Isolation and Identification of Low Molecular Weight Compounds Produced by Bacillus subtilis HJ927 and Their Biocontrol Effect on the Late Blight of Pepper (Capsicum annuum L.)

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A soil bacterium, Bacillus subtilis HJ927, exhibiting strong antagonistic property against pathogenic fungi was isolated from pepper fields infested with Phytophthora capsici. Pepper plants inoculated with P. capsici revealed severe root mortality while plants co-inoculated with B. subtilis HJ927 and P. capsici showed drastically reduced root mortality. Low molecular weight substances released by B. subtilis HJ927 mediated the plant protective effect. The anti-fungal compounds released by B. subtilis HJ927 were identified as 3-methylbutyric acid, 2-methylbutyric acid, and methyl 2-hydroxy, 3-phenylpropanoate by high-performance liquid chromatography and gas chromatography-mass spectrometry. In addition to these compounds, B. subtilis HJ927 also produced β-1,3-glucanase, a hydrolytic enzyme implicated in antifungal activity.

Key words: Bacillus subtilis, Phytophthora capsici, Pepper, Late blight of pepper, Antifungal activity

Introduction

The late blight of pepper, which is infected by Phytophthora capsici, is one of the most destructive soil-borne diseases of pepper (Capsicum annuum L.) in Korea. This disease occurs worldwide and causes root and crown rot, and aerial blight of leaves, stems and fruits. The control of Phytophthora blight has been accomplished mainly by fungicides such as metalaxyl and propamocarb (Hwang and Kim 1995; Kim et al., 1997). However, due to growing environmental concern relating to ecological damage and pesticide resistance, a great deal of attention has been devoted to searching biocontrol agents in recent years. Especially, the use of microorganisms to protect plants from pathogens have received increasing attention because they can be natural, safe, effective, persistent, and serve as durable alternative to pesticides (Dumas-Gaudot et al., 1996; Pozo et al., 2002).

Several studies have documented the protective effect of some microorganisms against soil-borne pathogens (Baker et al., 1983). For example, Singh et al. (1999) reported that Paenibacillus sp. 300 and Streptomyces sp. 385 suppressed wilt of cucumber. Similarly, Wei et al. (1991) reported the application of selected plant growth promoting rhizobacteria (PGPR), Psedomonas fluorescens G8-4, Psedomonas putida 34-13, and Serratia plymuthica 2-67 protected the cucumber plants against Colletotrichum orbiculare. In addition, Kobayashi et al. (1995) observed the control of summer patch disease in turfgrass by Xantomonas maltophilia and Serratia marcescens. In general, bacterial antagonistic effects against plant pathogens have been proposed to involve the production of sidrophores, antibiotics, and fungal cell wall hydrolytic enzymes such as chitinase and β-1,3-glucanase (Mavingui and Heulin, 1994).

The objectives of this work were to isolate antagonistic bacteria from pepper field infested by P. capsici and to isolate and identify compounds produced by the soil-bacteria that have anti-fungal activity.
Materials and Methods

Selection of antagonistic isolate  Soil samples were collected from *P. capsici* infested pepper field. Serially diluted soil samples were inoculated on HC agar medium containing dried *P. capsici* hyphae 0.5%, colloidal chitin 0.5%, NaHPO₄ 0.2%, KH₂PO₄ 0.1%, NaCl 0.05%, NH₄Cl 0.1%, MgSO₄·7H₂O 0.05%, CaCl₂·2H₂O 0.05%, yeast extract 0.05%, and agar 1.5%. The agar plates were incubated at 30°C for 5 days. Colonies bigger than 5 mm diameter were selected and successively examined for antifungal activity against *P. capsici* on CP agar plate containing colloidal chitin 0.5%, NaHPO₄ 0.1%, KH₂PO₄ 0.05%, NaCl 0.025%, NH₄Cl 0.05%, MgSO₄·7H₂O 0.025%, CaCl₂·2H₂O 0.025%, yeast extract 0.025%, agar 0.75% and potato dextrose agar 0.5%. One strain having strong anti-fungal activity was selected for further use and kept in 50% glycerol solution at -70°C.

Identification of antifungal compound produced by *B. subtilis* HJ927  Bacteria were cultured at 30°C for 3 days in LB broth and then centrifuged at 5,000 rpm for 10 min. The supernatant (15 L) was partitioned by ethylacetate (EtOAc). The aqueous phase was adjusted to pH 3.0 with 1 N HCl and extracted with EtOAc to obtain the EtOAc soluble acidic fraction. Anti-microbial activity, which was found in EtOAc soluble acidic fraction, was concentrated using a rotary evaporator under vacuum (Buchi, Switzerland). The acidic phase was further purified by silica gel adsorption chromatography (Kieselgel 60, Merck, 70-230 mesh, Darmstadt, Germany) and eluted with stepwise gradient of EtOAc-methanol (100:0, 90:10, 80:20, 70:30, 60:40, and 50:50, v/v). The 100% EtOAc fraction, which had strong anti-microbial activity, was further purified by silica gel adsorption chromatography and eluted with stepwise gradient of hexane-EtOAc-MeOH (12:2:1, 10:4:1, 8:6:1, 6:8:1, 4:10:1, and 2:12:1, v/v/v). The 12:2:1 fraction was again purified by sephadex LH-20 column chromatography (25-100 mesh, Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with methanol-chloroform (4:1, v/v). The final purification was carried out by HPLC (Waters, USA) with Bondapak C18 (7.8 x 300 mm, 10 μm, elution with 60% acetonitrile for 2 mL min⁻¹) and resulted in two pure fractions (tR 6.8 and 7.8 min).

Identification of unknown compounds isolated by Bondapak C18 was achieved using a GC-MS equipped with library search program (Wiley 7n). Oven temperature in GC (Hewlett Packard 6890N, Austria) was at 40°C for 2 min, then increased to 100°C at a rate of 2°C min⁻¹ and increased to 220°C at a rate 10°C min⁻¹, HP-5 MS column (30 m x 0.25 mm id., film thickness 0.25 μm) was used with helium as carrier gas, flow rate of 1 mL min⁻¹, injector volume of 1 μL and injector temperature of 220°C. MS (Hewlett Packard 5973N, Austria) conditions consisted of ionization voltage of 70 ev, ion source temperature of 220°C, mass range of 30-300 mass units, MS quadrupole temperature of 190°C, and interface temperature of 220°C. Active compounds were confirmed by comparing spectral and retention data obtained from the samples and authentic chemicals. During purification, antimicrobial activity of each elute was measured against *Escherichia coli* as test microorganism by a paper disc diffusion method (Hwang and Kim, 1995).

Anti microbial activity against *P. capsici*  The antimicrobial activity of the compounds purified by sephadex LH-20 column was tested against *P. capsici* according to Michener and Snell (1949). Paper disc (6 mm) loaded with 1 mg of purified compound was incubated on PDA plate with *P. capsici*. After 5 day incubation at 30°C, percentage of mycelia growth was calculated according to Hwang et al.[9] formula [1 - (diameter of mycelia growth in the chemical treated plate)/(diameter of mycelia growth in the untreated control)] x 100.

Preparation of bacterial and pathogen inoculum  Bacterial inoculum (*B. subtilis* HJ927) was cultured at 30°C in chitin broth for 3 days. The cultures were diluted in sterile distilled water to a concentration of 4 x 10⁶ colony mL⁻¹. A virulent pathogen, *Phytophthora capsici* (KACC 40480) was obtained from Korean Agricultural Culture Collection (KACC, Suwon, Korea) and then grown on the V8 juice agar in the dark at 30°C for 3 days. A portion of the medium containing the fungal hyphae was flooded with sterile de-ionized water and incubated under continuous fluorescent light for 5- days at 30°C for sporangial production. After 5 day incubation, it was chilled at 4°C for 30 min to release zoospores. Mycelia and sporangial debris were removed from zoospore suspension by filtration through sterile cheesecloth and the filtrate was diluted with sterile water to a concentration of 2 x 10⁸ zoospores mL⁻¹ (Kim et al., 1997).
Plant cultivation and inoculation Pepper (*Capsicum annuum* L.) seeds were sterilized with alcohol and then planted in the pot (600 mL) containing autoclaved soil mixture (soil: quartz sand: vermiculite, 2:1:1, v:v:v). Chemical characteristics of the soil consisted of 0.45% of organic matter, 72 mg L$^{-1}$ of P, 0.34 cmol of K, 11.10 cmol of Ca, 4.68 cmol of Mg, 12.67 cmol of cation exchangeable capacity, 6.29 of pH, and 0.65 dsm 1 of electronic condition. Peppers are grown at 24°C in an artificially illuminated room (12,000 lux at plant height) with a 16 hr photoperiod and thinned to one plant in each pot at 2 weeks after planting.

One hundred fifty mL of bacterial suspension (4 × 10$^6$ colony mL$^{-1}$) was inoculated three times (each 50 mL) by pouring into each pot at 5, 6, and 7 weeks after planting. Control plants were treated with chitin broth. At 7 weeks after planting, 50 mL of *P. capsici* zoospore suspension (2 × 10$^8$ zoospores mL$^{-1}$) was injected at the half of both bacterium inoculated plants and non-inoculated plants. Plants were watered with nutrient solution (KNO$_3$: 2.57 mM, MgSO$_4$·7H$_2$O: 1.01 mM, Ca(NO$_3$)$_2$·4H$_2$O: 3.6 mM, KH$_2$PO$_4$: 0.51 mM, ZnSO$_4$·7H$_2$O: 0.11 M, H$_3$BO$_3$: 4.58 µM, MnCl$_2$: 4H$_2$O: 1.01 µM, CuSO$_4$: 5H$_2$O: 0.08 µM, Na$_2$MoO$_4$: 2H$_2$O: 0.05 µM, and CoCl$_2$:Fe(NH$_4$)$_2$O$_6$: 62.7 µM) during the plant growth period. Plants were harvested at 0, 1, 2, 3, 6, and 9 days after infection with *P. capsici* zoospores. Plants were then carefully washed in running tap water and fresh weight of roots and shoots were measured.

Root mortality Root mortality was measured by the method of Liu and Huang (2000). Fresh root (0.5 g) was incubated in 5 mL 50 mM phosphate buffer (pH 7.4) containing 0.6% 2,3,5-triphenyltetrazolium chloride for 24 hr in the dark at 30°C. Roots were then rinsed with distilled water thoroughly. Formazan was extracted from the roots twice with 95% ethanol at 70°C for 4 hr. Combined extracts from the two extractions were adjusted to a final volume of 30 mL with 95% ethanol. Extracted solution was measured at 490 nm. A standard curve was developed using different proportions of living roots and roots killed in an autoclave. Root mortality was expressed as percentage of dead root fresh weight for total root fresh weight.

**Results**

Selection of antagonistic bacteria Bacterium having strong anti-fungal property was isolated from pepper field infested with *P. capsici*. This strain was identified as *Bacillus subtilis* based on 16s ribosomal RNA gene sequence and named as *Bacillus subtilis* HJ927 (data not shown). *B. subtilis* HJ927 inhibited the mycelial growth of *P. capsici* on the CP agar plate. Antifungal activity of *B. subtilis* HJ927 against *P. capsici* is shown in Fig. 1.

![Fig. 1. Formation of inhibition zones of *B. subtilis* HJ927 against *P. capsici* on the CP medium at 30°C.](image)

Identification of anti-fungal substance produced by *B. subtilis* HJ927 *in vitro* Active fractions obtained from sephadex LH-20 column contained 708.3 mg of yellow-brown odorant materials. The active fractions were further purified by HPLC, resulting in two distinct fractions, tR 6.8 and 7.8 (Fig. 2). When the fraction of tR 6.8 was analyzed by GC-MS, it contained two low molecule weight compounds, 3-methylbutyric acid (tR 6.8) and 3-methylbutanoic acid (tR 7.8).
Plant fresh weight The changes in plant fresh weight are shown in Fig. 5. Fresh weights of root and shoot were highest in *B. subtilis* HJ927 treated plants, followed by non-treated, *B. subtilis* HJ927 + *P. capsici* treated, and *P. capsici* treated plants at 9 days after *P. capsici* infection. Infection of *P. capsici* resulted in sudden wilt of entire plants due to rotting of the stems near the soil surface, root decay, and leaf abscission. Progressive development of disease eventually led to marked decrease of leaf fresh weight in *P. capsici*-treated plants at 6 days after infection (Fig. 5A). However, *B. subtilis* HJ927 protected plants from *P. capsici*, which was reflected in root fresh weight (Fig. 5B).

Root mortality Root mortality was almost constant in all treatments till 3 days after inoculation. However, root mortality in *P. capsici*-treated plants markedly increased thereafter with 78% of maximal value at 9 days after inoculation. In *B. subtilis* HJ927 + *P. capsici*-treated plants, there was no change in root mortality for 6 days, but slightly increased to 25% at 9 days after the treatment (Fig. 6).
Discussion

Various bacteria and fungi antagonistic to *P. capsici* are known to exist in soils (Hwang and Kim, 1995; Kobayashi et al., 1995). In this experiment, we isolated a bacterium having strong anti-fungal activity from heavily infected pepper field by *P. capsici* (Fig. 1). The bacterium was identified as *Bacillus subtilis* HJ927. There have been many previous reports on the antifungal activity of microorganisms and their use in controlling a number of plant pathogens. In particular, *Bacillus* spp., including *B. subtilis* have been used as biocontrol agents in many studies (Hwang et al., 2001; Ryder et al., 1999). Melent’ev et al. (2001) suggested that the antagonism between *Bacillus* sp. 739 and phytopathogenic fungi is mediated by non-enzymatic low molecular weight substances. Antifungal substances such as polymycin, colistin, edeine, mycobacin and bacitracins have been reported to be produced by *Bacillus* spp. (Babad et al., 1952; Fravel, 1998; Katz and Deamain, 1977; Mceen et al., 1986). Michener and Snell (1949) reported two factors, toximycin and mycosubtilin produced by a strain *B. subtilis*, which exhibited broad-spectrum antifungal activity *in vitro* against *M. fructicola* at low concentrations.

In this present study, we have identified three low molecular weight compounds (3-methylbutyric acid, 2-methylbutyric acid, and methyl 2-hydroxy, 3-phenylpropanoate) from yellow-brown odorant material produced by *B. subtilis* HJ927. The odorant material containing these compounds strongly inhibited the growth of *P. capsici* (Fig. 4). In earlier reports, the 3-methylbutyric acid and 2-methylbutyric acid have shown to inhibit the growth of *Ceratocystis ulmi* (Asante and Neal, 1964). The methyl 2-hydroxy, 3-phenylpropanoate has not been reported yet and appears to be a new antifungal compound. We tried to demonstrate the ability of 2-hydroxy, 3-phenylpropanoate, but we failed to obtain enough amount to test.

Furthermore, *B. subtilis* HJ927 also produced β-1,3-glucanase (data not shown), a hydrolytic enzyme that can inhibit pathogen through hydrolysis of fungal cell wall (Fravel, 1998). Our observation is consistent with results of Larena and Melgarejo (1996), who found that high levels of β-1,3-glucanase were produced by *Penicillium purpureogenum* against *Monilinia laxa*. We speculate that *B. subtilis* HJ927 may act synergistically against *P. capsici* by producing anti-fungal substances and hydrolytic enzymes. This suggestion is consistent with previous finding of Fogliano et al. (2002) who reported that the synergistic interaction between cell wall degrading enzymes and lipodesipatides secreted by *Pseudomonas syringae* was involved in the antagonistic mechanism against several pathogenic fungi. Furthermore, Weller et al. (1998) theorized that the production of antibiotics and hydrolytic enzyme appeared to be important characteristics in the suppression of root diseases.

We have also demonstrated a reduction of disease severity by *P. capsici* by co-inoculation with *B. subtilis* HJ927 (Fig. 6). Roots of plants co-inoculated with *B. subtilis* HJ927 and *P. capsici* were protected when compared to *P. capsici*-treated plants. These results suggest that *B. subtilis* HJ927 was capable of suppressing pepper disease by inhibiting the *P. capsici* directly in rhizosphere. In addition to its ability to reduce the severity of phytophthora blight, *B. subtilis* HJ927 showed consistent plant growth promotion effects (Fig. 5). Root and shoot growth of *B. subtilis* HJ927 treated plants were greater than those of non treated plants. Our results are very similar to those presented by Tang (1994), who reported that *B. subtilis* B908 has ability to not only promote plant growth but also suppress disease caused by *Rhizoctonia solani*. He suggested that *B. subtilis* directly protected plants by suppressing pathogen with antibiotic production, or indirectly protected plants where bacterium caused plants to induce defense mechanisms.
against pathogen attack. However, Hwang et al. (2001) suggested that it would be difficult to determine whether antagonistic compound, phenylacetic acid produced by *Streptomyces humidis* can trigger induced systemic resistance in pepper plant, because application of phenylacetic acid resulted in the reduction of the primary inoculum density of *P. capsici* in soils of pepper growing fields.

In conclusion, *B. subtilis* HJ927 produced anti-fungal compounds and hydrolytic enzymes *in vitro* and reduced the severity of phytophthora blight disease of pepper plant *in vivo*. The plant protective effect is presumably mediated by the production of anti-fungal substances and hydrolytic enzymes by *B. subtilis* HJ927 in rhizosphere.

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**References**


Bacillus subtilis HJ927에 의해 생성된 화합물의 분리, 동정 및 고추(Capsicum annuum L) 역병방제 효과

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병원성 곰팡이에 대해 강한 잔여성을 보이는 토양미생물 Bacillus subtilis HJ927를 Phytophthora capsici에 감염된 고추에서부터 분리하였다. B. subtilis HJ927를 P. capsici와 함께 고추에 처리한 결과 P. capsici를 접종한 처리군에 비해 크게 식물을 보호하는 것을 root mortality 측정결과 확인하였다. B. subtilis HJ927는 항균팀성 물질로 3-methylbutyric acid, 2-methylbutyric acid, 그리고 methyl 2-hydroxy, 3-phenylpropanoate를 분비해 내는 것을 HPLC와 GC-MS를 통해 분리 동정하였다. 또한 B. subtilis HJ927는 가수분해효소인 β-1,3-glucanase를 분비해 냉으로서 위 화합물과 함께 식물을 보호하는 것으로 분석되었다.