Biocontrol Activity of *Bacillus amyloliquefaciens* CNU114001 against Fungal Plant Diseases

Seung Hyun Ji¹, Narayan Chandra Paul¹, Jian Xin Deng^{1,2}, Young Sook Kim³, Bong-Sik Yun⁴ and Seung Hun Yu^{1,*}

¹Laboratory of Plant Pathology, Department of Agricultural Biology, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 305-764, Korea

²Department of Plant Protection, College of Agriculture, Yangtze University, Jingzhou 434025, China

³Research Center for Biobased Chemistry, Korea Research Institute of Chemical Technology, Daejeon 305-600, Korea

⁴Division of Biotechnology, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan 570-752, Korea

Abstract A total of 62 bacterial isolates were obtained from Gomsohang mud flat, Mohang mud flat, and Jeju Island, Republic of Korea. Among them, the isolate CNU114001 showed significant antagonistic activity against pathogenic fungi by dual culture method. The isolate CNU114001 was identified as *Bacillus amyloliquefaciens* by morphological observation and molecular data analysis, including 16SrDNA and gyraseA (*gyrA*) gene sequences. Antifungal substances of the isolate were extracted and purified by silica gel column chromatography, thin layer chromatography, and high performance liquid chromatography. The heat and UV ray stable compound was identified as iturin, a lipopeptide (LP). The isolate CNU114001 showed broad spectrum activity against 12 phytopathogenic fungi by dual culture method. The semi purified compound significantly inhibits the mycelial growth of pathogenic fungi (*Alternaria panax*, *Botrytis cinera*, *Colletotrichum orbiculare*, *Penicillium digitatum*, *Pyricularia grisea* and *Sclerotinia sclerotiorum*) at 200 ppm concentration. Spore germ tube elongation of *Botrytis cinerea* was inhibited by culture filtrate of the isolate. Crude antifungal substance showed antagonistic activity against cucumber scleotiorum rot in laboratory, and showed antagonistic activity against tomato gray mold, cucumber, and pumpkin powdery mildew in greenhouse condition.

Keywords Antifungal substance, *Bacillus amyloliquefaciens*, Fungal plant pathogen, Iturin

In the past century, farmers often relied heavily on chemical fertilizers and pesticides to increase the productivity of plants. However, the environmental pollution caused by excessive use and misuse of agrochemicals, as well as concern by some opponents of pesticides, has led to considerable changes in people's attitude toward the use of pesticides in agriculture. Consequently, some pest management researchers have focused their efforts on developing alternative inputs to synthetic chemicals for

control pests and diseases [1]. The organism that suppresses the pest or pathogen is referred to as the biological control agents (BCA). According to the members of the U.S. National Research Council, 'Biological control is the use of natural or modified organisms, gene, or gene products, to reduce the effect of undesirable organisms and to favor desirable organisms such as crops, beneficial insects, and microorganisms.'

Plants and pathogens interact with a wide variety of microorganisms. These interactions can significantly affect plant health in various ways. Some microorganisms nearby rhizosphere help plant health from pathogen attack, directly or indirectly. The types of interactions include mutualism, protooperation, commensalism, neutralism, competition, amensalism, parasitism and predation [2]. Microbes that contribute most to disease control are most likely those that could be classified competitive saprophytes, facultative plant symbionts, and facultative hyperparasites. In previous research, numerous studies have demonstrated that several other metabolites, including antibiotics, enzymes, and volatiles produced by antagonistic bacteria, play key roles in the control of various plant pathogens [3, 4].

Bacillus amyloliquefaciens was separated from *Bacillus subtilis* as a new species by Priest *et al.* [5] of Heriot-Watt

Mycobiology 2013 December, 41(4): 234-242
<http://dx.doi.org/10.5941/MYCO.2013.41.4.234>
pISSN 1229-8093 • eISSN 2092-9323
© The Korean Society of Mycology

***Corresponding author**

E-mail: shunyu@cnu.ac.kr

Received August 13, 2013

Revised October 21, 2013

Accepted October 28, 2013

©This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

University. *B. amyloliquefaciens* produces a variety of antibacterial and antifungal antibiotics zwittermicin-A [6], kanosamine [7] and lipopeptides (LPs) from the surfactins, iturin and fengycin, etc. involvement of iturins and fengycins was demonstrated in the antibiosis based biocontrol activity of *Bacillus* strains against various pathogens and in different plants species. Both iturins and fengycins are recently shown the antagonism toward *Podosphaera fusca* infecting melon leaves [8]. This was demonstrated by identifying iturins and fengycins as the main antibiotic products excreted by the *B. subtilis* strains, by showing the strong inhibitory effect of these LPs on *P. fusca* conidia germination, and by recovering LPs from bacterial-treated leaves and use of LP-deficient transformants. Spore forming ability of *Bacillus* makes these bacteria the best candidates for developing efficient biopesticide products from a technological point of view. First, their antagonistic effect is caused by compounds, antibiotics (bacilysin, iturin, mycosubtilin) and siderophores [9]. Second, they are able to induce growth and defense responses in the host plant [10]. Furthermore, *Bacillus* produce spores resistant to UV light and heat which allows them to resist adverse environmental conditions, and permits easy formulation for commercial purposes [11]. The objectives of the study were 1) to isolate antagonistic bacteria from soil and find antifungal compound against plant pathogens 2) extraction, purification and identification of antifungal compound from the selected bacteria 3) to evaluate the antifungal activity of the isolate CNU1140001 against powdery mildew and gray mold.

MATERIALS AND METHODS

Isolation and identification of bacteria. Soil bacteria were isolated from Gomsong mudflat, Mohang mudflat and Jeju Island, Korea in 2011. One gram soil sample was mixed with 10 mL of sterile water in a 50 mL tube. The soil mixture was diluted 1 : 10 ratio with distilled water. One hundred microliters of sample dilutions was spread in a nutrient agar (NA; beef extract 3 g, peptone 5 g, agar 15 g, D.W 1 L) media and tryptic soy agar (TSA; tryptone 15 g, soytone 5 g, sodium chloride 5 g, agar 15 g, D.W 1 L) and then incubated at 27°C for 1~3 days. After 2 days, bacterial colonies were transferred to NA media and incubated at 27°C. Pure culture was confirmed after several culturing and assigned isolate number. The bacterial culture suspension grown on nutrient broth media was stored in 30% glycerol stock solution at -70°C.

Dual culture antifungal activity of bacteria. To test the antifungal activity of bacteria, mycelial disk (5 mm diameter) of each plant pathogenic fungus was placed on the edge of potato dextrose agar media 30 mm from streak inoculation of each bacterium in the middle of plates. Plates were incubated at 25°C for 5~8 days. Inhibition of mycelia growth of each fungus was measured 5~8 days after inoculation.

Morphological and physiological observation of the selected isolate. The bacterial colony was grown on NA plate for 1 day. A colony taken from NA plate was transferred to a fresh Mueller-Hinton (MH) agar plate and TSA plate. Petri-dishes were kept in incubator for 2 days. Cells from single colony were used for gram staining and observed through microscope. The bacterium CNU114001 was also identified by API50 CHB kit using carbon sources.

Genomic DNA extraction, purification, PCR, and phylogenetic analysis. Genomic DNA was extracted by following the laboratory method [1]. Primers 27F and 1492R were used for the amplification of the bacterial 16SrDNA which was carried out in i-cycler (Bio-Rad, Hercules, CA, USA) for 30 cycles of 94°C for 1 min denaturing, 55°C for 40 sec annealing, and 72°C for 1 min extension. Initial denaturing at 94°C was extended to 5 min and the final extension was for 10 min at 72°C. The PCR product was purified using Wizard PCR Prep Kit (Promega, Madison, WI, USA). Purified double stranded PCR fragments were directly sequenced with BigDye terminator cycle sequencing kits (Applied Biosystems, Forester City, CA, USA), by following the manufacturer instructions. Additionally, *gyrA* primer set (*gyrA-f* and *gyrA-r*) was used to identify them more specifically. In order to amplify rDNA, PCR was carried out by the following conditions: initial denaturing at 94°C for 5 min, 30 cycles at 94°C for 40 sec denaturing, 55°C for 40 sec annealing, and 72°C for 1 min extension. The final extension was for 15 min at 72°C. Then the purified PCR product was sent to the company (Macrogen, Seoul, Korea) for sequencing. The 16s rDNA gene sequences were compared by EZ taxon search and BLAST search and the *gyrA* gene sequence was compared with the BLAST search available in the GenBank database. Maximum Likelihood trees were constructed using the MEGA5 program; the bootstrap analysis using 1,000 replications were performed to assess the relative stability of the branches.

Screening, extraction and purification of antifungal compound. After cultivating the bacterium in MH medium at 30°C for three days with 150 rpm, culture supernatant was collected from culture broth by centrifuging at 8,000 rpm for 30 min at 4°C (culture conditions was optimized, data not shown). The supernatant was mixed with equal volume of hexane, chloroform, ethyl acetate, and butanol, successively. Each fraction was collected and concentrated and dissolved with methanol. It was used for antifungal activity by paper disk method against four pathogenic fungi *Botrytis cinerea*, *Colletotrichum acutatum*, *Fusarium* sp., and *Penicillium digitatum*.

The crude extract from butanol layer was dried to remove methanol *in vacuo*, the crude extract was separated by silica gel column chromatography (70~230 mesh; Merck, Darmstadt, Germany) with chloroform-methanol (20 : 1), chloroform-methanol (10 : 1), chloroform-methanol (5 : 1),

chloroform-methanol (2 : 1), chloroform-methanol (1 : 1), methanol (100%) as a mobile phase, and antifungal fraction was collected. All fractions were checked to find an antifungal substance by the paper disk method against *Botrytis cinerea*.

Preparative thin-layer chromatography (PTLC) and high performance liquid chromatography (HPLC).

PTLC analysis of antifungal fractions was performed on glass-backed PTLC plates coated with silica gel 60 F₂₅₄, 20 cm × 20 cm (Merck). The antifungal fraction was read as a strip near the bottom edge of the plates and PTLCs were developed in chloroform/methanol/H₂O (65 : 25 : 4, v/v) as a mobile phase. The chromatograms were air-dried and then used for antifungal bioassay. The compounds on the PTLC chromatograms were revealed under UV light and by spraying with water. The PTLC chromatogram strips were divided by scraping from the TLC glass. And each section was extracted with chloroform-methanol (2 : 1) from striped silica gel. Extract solution was used to check the antifungal activity by the paper disk method. The fraction was purified by HPLC.

Stability tests.

Heat: To evaluate the stability of culture broth (cell free culture), different temperatures were used and they were -25°C, 5°C for 24 hr, 5°C, 55°C for 4 hr, -80°C, 100°C, 121°C for 20 min and at 25°C for 24 hr as a control. Antifungal activity was measured by the diameter of the inhibition zone.

Light: Each paper disk was loaded with 50 µL concentrated culture broth (cell free culture) and dried in laminar air flow chamber. The paper disks were exposed directly under UV light of 254 or 365 nm wavelengths, respectively, at a distance of 15 cm for 30 min. Disk stored in the dark served as a control. Disks were used for the measurement of antifungal activity. After 3~5 days, inhibition zones were checked and recorded.

Antifungal activity of the CNU114001. Antifungal activity of the isolate was evaluated using dual culture method against 12 pathogenic fungi- *Alternaria panax*, *Botrytis cinerea*, *Colletotrichum acutatum*, *C. orbiculare*, *Corynespora cassicola*, *Fusarium oxysporum*, *Penicillium digitatum*, *Phytophthora capsici*, *Rhizoctonia solani*, *Stemphylium lycopersici*, *Pyricularia grisea*, *Sclerotinia sclerotiorum*. Dual culture plates were incubated at 25°C for 5~8 days. Antifungal activity was indicative as mycelial growth inhibited in the direction of active bacteria.

Inhibition zone was calculated by the following formula [1].

$$\text{Inhibition percentage (\%)} = \frac{A_1 - A_2}{A_1} \times 100$$

Where, A_1 = radial growth of pathogenic mycelia without CNU114001, A_2 = radial growth of pathogenic mycelia with

CNU114001.

The isolate was grown on 40 mL of MH media for 3 days at 30°C with 150 rpm. After centrifuging, bacterial culture suspensions were filtered with syringe filter (0.22 µm pore size) for cell free culture suspension. *B. cinerea* was grown on potato dextrose agar (PDA) media for 7 days at 25°C. Mycelia were scraped and kept under near ultraviolet ray for 2 days (12 hr light/12 hr dark). Conidia were collected and the concentration adjusted to 5×10^5 conidia/mL. The conidial suspension was mixed with different concentrations (0, 2×, 5×, 10×, 50×, and 100×) of cell free suspension of CNU114001. Then, treated suspension was dropped on PDA media and kept at 25°C. After 24 hr, at least 30 germ tube lengths were measured and recorded per treatment.

Agar plugs (5 mm) of all pathogenic fungi, *Alternaria panax*, *Botrytis cinerea*, *Corynespora cassicola*, *Collectotrichum orbiculare*, *Penicillium digitatum*, *Fusarium oxysporum*, *Pyricularia grisea*, and *Sclerotinia sclerotiorum* were taken from a freshly growing colony and placed on PDA (6 cm) contained various concentrations of antifungal compound (50 ppm, 100 ppm, 200 ppm, 250 ppm, and 500 ppm) and dimethyl sulfoxid (DMSO) was used as a control. They were kept in incubator at 25°C for 3~7 days. Diameters of the fungal colonies were measured and recorded.

Bioassay.

Against sclerotium rot, gray mold, and powdery mildew: *Sclerotinia sclerotiorum* was grown on PDA media for 7 days at 25°C. Cucumber fruits (cultivar-bagdadagi) were washed in 2% NaOCl (sodium hypochlorite) for 5 min and then washed three times with sterilized water. Cucumbers were allowed to dry on paper towel in a laminar air flow chamber. Then, the cucumbers were wounded in depth 5 mm diameter at both ends by cork borer. Crude antifungal substance was adjusted with DMSO at 10 ppm, 50 ppm, 100 ppm. On each wound spot, crude substance was inoculated and DMSO was inoculated opposite spot as a control. They were dried naturally for 5 hr. After drying, agar plug (5 mm) of *Sclerotinia sclerotiorum* was inoculated in two spots of treated cucumber and they were placed on sterilized plastic box with moistened paper towels and incubated at 25°C. After 5 days of treatment, disease incidence were measured and recorded. Lesion length and reduction rate were calculated.

Tomato seeds were germinated in a cell tray containing soil. After 20 days, seedlings were transplanted to plastic pots (9 cm) in glass house. The bacterial suspension was diluted 10, 20, 50 times with sterilized water and sprayed onto whole tomato plants. Water was used as untreated control. The conidial suspensions (5×10^5 conidia/mL) were sprayed onto whole plants 1 day before treatments with various concentrated bacterial suspension. All the bacterial culture treatments were sprayed every seven days, 3 times. After 7 days from the final spray, disease incidence of infected leaves was calculated. Rate of infection was also

Table 1. Morphological characteristics and carbohydrate fermentation of the isolate CNU114001

	Activity	Properties	Activity	Properties	Activity
Physiological property		Carbohydrate		Carbohydrate	
Colony type	Undulant round, ivory	Sorbose	–	Trehalose	+
Cell shape	Rods	Rhamnose	–	Inulin	–
Endospore	+	Dulcitol	–	Raffinose	+
Carbohydrate		Inositol		Starch	+
Glycerol	+	Mannitol	+	Glycogen	+
Erythritol	–	Sorbitol	–	Xylitol	–
D-Arabinose	–	Adonitol	–	Gentiobiose	+
L-Arabinose	+	Galactose	+	D-Turanose	–
Ribose	+	Glucose	+	D-Lyxose	–
D-Xylose	+	Amygdalin	+	D-Lyxose	–
L-Xylose	–	Arbutin	+	D-Tagatose	–
Methyl-B-D-Xylopyranside	–	Esculin	+	D-Fucose	–
Methyl- α , D-Mannopyranside	–	Salcin	+	L-Fucose	–
Methyl- α , D-Glucoside	+	Cellobiose	+	D-Arabitol	–
N-Acetyl-glucosamine	–	Maltose	+	L-Arabitol	–
2-Keto-gluconate	–	Lactose	+	Gluconate	+
5-Keto-gluconate	–	Melibiose	+	Fructose	+
		Sucrose	+	Mannose	–

calculated.

$$\text{Disease severity (\%)} = \frac{A}{B} \times 100$$

Gray mold development on each leaf was recorded using visible symptom, where, 0 = no visible, 1 = gray mold development, A = sum of the number of infected leaf, B = sum of the number of leaf on plant.

Seedlings of cucumber and pumpkin were transplanted to plastic pots (9 cm) in glass house. Infections of powdery mildew were observed naturally from artificially infected plants. When powdery mildew symptoms were observed, 10, 20, and 50 times diluted bacterial suspensions were sprayed onto whole cucumber and pumpkin plants. All the treatments were sprayed after 7 days, 3 times. Rate of infection was calculated for disease incidence by the following formula.

$$\text{Disease severity (\%)} = \frac{5A + 4B + 3C + 2D + E}{5F} \times 100$$

Mildew development on each leaf was recorded using 1~5 scale developed, where 0 = no visible, mildew development, 1 = 1~5%, 2 = 6~25%, 3 = 26~50%, 4 = 51~75%, and 5 = $\geq 76\%$ of the leaf surface covered with mildew where A, B, C, D, and E are the number of leaves corresponding to the scale 5, 4, 3, 2, and 1, respectively, and F is the total number of assessed leaves.

RESULTS

Isolation and identification of antagonistic bacteria.

A total of 62 isolates were investigated for their antifungal activity against pathogenic fungi by dual culture method. Among all, the most effective isolate was CNU114001 from mud flat of Gomsohang in Korea.

Morphology and physiology. Cells of the strain CNU114001 were gram positive, motile and length varied from 1.73 to 2.62 μm and width 0.65 to 1.14 μm . Based on the morphological and physiological tests, this isolate might be *B. subtilis* or *B. amyloliquefaciens* (Table 1).

Molecular phylogeny. The genomic DNA of the isolate CNU114001 was amplified using 27F & 1492R and *gyrA*-F and *gyrA*-R as described previously [1]. After alignment of

Fig. 1. Phylogenetic tree of the isolate CNU 114001 and other *Bacillus* species from Gene Bank based on 16S rRNA gene. Phylogenetic tree was constructed by the MEGA 5.0 program using maximum likelihood method. The bootstrap analysis was performed with 1,000 replications. The present isolate is shown in bold (T, type strain).

the sequence of the isolate CNU114001 and other isolate of *Bacillus* species from GeneBank with maximum likelihood analysis, phylogenetic tree were constructed using the MEGA5

program. The tree showed that the isolate CNU114001 formed a clade with reference *Bacillus amyloliquefaciens* sequences at a bootstrap value of 70% (Fig. 1). The tree,

Fig. 2. Antifungal activity of crude substance of CNU114001 against four pathogenic fungi. A, *Botrytis cinerea*; B, *Colletotrichum acutatum*; C, *Fusarium* sp.; D, *Penicillium digitatum* (a, hexane; b, chloroform; c, ethyl acetate; d, buthatnol; e, water).

Fig. 3. Antifungal activity of different fractions against *Botrytis cinerea* (A). Fractions from silica gel column chromatography on thin layer chromatography plate eluted with CHCl_3 : MeOH : water = 65 : 25 : 4 v/v. B, 254 nm UV light; C, 365 nm UV light; D, Sprayed with water.

Fig. 4. High performance liquid chromatography profiles of isolated compounds from *Bacillus amyloliquefaciens* CNU114001 compares with iturin and fengycin.

constructed with *gyrA* gene sequence, showed that the isolate CNU114001 belonged to the group *Bacillus amyloliquefaciens* with a high bootstrap value of 99% (data not shown). The isolate CNU114001 was identified as *Bacillus amyloliquefaciens* with 16s rDNA and *gyrA* gene sequence analysis.

Extraction and identification of antifungal compound.

From 3 L of bacterial culture, antifungal substance was collected. After checking the antifungal activity against *B. cinerea* with hexane, chloroform, ethyl acetate and butanol layer it was confirmed that only butanol layer contained antifungal substances (Fig. 2). Then butanol extract was concentrated under vacuum at 40°C and the residue was dissolved with chloroform and methanol and separated by silica gel column chromatography. Each fraction was concentrated and dissolved with methanol and were confirmed against pathogen. Three fractions (48~50) showed antifungal activity (Fig. 3A). The active fractions were combined, concentrated and spotted on preparative silica gel TLC (Merck, 60 F₂₅₄). chloroform: methanol (2 : 1, v/v) was used as a mobile phase. The antifungal band was shown by water as a mobile phase (Fig. 3B~D) whose R_f value is 0.41. The fraction was dried using an evaporator, then performed HPLC analysis. HPLC profiling confirmed the compound as iturin comparing with standard iturin (Fig. 4).

Stability test. The stability of CNU114001 culture filtrate was checked on heat and UV light. The antifungal activity was stable at 100°C but did not show activity at 121°C (data not shown). The inhibition zone was 16 mm. When the antifungal compound treated with 254 and 365 nm UV light, the activity was the same as control. It concluded that the compound is stable to heat and ultraviolet ray.

Antifungal activity of CNU 114001. The isolate CNU114001 showed antifungal activity against all 12 different plant pathogenic fungi by dual culture. Against *C. orbiculare*, *F. oxysporum*, *P. digitatum* and *P. grisea* the

Table 2. Antifungal activity of CNU114001 against 12 pathogenic fungi

Pathogenic fungi	Growth of mycelia (mm)	Inhibition (mm)	
		Length	Reduced rate (%)
<i>Alternaria panax</i>	30.14	14.74	51.09
<i>Botrytis cinerea</i>	36.72	12.20	66.79
<i>Colletotrichum acutatum</i>	22.10	8.24	62.74
<i>C. orbiculare</i>	30.08	8.75	70.91
<i>Corynespora cassicola</i>	21.49	6.91	67.86
<i>Fusarium oxysporum</i>	37.42	10.92	70.82
<i>Phytophthora capsici</i>	36.90	26.75	27.50
<i>Penicillium digitatum</i>	29.33	5.25	82.10
<i>Rhizoctonia solani</i>	39.32	15.86	59.66
<i>Stemphylium lycopersici</i>	22.52	10.80	52.07
<i>Pyricularia grisea</i>	22.84	3.89	82.97
<i>Sclerotinia sclerotiorum</i>	24.09	11.80	51.02

CNU114001 showed strong inhibition, over 70% mycelial growth reduced compare to control plate. Although most of the mycelial growth reduction was over 50% but *P. capsici* showed 27.5% reduction (Table 2). Between the isolate CNU114001 and plant pathogenic fungi, abnormal mycelia, swollen and curved, at the boundary through microscope (×200) were observed (Fig. 5).

Eight plant pathogenic fungi were treated with different concentrations (0, 50, 100, 200, 250, and 500 ppm) of crude antifungal substance (Fig. 6). Eight pathogenic fungi couldn't grow on the PDA with 200 ppm antifungal substance. Mycelial growth of *A. panax*, *B. cinerea*, *P. grisea* and *S. sclerotiorum* was strongly reduced by crude extract in high concentration (50 ppm), except *Fusarium oxysporum*. It means low concentration of compound showed weak activity only against *F. oxysporum*.

The inhibitory activity of the isolate CNU114001 on spore germ tube elongation of *B. cinerea* was tested on PDA plate. Although most of the spores were germinated regardless of treatment, the germ tube elongation of spore reduced about 50% treated with cell filtrate (diluted 100×).

Fig. 5. Morphological changes of *Botrytis cinerea* by dual culture. A, Normal mycelia; B, Mycelia from co-inoculated with CNU114001. The arrows indicate the swollen balloon-like structure (scale bars: A, B = 50 µm).

Fig. 6. Antifungal activity of the compound against 8 pathogenic fungi in potato dextrose agar. Ap, *Alternaria panax*; Bc, *Botrytis cinerea*; Cc, *Corynespora cassicola*; Co, *Colletotrichum orbiculare*; Fo, *F. oxysporum*; Pd, *Penicillium digitatum*; Pg, *Pyricularia grisea*; Ss, *Sclerotinia sclerotiorum*.

Bioassay.

Against sclerotiorum rot, gray mold, and powdery mildew: The ethyl acetate extract of CNU114001 was diluted with DMSO at 10 ppm, 50 ppm and 100 ppm in order to evaluate against sclerotiorum rot of cucumber. Only DMSO treated control showed very high symptom (Fig. 7). Lesion length of control was 46.22 mm in

Fig. 7. Effect of crude antifungal substance to control sclerotinia rot of cucumber fruit. A, 10 ppm; B, 50 ppm; C, 100 ppm.

Table 3. Effect of CNU114001 on gray mold of tomato

Dilution	Disease incidence (%)	Control effect (%)
ND	20	68
20×	22	65
50×	30	52
Control	62	-

diameter. No disease infections were observed in all treated cucumber. The results showed that antifungal substance could reduce sclerotiorum rot, effectively.

The bacterial suspension was diluted 10, 20, and 50 times with sterilized water and sprayed onto tomato seedlings in green house. All the treatments were sprayed every 7 days, 3 times. The disease incidence in control was 62.1%, and was 20%, 22% and 30%, respectively when 10, 20 and 50 times diluted suspensions were used. The disease reductions were over 60% in 10 and 20 times and 50 times it was 52% (Table 3).

In case of cucumber plants, disease incidences were 24%, 35% and 48%, when applied 10, 20, and 50 folds, respectively. The disease reduction was over 60% when high concentrations of bacterial suspension were used. In case of pumpkin, the results shows that antifungal bacteria treated plants reduced the disease symptom significantly compare to nontreated plants. The disease incidence value in control, fungicide, and CNU114001 treated were 76%, 12%, and 34%, respectively. Both CNU114001 and fungicide inhibited the disease by 50% (data not shown).

DISCUSSION

The antagonistic bacterial isolate CNU114001 was obtained as a BCA from mud flat soil and identified as *Bacillus amyloliquefaciens*. *Bacillus* species are common bacteria from soil, fermented foods, and as endophytes. Some *Bacillus* species has the ability to inhibit potential plant pathogens as BCAs. *Bacillus amyloliquefaciens* was studied as a BCA to reduce green mold and blue mold rot on postharvest citrus [12], strawberry anthracnose [13] and damping off of soybean [14]. In the present study, the CNU114001 was tested for antifungal activity against 12 plant pathogenic fungi by dual culture method. Results showed that only against *Phytophthora capsici*, it showed low activity below 50% and against other fungi inhibition activity were high. Inhibitory germ tube elongation of *Botrytis cinerea* spore was investigated with CNU114001 culture filtrate. That spore germ tube elongation was inhibited and was delayed by bacterial culture filtrate. This result suggests that CNU114001 secreted secondary metabolite and antibiotic substances.

Numerous antibiotics have been isolated from various biocontrol strains representing different bacterial genera. According to Whipps [4], antibiotics produced by *Pseudomonas* spp. not only exhibit a wide range of diversity in the type but also in the number. *Bacillus cereus* strain UW85,

Pseudomonas fluorescens strains CHA0 and Pf5 [15] and *Burkholderia cepacia* CNU082111 [1] produce multiple antibiotic or different degrees of activity against specific pathogenic fungi. Antibiotic production by some bacteria plays a major role in disease suppression [16]. So far, Gram-negative bacteria, especially, *Pseudomonas* strains, have been intensively investigated with regard to the production of antimicrobial metabolites [17]. However, Gram-positive bacteria and especially strains of *B. subtilis*, and particularly, to the closely related *B. amyloliquefaciens* species were reported effective for the biocontrol of multiple plant diseases caused by soilborne [18] or post-harvest pathogens [19].

Numerous *Bacillus* species secreted volatile compounds [20] or cell wall degradation enzymes [21]. However the CNU114001 didn't show activity as volatile compound production. LPs are known to act in a synergistic manner, as suggested by several studies on surfactin and iturin [22], surfactin and fengycin [23], and iturin and fengycin [8]. Antifungal substance was extracted from cell free supernatant and then isolated by TLC, silica gel column chromatography. Total 60 fractions collected after silica gel chromatography. Activity of obtained fraction was confirmed by comparing with standard compounds as iturin. Iturins are a group of antifungal lipopeptides for a wide range of potential phytopathogens that consist of iturin A~E, bacillomycin D, F and mycosubtilin [24]. Lipopeptides relied on their membrane permeabilization properties [25]. However, the underlying mechanism is based on osmotic perturbation owing to the formation of ion-conducting pores and not membrane disruption or solubilization as caused by surfactins. Mycelia of phytopathogenic fungi were damaged by Lipopeptides. Abnormal mycelia were observed as changed cell wall structures like as swelling, lysis [26]. CNU114001 culture filtrate caused formation of abnormal mycelia of *Botrytis cinerea* as balloon like structures.

Several reports have shown that the success of control by antagonistic bacteria is defined mainly by their ability to affect this early step in the establishment of the disease [27, 28]. In this investigation, different fungal species, including gray mold, powdery mildew have been shown to be inhibited by diluted CNU114001 culture broth. Tomato plants were treated for the reduction of gray mold at 50 times diluted culture suspension and the control value was 52% and the reduction of powdery mildew was 55% when applied 20 times diluted culture suspension.

ACKNOWLEDGEMENTS

This study was supported by the National Institute of Biological Resources, Republic of Korea.

REFERENCES

- Paul NC. Diversity and biological control activity of endophytes from chili pepper (*Capsicum annuum* L.) in Korea [dissertation]. Daejeon: Chungnam National University; 2012.
- Odum EP. Fundamentals of ecology. Philadelphia: WB Saunders; 1953.
- Weller DM, Raaijmakers JM, Gardener BB, Thomashow LS. Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev Phytopathol 2002;40:309-48.
- Whipps JM. Developments in the biological control of soil-borne plant pathogens. Adv Bot Res 1997;26:1-134.
- Priest FG, Goodfellow M, Shute LA, Berkeley RC. *Bacillus amyloliquefaciens* sp. nov., nom. rev. Int J Syst Bacteriol 1987; 37:69-71.
- He H, Silo-Suh LA, Handelsman J, Clardy J. Zwittermicin A, an antifungal and plant protection agent from *Bacillus cereus*. Tetrahedron Lett 1994;35:2499-502.
- Stabb EV, Jacobson LM, Handelsman J. Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils. Appl Environ Microbiol 1994;60:4404-12.
- Romero D, de Vicente A, Rakotoaly RH, Dufour SE, Veening JW, Arrebola E, Cazorla FM, Kuipers OP, Paquot M, Pérez-García A. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Mol Plant Microbe Interact 2007;20:430-40.
- Shoda M. Bacterial control of plant diseases. J Biosci Bioeng 2000;89:515-21.
- Raupach GS, Kloepper JW. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 1998;88:1158-64.
- Raaijmakers JM, Vlami M, de Souza JT. Antibiotic production by bacterial biocontrol agents. Antonie Van Leeuwenhoek 2002;81:537-47.
- Yu SM. Biological control of postharvest green and blue mold rots of citrus fruits by *Bacillus amyloliquefaciens* BCL251 [dissertation]. Daejeon: Chungnam National University; 2009.
- Lee DG. Biological control of strawberry anthracnose using endophytic bacteria, *Bacillus amyloliquefaciens* CP1 [dissertation]. Daejeon: Chungnam National University; 2010.
- Yu GY, Sinclair JB, Hartman GL, Bertagnolli BL. Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biol Biochem 2002;34:955-63.
- Keel C, Weller DM, Natsch A, D'efago G, Cook RJ, Thomashow LS. Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. Appl Environ Microbiol 1996;62:552-63.
- Raaijmakers JM, Weller DM, Thomashow LS. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Appl Environ Microbiol 1997;63:881-7.
- Rosales AM, Thomashow L, Cook RJ, Mew TW. Isolation and identification of antifungal metabolites produced by rice-associated antagonistic *Pseudomonas* spp. Phytopathology 1995;85:1028-32.
- Asaka O, Shoda M. Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Appl Environ Microbiol 1996;62:4081-5.
- Mari M, Guizzardi M, Brunelli M, Folchi A. Postharvest biological control of grey mould (*Botrytis cinerea* Pers.: Fr.) on fresh-market tomatoes with *Bacillus amyloliquefaciens*. Crop Prot 1996;15:699-705.
- Fiddaman PJ, Rossall S. The production of antifungal volatiles

- by *Bacillus subtilis*. J Appl Bacteriol 1993;74:119-26.
21. Mathur A, Rawat A, Bhatt G, Baweja S, Ahmad F, Grover A, Madhav K, Dhand M, Mathur D, Verma SK, et al. Isolation of *Bacillus* producing chitinase from soil: production and purification of chito-oligosaccharides from chitin extracted from fresh water crustaceans and antimicrobial activity of chitinase. Recent Res Sci Technol 2011;3:1-6.
 22. Maget-Dana R, Thimon L, Peypoux F, Ptak M. Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. Biochemie 1992;74:1047-51.
 23. Ongena M, Jourdan E, Adam A, Paguot M, Brans A, Joris B, Arpigny JL, Thonart P. Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ Microbiol 2007;9:1084-90.
 24. Besson F, Peypoux F, Michel G, Delcambe L. Characterization of iturin A in antibiotics from various strains of *Bacillus subtilis*. J Antibiot (Tokyo) 1976;29:1043-9.
 25. Bonmatin JM, Laprvte O, Peypoux F. Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity-structure relationships to design new bioactive agents. Comb Chem High Throughput Screen 2003;6:541-56.
 26. Moyne AL, Shelby R, Cleveland TE, Tuzun S. Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. J Appl Microbiol 2001;90:622-9.
 27. Bker CJ, Stavelly JR, Thomas CA, Sasser M, MacFall JS. Inhibitory effect of *Bacillus subtilis* on *Uromyces phaseoli* and on development of rust pustules on bean leaves. Phytopathology 1983;73:1148-52.
 28. Yoshida S, Hiradate S, Tsukamoto T, Hatakeda K, Shirata A. Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology 2001;91:181-7.