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Antifungal activity of various chitinolytic bacteria against *Colletotrichum* in pepper

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Abstract: *Colletotrichum gleosporioides* causes the anthracnose disease in plants including vegetables and fruits. The pathogenicity of the strains was confirmed by using pepper fruit inoculation assays. The chitinolytic bacterial strains *Paenibacillus elgii* HOA73, *Lysobacter capsici* HS124, *Streptomyces griseus*, *Pseudomonas fluorescens,* and *Paenibacillus ehimensis* MA2012 were evaluated against the phytopathogenic fungal strains. The bacteria significantly inhibited *C. gleosporioides* strain 40003, the inhibition ranging from 17% to 37%. Similarly, 5–41% inhibition of *C. gleosporioides* 40896 was noticed. Moreover, *C. gleosporioides* 40965 and 42113 were also inhibited. The *n*-butanol extracted crude compound of *P. ehimensis* MA2012 completely inhibited the spore germination of the phytopathogen. Hence the chitinolysis may be considered as an important trait for screening the biocontrol bacteria against anthracnose.

Keywords: anthracnose; *Colletotrichum gleosporioides*; chitinolytic; biocontrol

Anthracnose is a fungal disease widely spread in all types of vegetation including fruits (cherries, apples, grapes, mangoes, strawberries) and vegetables (tomato, cucumber, spinach, pepper, beans). The symptoms begin as small pale yellow or water-soaked lesions on foliage and fruits. The lesions may turn tan, brown black in colour (Waller 1992). The pathogen is present in soil, weeds and diseased debris from previous crops and infected seeds. The disease is principally caused by a complex of *Colletotrichum* spp. (Teleomorph: *Glomerella* spp.). Significant losses were observed in pepper, strawberries, tomato and apple (Kim *et al.* 2007). *C. acutatum* was shown to produce sunken dark brown lesions on maturing fruits causing anthracnose in strawberries in Korea and Egypt (Nam *et al.* 2008; Embaby *et al.* 2009). Variable *Colletotrichum* species, i.e. *C. capsici, C. coccodes, C. gleosporioides,* and *C. acutatum*, were reported to cause the disease in pepper (Lee *et al.* 2007; Wei *et al.* 2008).

Chemicals are extensively used to control the fungal diseases. The excessive use of these chemical fungicides may, however, lead to environmental degradation and pollution (Leroux 2003). Moreover, they may enter the food chain (Bartlett *et al.* 2002). The chemical control efficiency may also decrease due to the development of resistance in the pathogens (Rosenberger 1981). Most of the fungal phytopathogens are soil-borne due to which

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the fungal diseases impart an intrinsic complexity in their chemical control and need systemic approaches. Therefore, the alternative control strategies against the fungal phytopathogens are always welcomed. The biocontrol microorganisms reportedly utilise a variety of mechanisms such as root colonisation, competition, enzymatic activity or degradative parasitism and antibiosis based antagonism (Harman 2006).

The cell wall-degrading enzymes (CWDEs) play an important role in the biocontrol activities of competent antagonists. Fungal cell walls are principally composed of chitin and glucan. The CWDEs including chitinases and glucanases have been reportedly involved in the biocontrol of fungal phytopathogens (Lim *et al.* 1991; Das *et al.* 2010). Chitin is a major structural polysaccharide and is abundant in the cell walls of the majority of fungi. The β-1-4 glycosidic bonds in chitin are responsible for the cell wall integrity and are targeted by chitinases, the chitin degrading enzymes. Chitin degradation is an important biological control mechanism for the fungal phytopathogens (Manjula & Podile 2005). *P. elgii* strain SMA-SDCH02 was shown to produce extensive chitinolytic enzymes as an active antifungal biocontrol agent (Das *et al.* 2010).

A number of chitinolytic bacteria have been cited in the literature as biocontrol agents. *Paenibacillus* spp. were reported to belong among the feasible biocontrol agents (BCA) owing to their abilities to produce endospores that are tolerant to thermal stresses, their rapid growth rates, high survival rates for longer periods of times during storage, and thence making them favourable for formulation as BCA (Kim *et al.* 2005). Many species of this genus produce antibacterial compounds including polymyxin, octopytin, and baciphelacin as well as antifungal compounds (Chung *et al.* 2000; Slepecky & Hemphill 2006). Furthermore, other species of this genus produced antimicrobial compounds including jolipeptin, gavaserin, saltavalin, fusaricidin A-D, and gatavalin (Li *et al.* 2007). Similarly, plant growth promoting abilities of *P. elgii* were frequently reported owing to its antagonistic behaviour against the fungal and bacterial plant diseases. Different strains of *P. elgii* were shown to control diverse fungal diseases caused by *Pythium aphanidermatum, Rhizoctonia solani, Botrytis cinerea, Chaetomium globosum, Cladosporidium resinae, C. gleosporioides* etc. (Kim 2004; Ding *et al.* 2011). Wu *et al.* (2010) showed that *P. elgii* B-69 produced an antibiotic characterised as Pelgipeptin A (polypeptin family) with strong antifungal activity against both the bacteria and the fungi even at minor doses. Moreover, *P. elgii* SD 17 produced antimicrobial compounds of the polypeptin family (Kim *et al.* 2005). *Pseudomonas fluorescens* is a well reported Gram negative biocontrol agent capable of producing various mechanisms against different phytopathogens (Cole *et al.* 2015). Various strains of *Streptomyces griseus* have also been reported possessing the biocontrol abilities (Zacky & Ting 2015).

In the present study, we tested the antagonistic activity of various strains of bacteria isolated from the seashore soil belonging to various taxonomic groups and possessing the chitinolytic abilities. The crab shell powder had routinely been used to fertilise these areas for many years. These chitinolytic bacterial strains were assayed here against various strains of the economically important anthracnose disease pathogen, *C. gleosporioides*. The fungal strains were collected from the culture bank and their pathogenicity was evaluated through fruit inoculation assays. Moreover, the spore inhibition assays were also performed.

MATERIAL AND METHODS

Bacterial strains. Five chitinolytic strains were used in the study: *Paenibacillus elgii* HOA73 effective against nematodes, fungi (Nguyen *et al.* 2015); *Lysobacter capsici* HS124 effective against nematodes (Lee *et al.* 2013); *Streptomyces griceus* H7602 effective against fungi (Nguyen *et al.* 2012); *Pseudomonas fluorescens* HN1205; *Paenibacillus ehimensis* MA2012 (Naing *et al.* 2015). All these strains were isolated from the soils that were collected from vegetable gardens located near to the seashores of Bo Seong and Sun cheon in Jeollanam Province, Korea, where the farmers used to apply crab shells as fertiliser (Hong *et al.* 2012).

The different strains were maintained in LB broth and stored in a laboratory of the Institute of Environmentally Friendly Agriculture, College of Agriculture and Life Sciences, Chonnam National University, Gwangju, South Korea. For long-term storage at –80°C, the bacteria were cultured in LB broth at 30°C for 3 days with shaking at 140 rpm. The LB broth culture was then mixed with an equal volume of 30% glycerol in Eppendorf tubes and stored until used.

Fungal strains. The fungal strains were obtained from Korean Agricultural Culture Collection (KACC).

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The different phytopathogenic strains used in the study included: *Colletotrichum gleosporioides* KACC40003; *C. gleosporioides* KACC40896; *C. gleosporioides* KACC42113, *C. gleosporioides* KACC40695. The strains were maintained on a potato dextrose agar (PDA) medium.

Pathogenicity of Colletotrichum strains against pepper using fruit inoculation assays. Detached pepper fruit inoculation assays were conducted to assess the phytopathogenicity of the different *C. gleosporioides* strains. For this purpose, commercial healthy fruits were surface sterilised with 1% NaOCl for 5 minutes. The fruits were wounded by pin-pricking at two places and placed in 12 cm square Petri boxes on moistened tissue papers. The conidial suspension was sprayed over the surface until run-off and boxes were taped with parafilm, and incubated at 30°C for 9 days. Only sterile water was applied in the control plates. The disease occurrence was assessed by appearance of blackish brown rotting fruit parts after 9 days.

Antagonistic activity of bacterial strains against Colletotrichum strains using dual culture assays. Chitin PDA medium (CP Agar medium; 0.1% Na_2HPO_4, 0.05% KH_2PO_4, 0.025% NaCl, 0.05% NH_4Cl, 0.025% $MgSO_4 \cdot 7H_2O$, 0.025% $CaCl_2 \cdot 2H_2O$, 0.005% yeast extract, 1.2% potato dextrose broth and 2% agar) was used to perform the confrontation assays between bacterial and fungal strains.

The fresh bacterial culture was prepared by streaking the bacterial strain on the nutrient agar plates followed by incubation at 28°C for 3 to 7 days. The fresh culture of the fungal strains was prepared on PDA plates and incubated at 25°C for 7 days.

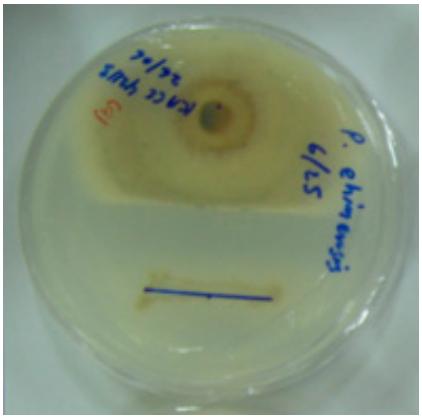

Figure 1. A dual culture assay plate: Inhibition of fungus *Colletotrichum gleosporoiodes* 42113 by *Paenibacillus ehimensis* MA2012 cultured on chitin-potato dextrose agar medium

The actively growing culture plugs (5 mm) were grown along with the bacterial strain on the same CP agar plate so that the distance between the two was 4.5 cm while the bacterial streak consisted of a 3-cm long line (Figure 1). The plates were then incubated at 25°C for 9 days. The control plates contained only the fungi. The experiments were performed in triplicate. The percentage inhibition was calculated by dividing the inhibition (the difference between the colony growth radii of the fungi growing towards the bacteria and the radii of the fungi growing in control plates) by the radial colony growth of the fungi in control and then multiplied by 100. The data were analysed by analysis of variance (ANOVA) and the means were compared by Fischer's least significant test (*LSD*; $P = 5\%$) using XLSTAT 2012.1.

Antifungal activity against C. gleosporioides of crude compound using spore inhibition assays. *P. ehimensis* MA2012 was grown in a chitin-gelatin medium [g/l; crab shell powder 1.0, gelatin powder 1.0, complete fertilizer (% $N/P_2O_5/K_2O$; 21 : 17 : 17) 3.0, sucrose 3.0, yeast extract 0.03, $FeCl_3$ 0.03] at 30°C for 5 days with shaking at 140 rpm. The culture broth was centrifuged at 7 000 *g* for 20 min and the supernatant was collected and filtered through Whatman filter paper No. 2. The crude compound was extracted using equal volumes of the culture filtrate and *n*-butanol. The organic solvent was mixed together and shaken well. The organic fraction was collected and evaporated using a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland). The crude organic compound thus obtained was weighed and dissolved in methanol and stored at 4°C until used.

C. gleosporioides was cultured in PDA plates for 2–3 weeks at 25°C. The conidial spores were collected from the PDA culture plates by adding sterile water onto the plate surface and then wiping off into a 100-ml flask containing 50 ml sterile water through a cotton cheese cloth to remove the mycelial fragments. The spore suspension thus obtained was standardised using a haemocytometer. The number of spores was adjusted to 10^6 per ml of the spore suspension by adding sterile water and used for the spore inhibition assays.

To assess the spore inhibitory activity of the extracted compound, 5 µl of the compound or methanol was added to the 1.5-µl sterilised Eppendorf microtubes. The tubes were kept open till the solvent evaporated inside the sterile chamber (laminar flow). Then the spore suspension (10 µl) was put into the tubes, mixed well and put onto glass slides which

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were placed in Petri plates containing damp tissues. The plates containing the slides were incubated at 25°C for 8 days. The germination of the spores was observed under the microscope.

RESULTS

Pathogenicity assays of Colletotrichum strains. The different strains of *C. gleosporioides* were pathogenic causing the disease symptoms in pepper fruits. The control plates showed no disease symptoms (Figure 2). The strains 40003 and 40896 showed the most aggressive disease symptoms.

Dual culture assays for screening of bacterial strains against Colletotrichum strains. The pathogen *C. gleosporioides* 40003 was inhibited by different strains of the antagonistic bacteria (Figure 3A). Fungal inhibition (17–37%) by different bacterial strains was observed. *L. capsici* inhibited the fungal strain by 27%, *P. ehimensis* inhibited by 23%, *P. elgii* inhibited by 17%, *P. fluorescens* inhibited by 37% and *S. griseus* inhibited by 33%.

Figure 2. Pathogenicity assays against pepper fruit of *Colletotrichum gleosporoiodes* strains obtained from Korean Agriculture Culture Collection

The pathogenic fungus *C. gleosporioides* 40896 was inhibited by different strains of the antagonistic bacteria (Figure 3B). Significant inhibition (5–41%) by different bacterial strains was observed. *L. capsici* inhibited the fungal strain by 5%, *P. ehimensis* inhibited by 14%, *P. elgii* inhibited by 14%, *P. fluorescens* inhibited by 41%, and *S. griseus* inhibited by 37%.

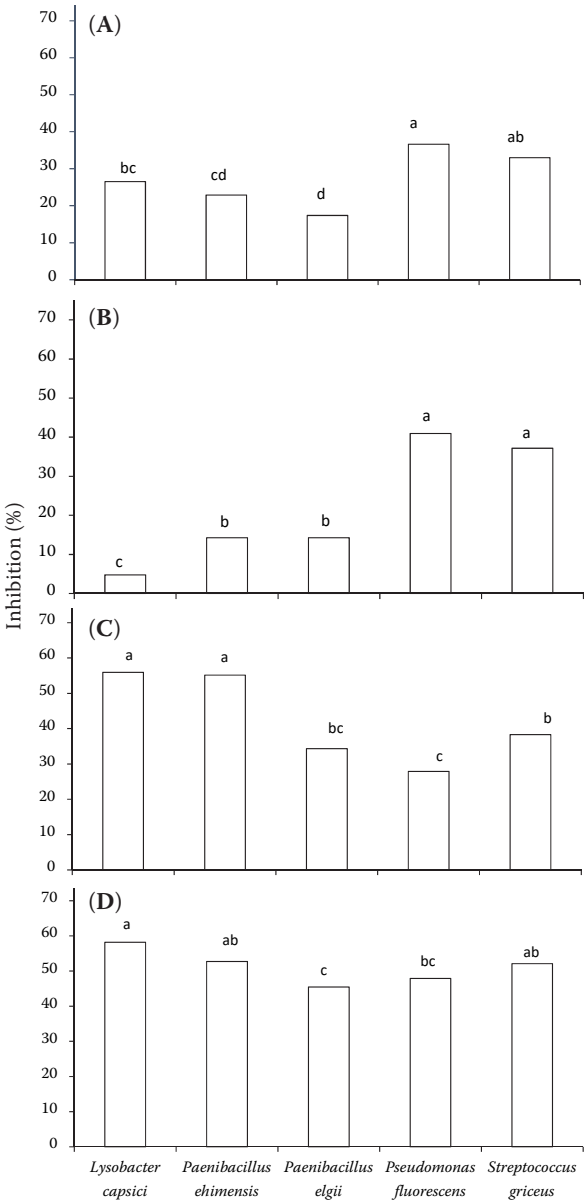

Figure 3. Inhibition of (**A**) *Colletotrichum gleosporioides* 40003, (**B**) *C. gleosporioides* 40896, (**C**) *C. gleosporioides* 40965, and (**D**) *C. gleosporioides* 42113 by different bacterial strains using dual culture assays

The data were analysed using analyses of variance (ANOVA) followed by Fisher's least significant difference (*LSD*) tests. The different small letters on the bars represent the significant differences ($P \leq 0.05$)

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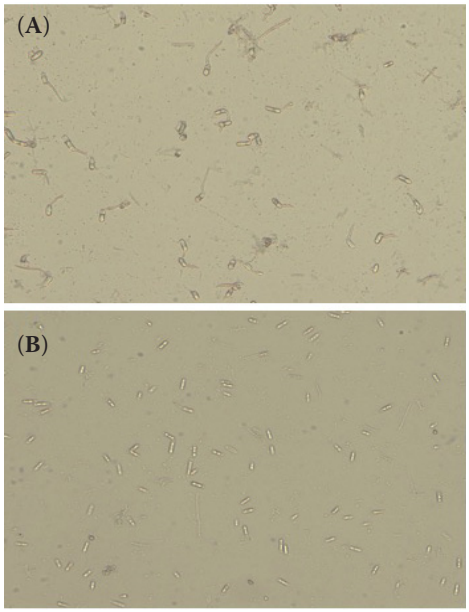

Figure 4. The *Colletotrichum gleospoioides* conidiospore inhibition by (**A**) methanol and (**B**) organic compound extracted with butanol from the cultural filtrate of *Paenibacillus ehimensis* MA2012 after 8 h of incubation at 40×

The strain *C. gleosporioides* 40965 was inhibited (28–56%) by different strains of the antagonistic bacteria (Figure 3C). *L. capsici* inhibited the fungal strain by 56%, *P. ehimensis* inhibited by 55%, *P. elgii* inhibited by 34%, *P. fluorescens* inhibited by 28%, and *S. griseus* inhibited by 38%. *C. gleosporioides* 42113 was inhibited (45–58%) by different strains of the antagonistic bacteria (Figure 3D). *L. capsici* inhibited the fungal strain by 58%, *P. ehimensis* inhibited by 53%, *P. elgii* inhibited by 45%, *P. fluorescens* inhibited by 48%, and *S. griseus* inhibited by 52%.

Spore inhibition assays of crude compound extracted with different organic solvents against C. gleosporioides. The germination of the spores was completely inhibited by the crude compound extracted with butanol. The inhibitory effect was clearly seen after 8 h of incubation (Figure 4). Most of the spores clearly germinated after 8 h of incubation at 25°C in the control (methanol) but the spores in compound extracted with butanol did not germinate.

DISCUSSION

Anthracnose is a fungal disease that occurs in a wide range of vegetation. Considerable losses are reported especially in fruits and vegetables because of this disease caused by different *Colletotrichum* species. It is a serious problem in red pepper, strawberry, wine and apple (Kim *et al.* 2007). Worldwide reports of *C. gleosporioides* pathogenicity can be found in the literature (Latinovic & Vucinic 2002). *C. gleosporioides* is a common anthracnose pathogen of fruit trees and chilli pepper in Korea (Hong *et al.* 2008). In the present study, different strains of *C. gleosporioides* were obtained from KACC and their pathogenicity to pepper was demonstrated using the fruit inoculation assays. The strains were found highly pathogenic to pepper. Fruit inoculation assays were previously used to determine the pathogenicity of *Colletotrichum* strains elsewhere (Janisiewicz & Korsten 2002).

Different chitinolytic bacterial strains in the present study showed antifungal activity against different strains of *C. gleosporioides*. *P. fluorescens* inhibited the colony growth of *C. gleosporioides* strains ranging from 28% to 41%. The ability of *P. fluorescens* to inhibit the plant pathogens is principally dependent on the production of antibiotics (Koche *et al.* 2013). Different antifungal compounds produced by *P. fluorescens* have been reported so far such as pyoluteorin, pyrrolnitrin, phenazine 1-carboxylic acid, and 2,4-diacetyl phloroglucinol (O'Sullivan & O'Gara 1992; Georgakopoulos *et al.* 1994; Maurhofer *et al.* 1995).

S. griseus also showed a significant inhibition in the present study. The same strain had shown an antagonistic potential against *Phytophthora capsici* elsewhere and the antifungal compound was also isolated (Nguyen *et al.* 2015). However, it was difficult to produce its inoculum in higher densities in field conditions, which is a prerequisite to develop a biocontrol (Kim K.Y., personal communication). *L. capsici* and *P. ehimensis* MA2012 showed similar inhibition profiles in the present study except the percentage inhibition of *C. gleosporioides* 40896 by *P. ehimensis* MA2012 was significantly higher than that by *L. capsici*. We have previously isolated an antifungal compound, polypeptin C, produced by *P. ehimensis* MA2012 (Naing *et al.* 2015). The chitinolytic enzymes produced by this strain were also found to be antifungal against *C. gleosporioides* (Seo *et al.* 2016). Here, we reported the 16S rRNA gene sequence used for the strain identification; the sequence was submitted to the Genebank database (accession No. JX997950). This strain bears a high potential of antifungal activity against the anthracnose pathogen and is a putative biocontrol agent. There are a few other reports about the biocontrol potential of some other strains of *P. ehimensis* (Hong *et al.* 2013; Huang *et al.* 2013; Naing *et al.* 2014). There-

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fore, the inhibitory assays against the fungal spore germination by its extract were performed. The *n*-butanol extracts of the culture broth of strain MA2012 inhibited the spore germination of *C. gleosporioides* by *P. ehimensis* MA2012 in the present study. Based on these results, we concluded that the compounds produced by this strain inhibited the conidial germination of the pathogen. *Paenibacillus* spp. including *P. ehimensis, P. elgii, P. alvei, P. tianmuensis,* are well known for producing lipopeptide antibiotics such as polymyxins, fusaricidins, battactin, permeatin, and analogues of polypeptin (Li & Jensen 2008; Shaheen *et al.* 2011; Qian *et al.* 2012; Huang *et al.* 2013).

It is concluded that all the chitinolytic strains of bacteria assayed in the present study inhibited *C. gleosporioides* strains that were pathogenic to pepper. The chitinolysis may be considered as a putative criterion for screening the biocontrol strains against the anthracnose disease. Such strains may have the ability to produce antibiotics that can inhibit the fungal spore germination.

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