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Biocontrol Ability of *Lysobacter antibioticus* HS124 Against *Phytophthora* Blight Is Mediated by the Production of 4-Hydroxyphenylacetic Acid and Several Lytic Enzymes

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Abstract Several rhizobacteria play a vital role in plant protection, plant growth promotion and the improvement of soil health. In this study, we have isolated a strain of *Lysobacter antibioticus* HS124 from rhizosphere and demonstrate its antifungal activity against various pathogens including *Phytophthora capsici*, a destructive pathogen of pepper plants. *L. antibioticus* HS124 produced lytic enzymes such as chitinase, β-1,3-glucanase, lipase, protease, and an antibiotic compound. This antibiotic compound was purified by diaion HP-20, silica gel, sephadex LH-20 column chromatography and high performance liquid chromatography. The purified compound was identified as 4-hydroxyphenylacetic acid by gas chromatography-electron ionization (GC-EI) and gas chromatography-chemical ionization (GC-CI) mass spectrometry. This antibiotic exhibited destructive activity toward *P. capsici* hyphae. In vivo experiments utilizing green house grown pepper plants demonstrated the protective effect of *L. antibioticus* HS124 against *P. capsici*. The growth of pepper plants treated with *L. antibioticus* culture was enhanced, resulting in greater protection from fungal disease. Optimum growth and protection was found when cultures were grown in presence of Fe(III). Additionally, the activities of pathogenesis-related proteins such as chitinase and β-1,3-glucanase decreased in roots, but increased in leaves with time after treatment compared to controls. Our results demonstrate *L. antibioticus* HS124 as a promising candidate for biocontrol of *P. capsici* in pepper plants.

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Introduction

Phytophthora blight is caused by a widespread soil-borne pathogen, *Phytophthora capsici*. It is responsible for serious damage to pepper plants, especially when soils contain excessive moisture after a heavy rainfall or irrigation. Many synthetic chemicals have been widely used for control of plant diseases such as *Phytophthora* blight, but their excessive use has caused several unintended problems. These problems include excessive environmental pollution and disease resistance against plant pathogens. Thus, researchers have focused on biological control of diseases and are considering antagonistic bacteria as ideal biocontrol agents. Antagonistic bacteria have been shown to inhibit the growth and proliferation of various phytopathogens with little or no side effects [1, 2].

Plant growth promoting rhizobacteria (PGPR) play a vital role in crop protection, plant growth promotion, and the improvement of soil health. The genus *Lysobacter*, one of the PGPR, has the ability to colonize roots after artificial inoculation and destroy other microorganisms, including pathogenic fungi and nematodes [3, 4]. A primary mechanism of pathogen inhibition used by PGPR includes the

production of antibiotics, lytic enzymes, volatile compounds, and siderophores [5, 6]. Antibiotics and lytic enzymes in particular are well known antimicrobial compounds that can directly or indirectly protect plants from pathogenic attack.

In general, plants are able to produce and accumulate enzymes, which aid defense against pathogen infection. Among these pathogenesis-related proteins (PRs), primarily chitinase and glucanase possess potential antipathogenic activities through degradation of fungal cell walls [7]. Glucanase from potato is reported to enhance resistance in flax against *Fusarium oxysporum* and chitinase from rice is effective against *Rhizoctonia solani* and *Magnaporthe grisea* [8, 9]. In addition, plant chitinase and glucanase could help the generation of signal molecules that may function as elicitors of further defensive mechanisms [10].

The aim of this work was to isolate antagonistic bacteria against various fungal pathogens especially against *P. capsici*, which causes serious damage in pepper, an important vegetable crop in Korea. Here, we report the isolation and characterization of *Lysobacter antibioticus* HS124, which strongly suppressed the growth of *P. capsici* as well as several other fungal pathogens. Our results demonstrate the biocontrol efficiency of *L. antibioticus* against *Phytophthora* blight and suggest its use in future field application.

Materials and Methods

Selection and Identification of Antagonistic Microorganism

Rhizosphere soils were collected from fields near Naju, Korea. Soils were serially diluted with sterilized water and inoculated on chitin medium containing 0.5% colloidal chitin, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.5% NaCl, 0.1% NH_4Cl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% $CaCl_2{\cdot}2H_2O$, 0.05% yeast extract, and 2% agar, pH 7.0. After 3 days incubation at 30°C, several colonies possessing strong clear zones were selected and then tested by dual culture assay against *P. capsici* on CP agar medium containing 0.25% colloidal chitin, 0.1% Na_2HPO_4, 0.05% KH_2PO_4, 0.25% NaCl, 0.05% NH_4Cl, 0.025% $MgSO_4{\cdot}7H_2O$, 0.025% $CaCl_2{\cdot}2H_2O$, 0.025% yeast extract, 0.5% potato dextrose broth, and 2% agar. One bacterial strain having the strongest antifungal activity was selected for further experiments and stored in 25% glycerol solution at −70°C. This isolate was identified by analysis of the 16S rRNA gene sequence and then by morphological and biochemical characteristics based on Christensen [11].

Antagonism Assay

The isolate was tested for antagonism toward several fungal pathogens: *P. capsici* KACC 40483, *F. oxysporum f.* sp *lycopesici* KACC 40032, *Diaporthe citri* ATCC 9054, *Pythium aphanidermatum* KACC 40156, *Pyricularia grisea* KACC 40414, *R. solani* AG-1 (IB) KACC 40111, and *R. solani* AG-2-2 (IV) KACC 40132. Antagonism was determined as % antifungal activity, equal to $[(A - B)/A \times 100]$; where A is the mycelia growth of control fungi, and B is the mycelia growth of the fungi with bacterial inoculation. Antifungal activity, reported as % inhibition, is as follows: (−), below 10%; (±), 10–20%; (+), 20–50%; (++), 50–80%; and (+++), 80–100%.

Purification and Identification of Antifungal Compound

The isolate was grown on Luria–Bertani broth at 30°C for 4 days and then centrifuged at 6,000 rpm for 15 min. The supernatant was acidified with concentrated HCl to pH 3 and extracted with ethyl acetate. The ethyl acetate soluble organic fraction was concentrated by a rotary evaporator and crude compound was obtained. It was dissolved in methanol and purified by diaion HP-20 column chromatography (Mitsubishi Chemical Co., Japan) with a stepwise gradient of water–methanol (100:0–0:100, v/v). Methanol fractions of 70 and 100% showing antifungal activity toward *P. capsici* by paper disc method were combined and purified further using a Kieselgel 60 silica gel column (Merk, Darmstadt, Germany) with stepwise elution on an increasing concentration of ethyl acetate–methanol (100:0, 50:50, 0:100, v/v). The 100% ethyl acetate fraction, having the highest antifungal activity, was purified further by silica gel column chromatography using hexane–ethyl acetate–methanol (20:40:40, v/v/v). Active fractions were further purified using a sephadex LH-20 gel filtration column (Sigma-Aldrich, St. Louis, MO, USA) with 100% methanol as a running phase.

One fraction showing antifungal activity was further purified using an HPLC system with C_{18} column (Waters, 10 μm, 7.8 × 300 mm). HPLC was performed with 80% acetonitrile in water at a flow rate of 1 ml min^{-1} and peaks were detected at 210 nm by a SPD-10 UV-VIS detector. The active compound separated by HPLC was analyzed by gas chromatography-mass spectrometry equipped with HP-5 MS column (30 m × 0.25 mm id. film thickness 0.25 μm).

Effect of the Purified Active Compound on *P. capsici* Hyphae

To examine the effect of isolated compound on hyphae of *P. capsici*, the active compound was first dissolved in

methanol to a final concentration of 500 ppm. One milliliter of *P. capsici* hyphae grown on PDB was placed into two separate test tubes and the active compound solution at final concentration of 100 and 200 ppm were added. Methanol was used as control treatment in the same volume of hyphal solution. The mixture of active compound solution and *P. capsici* was incubated at 30°C for 24 h and mycelia growth observed at various intervals using a BX41TF light microscope (Olympus Corp., Japan). All tests for observations of mycelial morphology were done in triplicate.

Lytic Enzyme Assay

To examine chitinase and β-1,3-glucanase activities, the bacterial supernatant was collected daily and assayed by method of Lingappa et al. [12] and Yedidia et al. [13], separately. Also, protease and lipase activities were determined using skim milk agar (MA) plate and LB plate supplemented with Tween 80, respectively [14].

Preparation of Bacterial and Pathogen Inocula

For pepper greenhouse trials, the bacterium was grown for 5 days at 30°C in culture media containing 1.5 g l^{-1} crab shell powder, 0.03 g l^{-1} yeast extract, 2 g l^{-1} glucose, 0.42 g l^{-1} N, 0.34 g l^{-1} P_2O_5, 0.34 g l^{-1} K_2O (pH 7.0) with (C1) or without (C2) the additional 27 mg l^{-1} $FeCl_3{\cdot}6H_2O$. Here, $FeCl_3{\cdot}6H_2O$ was used as a micronutrient for the bacterium. Media without the bacterium were used as controls, with (M1) or without (M2) the additional 27 mg l^{-1} $FeCl_3{\cdot}6H_2O$. For pathogen inoculum, zoospores were prepared by growing *P. capsici* (KACC 40483) on V8 juice agar medium by method of Kim et al. [15]. Zoospore suspension was filtrated by sterile cheesecloth and diluted with sterile water to a concentration of 1×10^6 zoospore ml^{-1}.

Plant Growth Condition and Preparation of Samples

Pepper seeds (*Capsicum annuum* L.) 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 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 planting, each pot was amended with 30 ml of a bacterial culture (C1 and C2) or media only (M1 and M2). At 6 weeks after planting, 30 ml of zoospore suspension of *P. capsici* (1×10^6 zoospore ml^{-1}) were poured into rhizosphere area. Plants were harvested at 0, 2, 4, 6 and 8 days after infection with *P. capsici* zoospores. The pepper plants were gently washed under running tap water and briefly dried. Plants were divided into vegetative portion and root portion, and then weighed. Each portion was ground to a powder using liquid nitrogen and proteins extracted with 3 ml of 10 mM potassium phosphate buffer (pH 7.0). Sample was then centrifuged at 12,000 rpm for 10 min at 4°C and supernatant retained and kept at −20°C until enzyme analysis performed.

Root Mortality Assay

Root mortality was measured by a modified method of Knievel [16]. A sample of fresh root (500 mg) was incubated with 10 ml of 0.6% 2,3,5-triphenyltetrazolium chloride in 0.05 M phosphate buffer (pH 7.4) at 30°C for 24 h. Roots were rinsed twice with distilled water, then extracted twice with 95% ethanol at 70°C for 4 h. Both ethanol extracts were combined and adjusted to a final volume of 20 ml with 95% ethanol. Absorbance of this solution 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 is expressed as percentage dead root of the total root assayed.

Enzyme Activity Assay in Soil and PRs Activity Assay in Plant

Chitinase and β-1,3-glucanase activity in soil and in plants were determined using the method of Tabatabai [17] and Yedidia et al. [13].

Results

Selection and Identification of Antagonistic Bacteria

A total of 60 bacterial strains showing a clear zone on chitin agar medium were isolated from the Naju area in Korea and sub-cultured on the same medium. Dual plate assay was performed for the selection of antagonistic bacteria against *P. capsici* on CP agar plate. One bacterium exhibited strong antifungal activity against *P. capsici* as well as various fungal pathogens as listed in Table 1. This bacterium was identified as *Lysobacter* strain by 16S rRNA gene analysis and furthermore morphological and biochemical characteristics of isolate were highly matched with *L. antibioticus* (KACC11383) (data not shown). Thus, this bacterium was designated as *L. antibioticus* HS124 (Accession No. FJ930928).

Table 1 Antifungal activity by *L. antibioticus* HS124 against various fungal pathogens

Fungi	Antifungal activity
Phytophthora capsici KACC 40483	+++
Fusarium oxysporum f. sp *lycopesici* KACC 40032	++
Diaporthe citri ATCC 9054	+++
Pythium aphanidermatum KACC 40156	–
Pyricularia grisea KACC 40414	++
Rhizoctonia solani AG-1 (1B) KACC 40111	+++
Rhizoctonia solani AG-2-2 (IV) KACC 40132	+++

Fungi were grown on CP medium at 30°C for 7 days. Antifungal activity is reported as (−) inhibition below 10%, (±) between 10 and 20%, (+) between 20 and 50%, (++) between 50 and 80%, (+++) between 80 and 100%

Identification of Antifungal Substance Produced by *Lysobacter antibioticus* HS124

The growth of *P. capsici* was strongly inhibited by the crude extract of *L. antibioticus* HS124. The extract (4.5 g) was purified by column chromatography using diaion HP-20, silica gel, sephadex LH-20, and HPLC. One compound demonstrating antifungal activity was obtained from one distinct fraction of Rt 13.41. This compound showed a strong antifungal activity toward *P. capsici* when only 2 mg of pure compound was applied by disc diffusion method (data not shown). This purified compound was identified as a 4-hydroxyphenylacetic acid by GC-EI and confirmed by GC-CI (Fig. 1).

Effect of Antifungal Substance on *P. capcisi* Hyphae Morphology

To determine the effect of antifungal substance on fungal pathogen, *P. capcisi* was grown on PDB with and without the presence of active compound. After 24 h incubation, *P. capcisi* incubated without the active compound showed normal morphology under the light microscope (Fig. 2a). However, *P. capcisi* incubated with either 100 or 200 ppm of 4-hydroxyphenylacetic acid revealed abnormal hyphae such as de-formation, lysis, and bending (Fig. 2b, c).

Production of Lytic Enzymes

Production of lytic enzymes, such as chitinase and β-1,3-glucanase activities, were examined from the culture supernatant of *L. antibioticus* HS124. Chitinase activity gradually increased over a time period of 4 days, eventually reaching a maximum value of 3.98 unit ml^{-1}. Thereafter, the chitinase activity rapidly decreased (data not shown). Conversely, β-1,3-glucanase activity slowly increased over roughly the same time period eventually reaching a maximum value of 1.79 unit ml^{-1} at 3 days. Thereafter, the decrease in activity was very gradual (data not shown). In addition, this strain showed lipase and protease activity as evidenced by the formation of precipitation zones on LB agar supplemented with 1% Tween and a clearing zone on MA medium, respectively (data not shown).

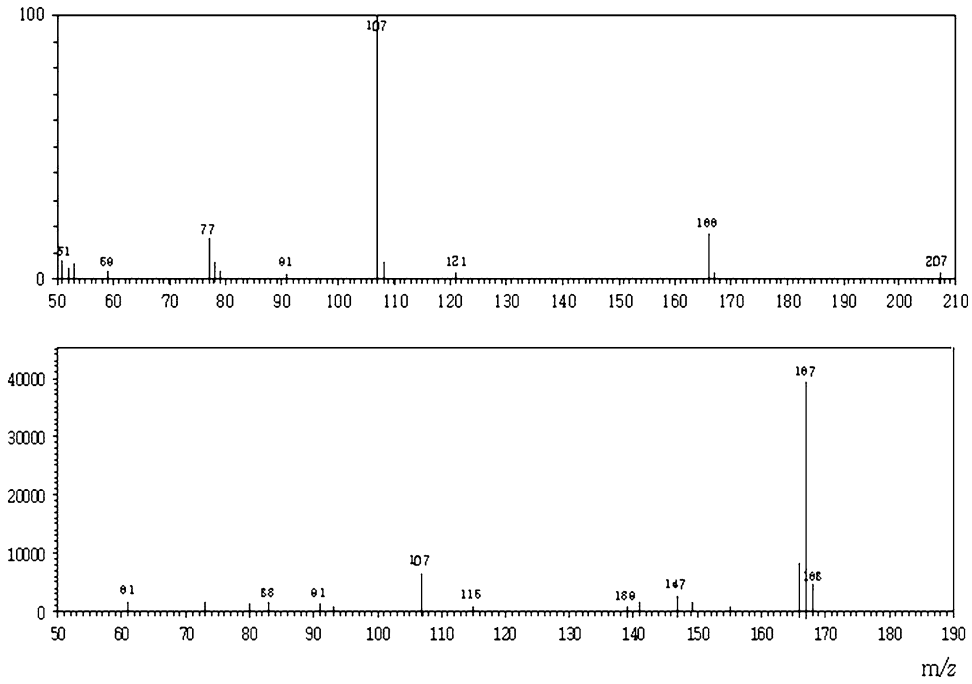

Fig. 1 GC-EI (*top*) and GC-CI (*bottom*) spectrums of 4-hydroxyphenylacetic acid from supernatant of *L. antibioticus* HS124 (Rt 13.3 min of GC)

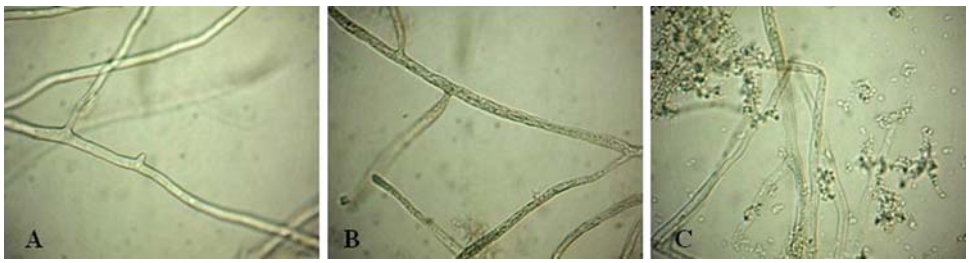

Fig. 2 Light microscopy examination of the effect of 4-hydroxyphenylacetic acid on hyphal morphology of *P. capsici* grown at 30°C for 24 h. Control (**a**); treated with 100 ppm of 4-hydroxyphenylacetic acid (**b**); treated with 200 ppm of 4-hydroxyphenylacetic acid (**c**)

Growth Promotion and Biocontrol Effect by *L. antibioticus* HS124

To examine the effect of *L. antibioticus* HS124 on pepper growth and disease control, pepper rhizosphere was applied with bacterial cultures that were grown either in presence (C1) or absence (C2) of Fe(III) or only medium with (M1) and without (M2) Fe(III) as controls and then infected with *P. capsici* zoospores. Pepper plants receiving only culture medium irrespective of the presence or absence of Fe(III) showed wilting and rotting of the stems within 5 days after infection. Progressive developments of disease led to a root mortality of roughly 79 and 90%, respectively, at 8 days after *P. capsici* infection. On the other hand, root mortality of pepper plants inoculated with *L. antibioticus* HS124 (C1 and C2 treatments) was markedly reduced compared to controls (M1 and M2 treatments) having lowest value of 30% in C1 treatment at 8 days after *P. capsici* infection (Fig. 3).

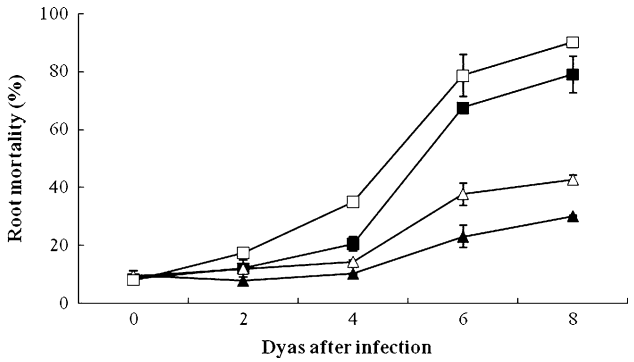

Fig. 3 Changes in root mortality of pepper plants infected with *P. capsici* after treatments of each bacterial culture with (C1, *filled triangle*) or without $FeCl_3 \cdot 6H_2O$ (**C2,** *open triangle*) and only medium with (M1, *filled square*) or without $FeCl_3 \cdot 6H_2O$ (M2, *open square*) as controls. Calculated mean values are from three replicates. *Error bars* represent standard error of the mean

Enzyme Activity in Soil and Pathogenesis-Related Proteins (PRs) in Plant

Microbial enzyme activities from rhizosphere soils of pepper are shown in Fig. 4. Chitinase activities of C1 and C2 treated soils were always higher than those of M1 and M2. β-1,3-glucanase activity demonstrated a similar pattern with chitinase activity. In pepper leaves, chitinase activities of M1 and M2 treatments gradually increased throughout the experimental period while C1 and C2 treated leaves showed an increase during the first 2 days and thereafter remained constant (Fig. 5). In contrast, the chitinase activity in roots decreased gradually in plants treated with M1 and M2 while plants treated with C1 and

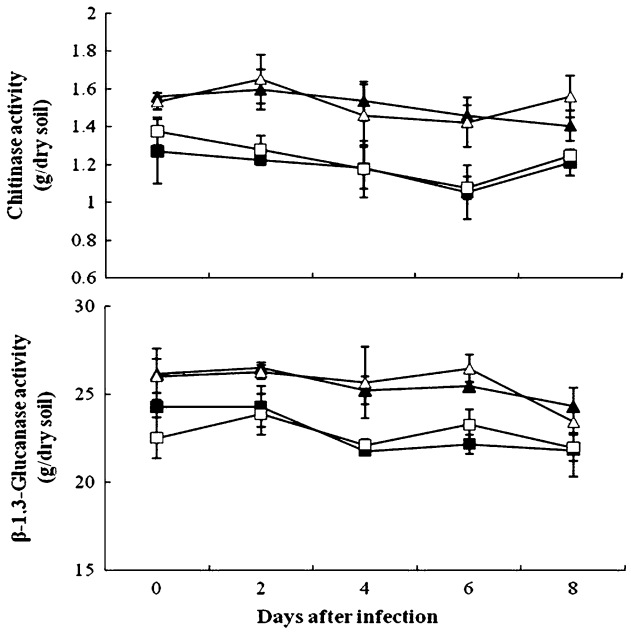

Fig. 4 Changes of chitinase activity (*top*) and β-1,3-glucanase activity (*bottom*) in soils infected with *P. capsici*. These changes were noted after treatments of each bacterial culture with (C1, *filled triangle*) or without $FeCl_3 \cdot 6H_2O$ (C2, *open triangle*) and only medium with (M1, *filled square*) or without $FeCl_3 \cdot 6H_2O$ (M2, *open square*) as controls. Calculated mean values are from three replicates. *Error bars* represent standard error of the mean

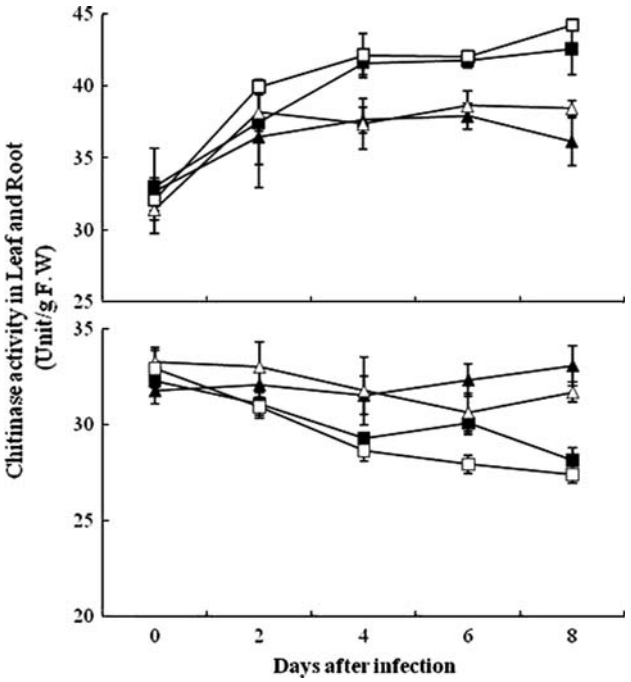

Fig. 5 Changes of chitinase activity in leaves (*top*) and roots (*bottom*) of pepper plants infected with *P. capsici*. These changes were noted after treatments of each bacterial culture with (C1, *filled triangle*) or without $FeCl_3 \cdot 6H_2O$ (C2, *open triangle*) and only medium with (M1, *filled square*) or without $FeCl_3 \cdot 6H_2O$ (M2, *open square*) as controls. Calculated mean values are from three replicates. *Error bars* represent standard error of the mean

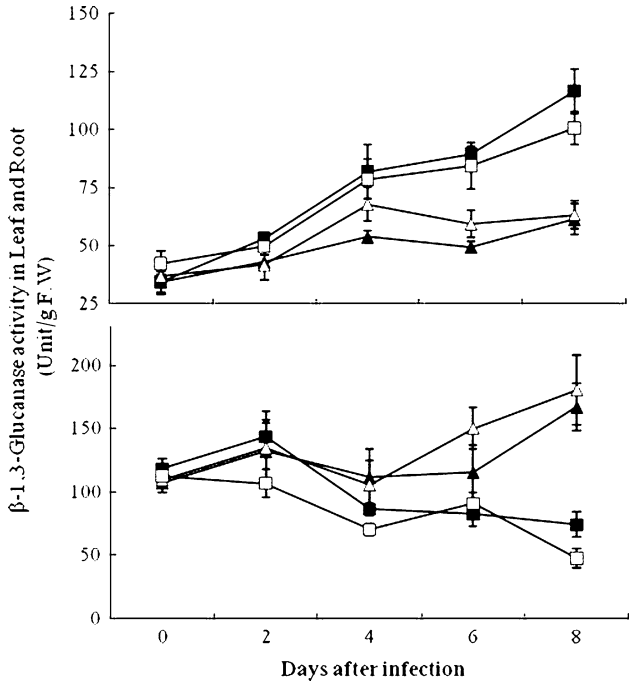

Fig. 6 Changes of β-1,3-glucanase activity in leaves (*top*) and roots (*bottom*) of infected pepper plants with *P. capsici*. These changes were noted after treatments of each bacterial culture with (C1, *filled triangle*) or without $FeCl_3 \cdot 6H_2O$ (C2, *open triangle*) and only medium with (M1, *filled square*) or without $FeCl_3 \cdot 6H_2O$ (M2, *open square*) as controls. Calculated mean values are from three replicates. *Error bars* represent standard error of the mean

C2 the chitinase activity showed little fluctuation (Fig. 5). β-1,3-Glucanase activity gradually increased in leaves of M1 and M2 treatments till 8 days after *P. capsici* infection. However, the activity in C1 and C2 slightly increased till 4 days and thereafter was almost constant. In roots, β-1,3-glucanase activity in M1 and M2 increased till 2 days and then decreased till the end of pot trials except at 6 days in M2. β-1,3-Glucanase activity in roots of C1 and C2 gradually increased till 8 days after *P. capsici* infection (Fig. 6).

Discussion

Many types of bacteria are associated with the rhizosphere of crop plants. Some of these bacteria have been shown to exert beneficial effects on the host plant. These bacteria typically colonize the root surface quite readily, stimulating overall plant growth by producing plant regulators and health by suppressing plant disease. In this experiment, an isolate of *Lysobacter antibioticus* HS124 demonstrated a broad spectrum of antifungal activity against various fungal pathogens including *P. capsici*.

Earlier reports have shown that *Lysobacter* sp. can produce lytic enzymes and antibiotics, which play a vital role in biological control of pathogens [18, 19]. In this study, we isolated 4-hydroxyphenylacetic acid from the culture supernatant of *L. antibioticus* HS124 and observed the abnormal hyphae of *P. capcisi*, which was deformed or swelled, and finally destroyed by the presence of 4-hydroxyphenylacetic acid. To our knowledge, this is the first report of 4-hydroxyphenylacetic acid secreted by a *Lysobacter* strain. Previously, our studies showed that the 4-hydroxyphenylacetic isolated from *Burkholderia* sp. strongly suppressed growth of several pathogenic fungal strains such as *Botrytis cinerea*, *Chaetomlum globosum*, *Didymella bryoniae*, *Pestalotiopsis* sp., *F. oxysporum f.* sp. *cucumerinum*, *P. capsici*, and *R. solani* [5]. Russell and Furr [20] reported that this compound contains phenol in its chemical structure, which damages cell membrane, resulting in release of intracellular constituents. Furthermore, one of the most effective mechanisms that *Lysobacter* sp. employs to prevent proliferation of phytopathogens is by the synthesis of lytic enzymes [21]. The strain HS124 produces chitinase, β-1,3-glucanase, lipase and protease (data not shown), which presumably associated with biochemical changes leading to structural modification of *P. capsici* cell walls. The fungal cell walls of oomycetes (especially *P. capsici*) are composed of glucans and other polymers with little chitin [22]. Therefore, our

results suggest that HS124 may act synergistically against *P. capsici* by the production of antifungal substance and lytic enzymes. This is consistent with previous findings by Fogliano et al. [23] who reported that inhibition of fungal pathogens was more synergistically effective by treatments of antibiotics (syringomycin E and syringopeptin 25A) and hydrolytic enzymes (chitinase and glucanase) from *Pseudomonas* strain.

Worldwide, the management of agricultural crops by inoculation with antagonistic bacteria has shown considerable promise for biocontrol of plant pathogens. When roots of peppers 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 [24]. Similar results have been reported in our previous findings with red pepper [25]. In our current study, growth of peppers treated with bacterial cultures (C1 and C2) was significantly enhanced, resulting in excellent protection from disease compared to controls (M1 and M2) where β-1,3-glucanase and chitinase in rhizosphere of bacterial cultures was always higher than those of controls during experiment periods. Most notably cultures that were grown in presence of Fe(III) provided the best control of *Phytophthora* blight. In previous our in vitro study, antifungal activity of HS124 against *P. capsici* gradually increased from 0 to 100 μM Fe(III), but above 150 μM Fe(III), slightly deceased. Especially, stain HS124 grown with 100 μM Fe(III) showed approximately 1.5 times higher antifungal activity against *P. capsici* compared to those grown in the absence of Fe(III) [26]. It seems that Fe(III) could promote the production of metabolites related to pathogen inhibition by the bacteria, resulting in protection of peppers from disease. This possibility is strengthened by an earlier report, which claimed that Fe(III) can influence the production of secondary metabolites by acting as a catalyst of many enzymes or exert its influence at the regulatory level by promoting the transcription of biosynthetic genes [27].

Pathogenesis-related proteins, commonly chitinase and β-1,3-glucanase, are generally known to aid the plant defense mechanism and inhibit pathogen infection. The constitutive expression of PRs in leaves and roots can lead to reduce pathogen growth and symptom expression [28]. In peppers treated with controls, the activities of PRs (chitinase and β-1,3-glucanase) increased in leaves, but decreased in roots with time while PRs of bacterial cultures treated peppers maintained rather constant. It seems that *P. capsici* infection may not activate PRs in root but activate in leave. Also, Jung et al. [29] reported that co-inoculation of antagonistic bacteria with *P. capsici* reduces PRs activity of pepper from biochemical aberration such as the drastic decrease and increase of PR proteins in roots and leaves, respectively. Thus, our result suggests that accumulation of PRs is not a prerequisite for plant protection. Similar conclusion was also made by Dassi et al. [30] who argued that accumulation of PRs may not always reflect an induction of resistance and only represent a response to pathogen attack.

In conclusion, the antagonistic bacterium, *L. antibioticus* HS124 produces 4-hydroxyphenylacetic acid, a secondary metabolite and several lytic enzymes including chitinase, β-1,3-glucanase, lipase and protease. In pot trials, treatment of pepper plants with HS124 greatly suppressed *Phytophthora* bight. Our results clearly demonstrate the potential of *L. antibioticus* HS124 for protection of plant disease and suggest this strain should be promoted as a biocontrol agent against *Phytophthora* blight.

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