 h and the mycelia were observed under the light microscope (Olympus BX41TF, Japan). All tests for observations of morphological mycelia were done in triplicate.

**Preparation of H7602 culture and pathogen inoculum.** For the pot trial, H7602 was grown in GC media (as mentioned above) for 5 days at 30°C. Zoospores were prepared by growing *P. capsici* (KACC 40483) on the V8 juice agar medium containing [V8 juice 10%; CaCO3 0.1%; and Agar 2%] at 30°C. After incubation for 5 days, the fungal media were cut and moved to an empty plate (9 × 9 cm). All fungal media were flooded with sterile water and replaced daily with the same amount of sterile water. The continuous incubation was performed under fluorescent light for 5 days at 30°C to produce sporangia and then chilled at 4°C for 30 min to release zoospores. Zoospore suspension was filtrated by sterile cheesecloth and diluted with sterile water to a concentration of 1 × 106 zoospore ml⁻¹ (Kim et al., 1997).

**Plant growth condition and sampling.** Pepper seeds (*Capsicum annuum L.*, Chungok) were sown in 3 × 3 cm plastic cell plug tray filled with commercial grade bedding soil. At 4 weeks after sowing, pepper seedlings were transplanted to plastic pots containing 600 g of non-sterilized soil mixture (soil: sand: vermiculite, 2:1:1, v:v:v). Pepper plants were grown at 24°C in an artificially illuminated room (12,000 lux at plant height) with a 16 h photoperiod. At 2, 3, 4 and 5 weeks after transplanting, each pot was amended with 50 ml of H7602 culture (GC+H7602), GC medium only (GC medium) or commercial fungicide 1% (Fungicide) named as Kkea Kkeu Tan (Korea). Thirty isolates showing clear zones on chitin agar medium were isolated from rhizosphere samples of Gunsan area in Korea. Dual culture assay was performed for the selection of antagonistic microorganism against various fungal pathogens on chitin potato dextrose (CP) agar plates. One isolate exhibited antifungal activities against *P. capsici*, *F. oxysporum* and *R. solani*. When this isolate and fungal pathogens were simultaneously inoculated for 7 days, the growth inhibitions were found to be 53.33% against *P. capsici*, 41.67% against *F. oxysporum* and 30.00% against *R. solani* (Table 1).

This isolate was identified as *Streptomyces griseus* based on 16S rRNA gene sequence analysis. The septic isolate

**Root mortality assay.** Root mortality was measured by modified method of Knievel (1973). Two hundred and fifty mg of fresh roots were incubated with 10 ml of 0.6% 2, 3, 5-triphenyltetrazolium chloride in 0.05 M sodium phosphate buffer (pH 7.4) for 24 h in the incubator at 30°C. Roots were rinsed twice with distilled water and then extracted twice with 95% ethanol at 70°C for 4 h. Combined extracts were adjusted to a final volume of 20 ml with 95% ethanol. Absorbance was measured using spectrophotometer at 490 nm. A standard curve was made using different proportions of living roots and killed roots to calculate root mortality. Root mortality was expressed as percentage dead root dry weight (D.W) of the total root D.W.

**Enzyme activity assay in soil and root.** Chitinase and β-1,3-glucanase activities in the rhizosphere soil samples and roots were determined using the modified method of Tabatabai (1982) and Yedidia et al. (2000), respectively.

**Statistical analysis.** The data were subjected to analysis of variance using SAS 9.1 software (SAS Institute, 2003). Mean values among treatments were compared by the least significant difference (LSD) test at 5% level (p = 0.05) of significance and presented as the mean values ± standard deviation (SD).

**Results**

**Isolation and identification of antagonistic microorganism.** Thirty isolates showing clear zones on chitin agar medium were isolated from rhizosphere samples of Gunsan area in Korea. Dual culture assay was performed for the selection of antagonistic microorganism against various fungal pathogens on chitin potato dextrose (CP) agar plates. One isolate exhibited antifungal activities against *P. capsici*, *F. oxysporum* and *R. solani*. When this isolate and fungal pathogens were simultaneously inoculated for 7 days, the growth inhibitions were found to be 53.33% against *P. capsici*, 41.67% against *F. oxysporum* and 30.00% against *R. solani* (Table 1).

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Sequence showed high identity when compared with other matching sequences (97-100%), and designated as *Streptomyces griseus* H7602 with GenBank accession No. JN827310 and BLAST analysis revealed strong homology with *Streptomyces griseus* EF687741.1 (Fig. 1).

Production of lytic enzymes. Production of lytic enzymes, such as chitinase and β-1,3-glucanase, was examined from the culture supernatant of H7602. Chitinase and β-1,3-glucanase activities rapidly increased in the time period of 4 days, and eventually reached a maximum value of 4.12 unit ml\(^{-1}\) and 5.20 unit ml\(^{-1}\), respectively. Thereafter, they gradually decreased from 4 to 7 days (Fig. 2). In addition, H7602 showed lipase and protease activity as evidenced by the formation of precipitation zones on LB agar supplemented with 1% of Tween 80 and a clear zone on MA medium, respectively (data were not shown).

Hyphae morphology of *P. capsici* affected by crude extract of H7602. To determine the effect of antifungal substance on fungal pathogen, *P. capsici* was grown on PDB medium with the presence of active crude extract.

### Table 1. Antifungal activity of *S. griseus* H7602 against various fungal pathogens

| Fungi                          | Inhibition (%) | Antifungal activity |
|-------------------------------|----------------|---------------------|
| *Phytophthora capsici* KACC 40483 | 53.33 ± 1.44   | ++++                |
| *Fusarium oxysporum f. sp lycopersici* KACC 40032 | 41.67 ± 3.82   | +++                |
| *Rhizoctonia solani* AG-2-2 (IV) KACC 40132 | 30.00 ± 2.50   | +++                |

Antifungal activity (inhibition zone): − no inhibition zone; + (very weak), 0-5 mm; ++ (weak), > 5-10 mm; +++ (moderate), > 10-15 mm; ++++ (strong), > 15-20 mm; ++++++ (very strong), > 20 mm as the distance between the fungal pathogen and the area of antagonist growth after 7 days. Calculated mean values are from five replicates.
After 72 h, *P. capsici* hyphae incubated with methanol showed normal morphology under the light microscope (Fig. 3A). However, Some *P. capsici* hyphae incubated with 500 ppm of crude extract revealed abnormal mycelia such as degradation, deformation, and lysis (Fig. 3B), and most of *P. capsici* hyphae were degraded in incubation with 1000 ppm (Fig. 3C).

**Growth promotion and biocontrol effect of H7602.** To examine the effect of H7602 on pepper growth and disease control, pepper rhizosphere was inoculated with H7602 culture (GC+H7602), GC medium only (GC medium), commercial fungicide 1% (Fungicide) or tap water (Control) and then infected with *P. capsici* zoospores. There were increases in root mortality percentages of all treatments during observation time, but they were different in each treatment. Pepper plants treated with tap water, GC medium and fungicide showed wilting and rotting of the roots at 6 days after *P. capsici* infection, and progressive development of disease led to a high root mortality percentage of 78.56, 70.62 and 53.99%, respectively, at 12 days after *P. capsici* infection. On the other hand, root mortality percentage of GC+H7602 treatment slowly increased, had the lowest value of 31.21% at 12 days after *P. capsici* infection (Fig. 4), and reduced 47.35% in comparison with control. There were not significant differences among treatments at 0 and 3 days, but they were found at 6, 9 and 12 days after *P. capsici* infection between treatments (GC+H7602 and fungicide) and others.

For measurement of plant growth, fresh root and shoot weights confirmed that pepper plants treated with H7602 were still increased from the *P. capsici* challenge. The fresh shoot/root weights of control, GC medium and fungicide treatments gradually increased from 0 to 6 days after *P. capsici* infection and then slightly decreased at 9 and 12 days due to reduced growth of pepper by the pathogen. However, the fresh root and shoot weights of H7602 treated pepper plants continuously increased during infection period (Table 2), and they had higher values of 56.37% and 17.56% than control at 12 days after *P. capsici* infection, respectively. Hence, pepper plants inoculated with H7602 showed better significant growth than others from *P. capsici* attack.

**Enzyme activity in soil and root.** Enzyme activities in rhizosphere of pepper plants were presented in Fig. 5A and 5B. During experiment period, chitinase activity in soil treated with H7602 was always higher than that of soil treated with others, and β-1,3-glucanase activity also presented a similar pattern with chitinase activity in all treatments. In pepper roots, chitinase activity gradually increased in H7602 treated pepper roots at 6, 9 and 12 days after *P. capsici* infection. In contrast, this activity gradually decreased at the same time in other treated pepper roots (Fig. 5C and 5D).
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5D). β-1,3-glucanase activity indicated little fluctuation in GC medium treatment during observation, while it gradually increased in pepper roots treated with H7602 and gradually decreased in others treated with tap water or fungicide.

**Discussion**

The need to produce innocuous food crops and reduce the pollution generated by synthetic chemicals has led to a search for biocontrol agents against plant pathogens which are safe for both the crops and human consumption. Several streptomycetes species have been described as biocontrol agents effective against numerous plant pathogens, and have shown the potential to produce bioactive compounds as well as to reduce or inhibit mycelial growth of several fungi (Bressan and Figueiredo, 2008; Ezziyyani et al., 2007; Mukherjee and Sen, 2006). In present study, H7602 strain was isolated from rhizosphere and demonstrated a broad spectrum of strong antifungal activity against various fungal pathogens including *P. capsici* (Table 1). It was identified as *S. griseus* (Accession No. JN827310) using 16S rRNA analysis (Fig. 1).

A primary mechanism of pathogen inhibition used by plant growth promoting rhizobacteria (PGPR) includes the production of antibiotics, lytic enzymes, volatile compounds, and siderophores (Mao et al., 2006). Antibiotics and lytic enzymes in particular are well known antifungal compounds that can directly or indirectly protect plants from pathogen attack. Previous results of Mukherjee and Sen (2006) indicated that lytic enzymes such as chitinase and β-1,3-glucanase were produced by strains of *Streptomyces* sp. and they would be used in controlling plant pathogens. Kim et al. (2003) also reported that the purified chitinase from *Streptomyces* sp. M-20 showed antifungal activity against *Botrytis cinerea*, and lysozyme activity against the cell wall of *B. cinerea*. In our study, lytic enzyme (chitinase and β-1,3-glucanase) activities had high values in mineral nutrient medium supplemented with mycelium powder of *P. capsici* (Fig. 2). Similarly, Trejo-Estrada et al. (1998) reported that chitinase and β-1,3-glucanase production from *S. violaceusniger* YCED-9 was induced by fungal cell wall from *Fusarium oxysporum*.

Particular *Streptomyces* species have exhibited potential in the biological control of plant fungal pathogens thorough production of antibiotics (Ezra et al., 2004). Lee et al. (2005) reported 4-phenyl-3-butenoic acid produced by *Streptomyces koyangensis* sp. nov., which inhibited the mycelial growth of several plant pathogenic fungi. In
The antagonistic activity of biocontrol microorganisms is often demonstrated by the inhibition of mycelial growth or a reduction in symptoms of infected plants (Khan et al., 2005). In the pot trial, our results demonstrated that the strain H7602 had a high potential to suppress PRR and enhance growth of pepper plants. Normally, when zoospores of *P. capsici* were inoculated in rhizosphere of pepper plants, root rot disease rapidly developed and reached high disease index. This is the reason why H7602 was inoculated before *P. capsici* infection. Moreover, it is important to stress the survival percentage of pumpkin plants inoculated with *P. capsici* after the pre-inoculation with H7602. The inoculation of pepper plants with H7602 significantly reduced the root mortality percentage, and improved pepper growth from the pathogen attack (Fig. 4 and Table 2). In this case, pepper plants were protected from root rot symptoms by the colonization of H7602. Similar conclusion was also made by Shen et al. (2002), when roots of pepper plants were treated with a suspension of *Serratia plymuthica* A21-4 and then inoculated with zoospores suspension of *P. capsici*, the number of diseased plants and disease severity were significantly reduced. Also, El-abyad et al. (1993) reported that plant growth was improved by the seed coating treated with *Streptomyces* spp.

Among pathogenesis-related proteins (PRs), primarily chitinase and glucanase possess potential antifungal activities through degradation of fungal cell walls (Dumas-Gaudot, 1996). In addition, chitinase and β-1,3-glucanase are a structurally and functionally diverse group of hydrolytic enzymes involved in defense reactions of plants against pathogens (Jackson and Taylor, 1996). Our investigation in pot trial studies demonstrated the chitinase and β-1,3-glucanase activities in soils and roots due to H7602 application (Fig. 5). Promotion of these enzymes in pepper roots and soils as the result of H7602 colonization could play a role in bioprotection against *P. capsici*. This is consistent with previous findings by Jung et al. (2005) who reported that the activities of β-1,3-glucanase and chitinase in *Paenibacillus illinoisensis* and *P. capsici* treated roots had increased by 54.8, and 52.8%, respectively, compared to *P. capsici* treated roots at seven days after inoculation. Yedidia et al. (2000) reported that cucumber roots treated with *T. harzianum* T-203 exhibited higher activities of chitinase (EC 3.2.1.14), β-1,3-glucanase (EC 3.2.1.6) up to 72 h post-inoculation compared to untreated control in cucumber. *Pseudomonas fluorescens* isolate Pf1 induced β-1,3-glucanase and chitinase in tomato roots inoculated with the pathogen (Ramamoorthy et al., 2002). Also, colonization of bean roots by rhizobacteria correlated with induction of PR proteins resulting in induced systemic resistance against *B. cinerea* (Zdor and Anderson, 1992). This clearly indicated that application of the antagonistic microorganisms induced PR proteins in roots of host plants.

Based on these results, we have found that, the antagonistic actinomycete, *S. griseus* H7602, showed strong antifungal activity against several pathogens consisting of *P. capsici*, and produced active substance as well as several lytic enzymes including chitinase and β-1,3-glucanase *in vitro*. In the pot trial, application of H7602 for pepper plants greatly suppressed PRR, enhanced pepper plant growth, and promoted enzyme activities in soil and roots *in vivo*. Thus, our results clearly demonstrated the potential of H7602 to be used for biocontrol of root rot disease caused by *P. capsici* in pepper. Further studies are required to confirm the efficacy of H7602 under field conditions, and more work is needed to purify and characterize the secondary metabolites produced by H7602 responsible for disease suppression.

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