Isolation and Biocontrol Potential of *Bacillus amyloliquefaciens* Y1 against Fungal Plant Pathogens

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This study was performed to investigate thermophilic bacteria from soil having broad antifungal spectrum against *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Phytophthora capsici*, *Fusarium oxysporum* f.sp. lycopersici, and *Botrytis cinerea*. One isolate selected could resist heat shock of 60°C for one hour, and had broad antifungal activity in dual culture assay against all tested fungal pathogens and was identified as *Bacillus amyloliquefaciens* Y1 using 16S rRNA gene sequence. Further investigation for antifungal activity of bacterial culture filtrate (BCF) and butanol crude extract (BCE) of various concentrations showed broad spectrum antifungal activity and fungal growth inhibition significantly increased with increasing concentration with highest growth inhibition of 100% against *R. solani* with 50% BCF and 11 mm of zone of inhibition against *R. solani* with 4 mg BCE concentration. Treatment of butanol crude extract resulted in deformation, lysis or degradation of *C. gloeosporioides* and *P. capsici* hyphae. Furthermore, *B. amyloliquefaciens* Y1 produced volatile compounds inhibiting growth of *R. solani* (70%), *C. gloeosporioides* (65%) and *P. capsici* (65-70%) when tested in volatile assay. The results from the study suggest that *B. amyloliquefaciens* Y1 could be a biocontrol candidate to control fungal diseases in crops.

**Key words:** Thermophilic bacteria, Dual culture assay, Bacterial crude extract, Crude extract, Volatile assay

### Antifungal potential (dual culture assay) of *Bacillus amyloliquefaciens* Y1.

| Pathogens                        | Inhibition zone (mm) | Antifungal activity |
|----------------------------------|----------------------|---------------------|
| *Rhizoctonia solani* KACC 40111  | 15                   | ++++                |
| *Colletotrichum gloeosporioides* KACC 4003 | 11              | +++                |
| *Phytophthora capsici* KACC 40483 | 9                    | ++                  |
| *Fusarium oxysporum* KACC 40032  | 6                    | ++                  |
| *Botrytis cinerea* KACC 40854    | 18                   | ++++                |

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Introduction

The desire to reduce the use of potential harmful chemical fungicides to control plant diseases has grown substantially over the past few decades. This desire, in turn, prompted the growth of research on the use of microbial agents to control plant diseases. To control plant disease using biocontrol has been considered a realizable alternative to manage plant diseases (Cook, 1993). To inhibit or stop growth, infection or reproduction of one microorganism using another microorganism is known to be biocontrol or biological control (Baker, 1987; Cook, 1993). Fungal plant pathogens are known to be the most important microbial agents causing serious diseases resulting economic losses in agriculture annually (Agrios, 1988). Botrytis is a major cause of bunch rot in wine and table grapes, reduction in profit due to its expenses for annual control in South Africa (SA R25 million/year), Australia (AUS $52 million/year) and Chile (US $22.4 million/year) (Scholefield and Morison, 2010; Esterio et al., 2009).

The Fusarium species collectively can infect more than 100 different hosts, provoking severe losses in crops such as tomato, cotton, banana, melon and others (Michielse and Rep, 2009). Rhizoctonia solani causing fruit decay, stem cankers, seed decay, foliage diseases and damping-off affecting different host plants including potato, grass, sugar beets and others. Important food crops including bananas, cassava and sorghum, grown by subsistence farmers in developing countries throughout the tropics and subtropics are at risk due to damaged caused by Colletotrichum spp. It is also known for successful as a post-harvest pathogen because latent infections, which are initiated before harvest, which become active until after the fruit has been harvested or appears on the market shelf. Up to 100% of the stored fruit can be at risk for damage as a result of Colletotrichum disease (Prusky, 1996). The term late blight is well known with Phytophthora, causing late blight in pepper causes severe yield loss worldwide. In order to reduce fungal contamination in agriculture, fungicides are used widely, which may cause environmental pollution. So, biological control is regarded as a most desirable practice, in view of the need for sustainable agriculture.

There have been considerable efforts to find biological control agents against fungal plant pathogens and several candidates have been reported including Pseudomonas spp., Streptomyces spp., and Bacillus spp. to use effectively to control plant diseases. Antagonistic bacteria can synthesize different types of antimicrobial compounds against fungi, and favor the growth and defense responses of host plants. In additions one like Bacillus spp. permits an easy formulation and storage of the commercial products due to their ability to survive adverse environmental conditions.

Antagonistic bacteria have been studied exclusively for their mechanisms of disease suppression and they involved producing antibiotic, extracellular enzymes, biosurfactant or cyanide production and or the preoccupation of infection sites (Mao et al., 2008; Sang, 2008).

Bacillus species have high phenotypically ecological diversity to interact with plant resulting both in epiphytic and endophytic colonization (McSpadden Gardener, 2004). Many strains of Bacillus subtilis, Bacillus cereus and Bacillus amyloliquefaciens have been found to interact with plants and produce beneficial effects including disease suppression (Choudhary and Johri, 2009). Plant-associated B. amyloliquefaciens has been shown to produce a variety of secondary metabolites involved in microbial antagonism (Chen et al., 2009) and enzymes like chitinase (Niazi et al., 2014), thus supporting disease suppression in plants.

In the present study we have tested the effect of the bacterial biocontrol agent (BCA) B. amyloliquefaciens Y1 on the growth inhibition of five agriculture important fungal pathogens, Rhizoctonia solani, Colletotrichum gloeosporioides, Phytophthora capsici, Fusarium oxysporum Lsp. lycopersici and Botrytis cinerea. Although the B. amyloliquefaciens, bacteria are not yet available as a commercial BCA, substantial research has been done on its plant growth effect as well as the underlying mechanisms of action (Danielsson et al., 2007; Sarosh et al., 2009). The objective of this study is to isolate thermophilic biocontrol agent from soil to use in field condition and to analyze its broad antifungal spectrum against important fungal plant pathogens.

Material and Methods

Isolation and identification of antagonistic microorganism

Bacillus amyloliquefaciens Y1 (Gene bank accession number KJ166752) was isolated from field soil of Chonnam National university (35.176° N, 126.9081° E), South Korea. Initially ten grams of soil was put it into the flask containing 100 mL of distill autoclaved water and incubate at 60°C for one hour (to isolate biocontrol agent which can be used in field conditions without autoclave) and then serially diluted and poured on LB (Laurie Britannia) agar plate. All isolates were tested for dual culture assay and with highest antifungal activity was selected for further study. All isolates were tested for antagonism against several fungal pathogens namely: Rhizoctonia solani AG-2-2 (IV) KACC 40132, Colletotrichum gloeosporioides KACC 4003, Phytophthora capsici KACC 40483, Fusarium oxysporum f.sp. lycopersici KACC 40032 and Botrytis cinerea KACC 40854 purchased from KACC (Korea Agriculture Culture Collection, 225 Seodun-dong, Suwon, and Gyeonggi Province, Korea). Bacterial isolate was inoculated on LP agar medium (Laurie Britannia mixed with Potato dextrose agar
50:50) one day before inoculation of the fungal pathogens. The antagonist and the test pathogen were placed 4 cm apart on the same LP plate, and all cultures were incubated at 26 °C. All tests were carried out in three replications. Growth of fungal pathogens was evaluated at 7 days after inoculation exceptionally for *Rhizoctonia solani* after 3 days by measuring colony radius from the original point of inoculation in the direction of the antagonist. Antifungal activity was defined in the terms of size of the inhibition zone and results were recorded. For identification of isolate 16S rRNA sequence was used. The strain was stored at -70 °C in 25% glycerol for future use.

**Antifungal assay of bacterial culture filtrate (BCF) of *B. amyloliquefaciens Y1***  
*B. amyloliquefaciens Y1* was grown in LB broth at 40 °C for 7 days with shaking at 170 rpm. The culture broth was centrifuged at 7,000 g for 20 min and the supernatant collected was then filtered serially through Whatman filter paper No. 2, filter paper 0.45 µm and finally 0.20 µm. Filterate was mixed with autoclaved PDA with final concentration of 0%, 10%, 30%, and 50% concentration. A mycelia plug of 5-mm mycelial plug from a 7-day-old plate culture of *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Phytophthora capsici*, *Fusarium oxysporum* f.sp. lycopersici and *Botrytis cinerea* was placed in the center of the PDA plate and incubated at 28 °C in the dark. Each assay was carried out in triplicate. Mycelial growth was measured after 3 days of inoculation for *R. solani*, and 7 days for *C. gloeosporioides*, *P. capsici*, *F. oxysporum* f.sp. lycopersici, and *B. cinerea* at 26°C using the following formula. Percentage of growth inhibition = (R − r)/R *100; where ‘R’ is the radius of the fungal colony on the control plates and ‘r’ is the radius of the fungal colony on the treatment plate.

**Antagonistic activity of bacterial crude extract (BCE) against plant pathogens** To prepare BCE, the BCF obtained from culturing isolate *B. amyloliquefaciens Y1*, as mentioned above, was acidified with concentrated 0.1 N HCl to pH 3.0 and then extracted with an equal volume of n-butanol. The soluble organic fraction was concentrated using a rotary evaporator (Buchi, Switzerland) to obtain BCE. To test antagonistic activity, the BCE was dissolved in methanol to make stock solution of 20% concentration. A mycelia plug from a 7-day-old plate culture of *B. amyloliquefaciens Y1* was prepared as above was checked against *Colletotrichum gloeosporioides* and *Phytophthora capsici* hyphae using light microscope. To examine the effect of the BCE on hyphae, *P. capsici* and *C. gloeosporioides* culture were grown on potato dextrose broth (PDB) medium at 30°C for 5 days. The BCE 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 tube containing mixtures of BCE and one fungus (*C. gloeosporioides* or *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.

**Antifungal volatile assay** The antifungal volatile compounds assays were performed following the method used by Raza et al. (2009) with some modifications. Bacterial strain was cultured 10⁶ by spreading 50 µL of bacterial culture on LB plate and incubated at 28°C for 24 h. Mycelial plug (5 mm) taken from the actively growing culture of *Rhizoctonia solani*, *Colletotrichum gloeosporioides* and *Phytophthora capsici* was placed in the center of another petri dish containing PDA. The dishes containing the mycelial plugs were inverted over the bacterial plates by replacing their covers and sealed together face to face with parafilm followed by incubation at 26°C. The diameter of fungal mycelium was measured every 6 h for *R. solani* while 24 h interval for *C. gloeosporioides* and *P. capsici*. The fungal plates with covers replaced by LB plates containing no bacterial cultures were used as controls. The data were analyzed by the student’s t-test using Microsoft excel 2007 program (p ≤ 0.05).

**Results and Discussion**

**Isolation and Identification** Five bacteria were isolated after heat of 60°C for 1 hour but among these only isolate Y1 exhibited antifungal activity on LP medium against all five tested fungal pathogens including *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Phytophthora capsici*, *Fusarium oxysporum* f.sp. lycopersici, and *Botrytis cinerea*, while others four did not show antifungal activity. When this isolate Y1 and fungal pathogens were incubated for 7 days, *B. cinerea* was found to be more affected as inhibition zone of 1.8 cm as followed by *R. solani* with inhibition zone 1.5 cm, *C. gloeosporioides* 1.1 cm, *P. capsici* 1.2 cm, *C. gloeosporioides* f.sp. lycopersici 1.6 cm and *Fusarium oxysporum* f.sp. lycopersici 1.7 cm as above was checked against *Colletotrichum gloeosporioides* and *Phytophthora capsici* hyphae using light microscope. To examine the effect of the BCE on hyphae, *P. capsici* and *C. gloeosporioides* culture were grown on potato dextrose broth (PDB) medium at 30°C for 5 days. The BCE 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 tube containing mixtures of BCE and one fungus (*C. gloeosporioides* or *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.
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Table 1. Antifungal activity of *B. amyloliquefaciens* Y1 against fungal pathogens.

| Pathogen                  | Inhibition zone (mm) | Antifungal activity |
|---------------------------|----------------------|---------------------|
| *Rhizoctonia solani* KACC 40111 | 15                   | ++++                |
| *Colletotrichum gloeosporioides* KACC 4003 | 11                   | +++                |
| *Phytophthora capsici* KACC 40483 | 9                    | ++                  |
| *Fusarium oxysporum* KACC 40032 | 6                    | ++                  |
| *Botrytis cinerea* KACC 40854 | 18                   | ++++                |

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

Fig. 1. Neighbor-Joining tree showing the position of *Bacillus amyloliquefaciens* Y1 (bold, accession No. KP967704) compared to related organisms in a 16S rRNA gene tree. The scale bar value (0.0002) at the bottom indicates genetic distance units based on Nei’s genetic distance.

capsici 0.9 cm and *F. oxysporum* 0.6 cm (Table 1). Isolate Y1 was identified as *Bacillus amyloliquefaciens* using 16S rRNA sequence analysis with accession no KP967704. Rini and Sulochana (2007) assessed antifungal potential of *Trichoderma* and *Pseudomonas* against *R. solani* and *F. oxysporum* using dual culture technique. The phylogenetic tree was constructed by using the CLUSTAL-W program comparing its 16S rRNA sequences with the published ones at Gene Bank Data base of National Center for Biotechnology Information (NCBI; Bethesda, MD) (Fig. 1).

Antifungal assay of bacterial culture filtrate (BCF) of *B. amyloliquefaciens* Y1. Tested fungal pathogens growth was inhibited at various concentrations of *B. amyloliquefaciens* Y1 cell free culture filtrate. Increasing concentration has invers effect on fungal growth. A mechanism of pathogen inhibition used by antagonistic microorganism is through production of antibiotics, lytic enzymes, volatile compounds and siderophores (Mao et al., 2006). Bacterial cultures filtrate of 10% concentration showing significant antifungal activity as shown in figure 2. Bacterial culture filtrate of *B. amyloliquefaciens* Y1 was found to be highly active against all tested fungi especially *B. cinerea* and *R. solani* were found very sensitive. *R. solani* cannot grow at 50% concentration with 100% of inhibition while *B. cinerea*, *C. gloeosporioides*, *P. capsici* and *F. oxysporum* showing inhibition of 66.2, 69.79, 54 and 65 % respectively (Fig. 2). On the basis of these results, a mode of action of antifungal activity in culture filtrate is through production of lytic enzymes and antifungal metabolites by *B. amyloliquefaciens* Y1 in media. Ehtesmoul-Haque and Ghaffar (1993) reported that antifungal activity by many antagonistic bacteria is possible through by production of secondary metabolites.

Antagonistic activity of bacterial crude extract (BCE) against plant pathogens. Growth of fungal pathogens was significantly affected by various concentrations of *B. amyloliquefaciens* Y1 loaded into paper discs. *R. solani* growth was affected by crude extract of 1 mg with
Fig. 2. Bacterial culture filtrate (BCF) of *B. amyloliquefaciens* Y1 mixed with potato dextrose agars showing growth inhibition of *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Phytophthora capsici*, * Fusarium oxysporum f. sp lycopersici*, and *Botrytis cinerea* and PDB without culture filtrate was used as control (0%). Error bars represent standard deviation of the mean from three replicates.

Fig. 3. Zone inhibition of *Bacillus amyloliquefaciens* Y1 crude extract against *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Phytophthora capsici*, *Fusarium oxysporum f.sp lycopersici*, and *Botrytis cinerea* on potato dextrose agar plates incubated at 26°C after 3 days of incubation for *Rhizoctonia solani* and 7 days for other fungi. Calculated mean values are from three replicates. BCE and Methanol were loaded into the paper discs on each sides of the same plate. Error bars represent standard deviation of the mean of three replicates.

inhibition zone of 6.6 mm and inhibition zone was found to increase with increasing amount of crude extract loaded into paper discs with maximum value of 11.33 mm at 4 mg of crude extract. Crude extract of 4 mg highly affect all fungal pathogens growth with 8.6, 6.0, 5.3 and 3.0 mm of zone inhibition against *P. capsici*, *B. cinerea*, *F. oxysporum* and *C. gloeosporioides* respectively (Fig. 3).

Additionally, various concentrations of butanol crude extract of *B. amyloliquefaciens* Y1 culture showed significant antifungal activity against all tested fungal plant pathogens. This prove that crude extract of *B. amyloliquefaciens* Y1 have secondary metabolites which inhibit growth of fungi. Previous study reported that *Bacillus* species produce various secondary metabolites that effectively suppress or control phytopathogenic fungi (Chao et al., 2003; Souto et al., 2004; Korenblum et al., 2005).

**Effect of butanol crude extract on fungal hyphae**

Changes were found in hyphae morphology of both fungal pathogens *C. gloeosporioides* (C.g) and *P. capsici* (P.c) as a result of butanol crude used in PDB with its final concentration of 500 ppm and 1000 ppm of active crude extract. Changes like degradation, lysis and deformation were found in both fungi mycelia after treatment with 500 ppm of crude extract but degradation was found more in *P. capsici* while 1000 ppm treatment resulting completely degradation of hyphae (Fig. 4). Biological control activity of antagonistic bacteria against fungal pathogens is through production of antibiotic (Ezra et al., 2004; Lee et al., 2005). In present study hyphae of *C. gloeosporioides* and *P. capsici* with abnormal structure was found by crude extract which may consider being antibiosis as defined by Baker and Cook (1982). Furthermore, antifungal potential of *B. amyloliquefaciens* Y1 crude extract affecting fungal and degrading fungal mycelia makes it biocontrol candidate for further study to identify its antifungal metabolite responsible for antifungal activity.

**Antifungal volatile assay**

Fungal pathogens growth was found to be affected by volatile compounds produced by *B. amyloliquefaciens* Y1 (7.6×10⁷ CFU) in volatile assay. These antifungal volatile compounds affect significantly that fungi growth was slow down and finally can no more grow. The colony diameters were significantly reduced even from the first day of observation in case of *R. solani* which only grown for 2.00 cm when controls have grown of 8.5 cm diameter (Fig. 5A). Nonetheless, the growth inhibition of the colonies was also significant in case of *P. capsici* visible from 48 h onwards (Fig. 5B) and with finally grown for 2.4 cm diameter. Similar results were observed in case of *C. gloeosporioides*, the growth of which grown only 2.8 cm diameter when finally growth diameter was measured (Fig. 5C). Naing et al. (2013) used volatile assay technique to investigate antifungal potential of antagonistic bacteria and reported that the volatiles produced by *Paenibacillus ehimensis* KWN38 could restrict the growth of the tested pathogenic fungi. Moreover, volatile compounds produced by *B. amyloliquefaciens* Y1 restricts growth of tested fungi is accordance to Fernando et al. (2005) and Kai et al. (2006) reported various volatile compounds produce by antagonistic bacteria and fungi showed antifungal activity. Furthermore, there is possibility of using volatile compounds produce by the *B.amyloliquefaciens* Y1 for post-harvest disease control especially against *C. gloeosporioides* causing post-
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Fig. 4. Light microscopy examination on effect of crude extract of *Bacillus amyloliquefaciens* Y1 on hyphal morphology of *Colletotrichum gloeosporioides* (C.g), and *Phytophthora capsici* (P.c) in potato dextrose broth incubated at 26°C after incubation for 96 hours. Treated with Methanol as control (A) treated with 500 ppm of crude extract (B) and treated with 1000 ppm of crude extract (C).

Fig. 5. Growth of *Rhizoctonia solani* (A), *Colletotrichum gloeosporioides* (B) and *Phytophthora capsici* (C) in presence of volatile compounds (VCA) produced by *Bacillus amyloliquefaciens* Y1 (Red shaded area) and Control (without VCA) is (Blue Shade) in terms of colony diameter. The unit for all axes in chart is millimeter. Means with different letters in each treatment within same axis are significantly different.

harvest disease in pepper.

**Conclusion**

Based on these results, we have found that, *Bacillus amyloliquefaciens* Y1, the antagonistic bacteria, showed strong antifungal activity against several pathogens consisting of *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Phytophthora capsici*, *Fusarium oxysporum* f. sp. lycopersici, and *Botrytis cinerea* by producing active metabolites in its culture filtrate and crude extract. Moreover, our results clearly demonstrated the potential of producing volatile compounds by *B. amyloliquefaciens* Y1, which make its application possible to control post-harvest diseases caused by fungal pathogens. Further studies are required to confirm the efficacy of *B. amyloliquefaciens* Y1 under field conditions, and more work is needed to purify and characterize the secondary metabolites produced by *B. amyloliquefaciens* Y1 responsible for disease suppression.
An important perspective of the present study is the isolation of *B. amyloliquefaciens* Y1 strain which is effective biocontrol agent to control soil born fungal plant pathogens.

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