World J Microbiol Biotechnol (2008) 24:3057–3062
DOI 10.1007/s11274-008-9852-x

ORIGINAL PAPER

Purification and partial characterization of antifungal metabolite from *Paenibacillus lentimorbus* WJ5

Young-Keun Lee · M. Senthilkumar · Jung-Hun Kim · K. Swarnalakshmi · K. Annapurna

Received: 26 March 2008 / Accepted: 29 August 2008 / Published online: 19 September 2008

Abstract *Paenibacillus lentimorbus* strain WJ5, a soil isolate showed in vitro antagonistic activity against several fungal phytopathogens belonging to the ascomycetes, basidiomycetes and oomycetes. The antifungal metabolite was extracellular and could be extracted with *n*-butanol. Its production was initiated at the end of the exponential phase, reaching a maximum after 5 days incubation at 30°C. Crude extract of the antifungal metabolite was thermostable (121°C for 3 h) and no loss of activity was recorded when exposed to proteinase K, sodium dodecyl sulphate (1%), Tween-80 (1%) and glycerol (1%). However, cationic hexadecyltrimethylammonium bromide and lysozyme inactivated the metabolite. The antifungal metabolite was purified by silica gel thin layer chromatography and Sephadex LH-20 size exclusion chromatography. Loss of activity during acid hydrolysis indicated the peptide nature of the antifungal metabolite. The FT-IR spectrum of the antifungal metabolite confirmed the presence of the peptide and glycosidic bonds.

Keywords *Alternaria alternata* · Antifungal metabolite · *Paenibacillus lentimorbus* WJ5 · Biocontrol

Y.-K. Lee · M. Senthilkumar (✉) · J.-H. Kim
Radiation Application Research Division, Advanced Radiation Technology Institute, Korean Atomic Energy Research Institute, Sinjeong, Jeongeup 580-185, Republic of Korea
e-mail: senthil8009@yahoo.co.in

Y.-K. Lee
e-mail: yklee@kaeri.re.kr

J.-H. Kim
e-mail: cremoris2000@hotmail.com

K. Swarnalakshmi
Division of Crop Physiology, Biochemistry and Microbiology, Indian Institute of Pulse Research, Kanpur 208024, India
e-mail: swarna_bga@yahoo.com

K. Annapurna
Division of Microbiology, Indian Agricultural Research Institute, New Delhi 110012, India
e-mail: annapurna96@yahoo.co.in

Introduction

Increased public concern about the accumulation of pesticide residues in the biosphere and the development of resistance among pathogens against conventional antibiotics have led scientists toward the development of alternative strategies for plant disease suppression. Many researchers have unarguably suggested that biological control (Pusey and Wilson 1984; Lemanceau and Alabouvette 1991) and use of microbial pesticides (Potera 1994) are the best alternatives. Many microorganisms are capable of suppressing plant diseases, but only a few have been commercialized as biofungicides. The complexity of the soil ecosystem makes biological control of root pathogens by introduced bacteria a challenge. However, the endospore-forming trait of *Bacillus* spp. made them suitable candidates for biological control of plant diseases. The activity of the soilborne fungus, *Pythium aphanidermatum*, that causes cottony cucumber leak was successfully suppressed by a Zwittermicin-producing strain of *Bacillus cereus* UW85 (Leifert et al. 1995). Production of anti-*Botrytis* and anti-*Alternaria brassicicola* antibiotics by *B. subtilis* CL27 and *B. pumilus* CL45 have been reported (Smith et al. 1993). Most of the antifungal metabolites characterized from *Bacillus* species were peptides, thus these now represent the predominant group. These peptide antibiotics are synthesized ribosomally during the growth

stage or non-ribosomally after the growth has ceased. *B. subtilis* strains are known to produce lipopeptide antibiotics which are either cyclic (iturin series) or a macrolactone (surfactins, fengycins and plipastatins), which contains a long hydrophobic tail. Cyclic peptide antibiotics such as mycosubtilin, eumycin, bacillomycin and the iturin series have been reported to inhibit several phytopathogenic fungi (Majumdar and Bose 1958; Besson et al. 1977; Isogai et al. 1982; Besson and Michel 1990). Budi et al. (1999) isolated *Paenibacillus* sp. strain B2 from the mycorhizosphere of *Sorghum bicolor* inoculated with *Glomus mosseae* and Selim et al. (2005) partially characterized the antifungal peptides.

Though the antibiotic-producing potential of *Bacillus* and *Paenibacillus* species has been recognized for more than six decades, very few reports are available on the antifungal trait of *P. lentimorbus* (Chen et al. 2003; Dasgupta et al. 2006). Recently, Nautiyal et al. (2006) reported that *P. lentimorbus* B-30488, isolated from the milk of Sahiwal cows showed antifungal activity against *Fusarium oxysporum* f. sp. *ciceri*. New strains of bacteria may potentially provide new biocontrol agents with novel mechanisms of diseases suppression in a range of environments. So it is necessary to continue the search for novel useful microbes from the untapped microbial diversity. In our laboratory, *P. lentimorbus* WJ5 was isolated from soil and its antifungal trait was established against phytopathogens (Lee et al. 2003). In the present investigation, we extracted and partially purified the antifungal metabolite(s) of *P. lentimorbus* WJ5 and studied their physicochemical properties.

Materials and methods

Bacterial and fungal strains

Paenibacillus lentimorbus WJ5 was previously isolated from the soil in Uljin province, Korea (Lee et al. 2003) and maintained on Nutrient Agar (NA) at 5°C. Phytopathogenic fungal isolates (Table 1) were obtained from Radiation Application Division, Korean Atomic Energy Research Institute (KAERI) Korea and maintained on Potato Dextrose Agar (PDA) at 5°C.

Activity spectrum and production of antifungal metabolite by *P. lentimorbus* WJ5

To study the spectrum of antifungal activity, agar plugs (5 mm diameter) were taken from actively growing regions of fungal phytopathogens cultures (Table 1) and placed at the centre of PDA plates. A loopful of cells of *P. lentimorbus* WJ5 was streaked 2 cm away from the fungal disc and the plates were incubated at 30°C for 7 days. Plates were observed for the presence as well as the size of inhibition zone. To study the growth and production of antifungal metabolites, a loopful of cells of *P. lentimorbus* WJ5 was inoculated into 200 ml of potato dextrose broth (PDB) and incubated at 30°C with shaking at 200 rev/min. At regular time intervals, an aliquot of the bacterial culture was taken to determine the population level and production of antifungal metabolite. Population was assessed on PDA plates by standard plate count method. To determine the antifungal activity, cell-free culture supernatant was obtained by centrifugation at 10000 rev/min for 15 min) followed by membrane filter (0.2 μm pore size) sterilization. About 100 μl of cell-free culture supernatant was loaded onto paper discs (5 mm diameter) placed on PDA plates. An agar plug, taken from actively growing regions of *Alternaria alternata* culture was placed at the centre of PDA plates and incubated at 30°C for 4 days. Bioassay experiments were repeated three times with five replications.

Table 1 Antagonistic activity of *P. lentimorbus* WJ5 towards plant fungal pathogens

Fungus	Inhibition zone (mm)[a]
Alternaria alternata	8.00 ± 1.22
Alternaria solani	10.25 ± 0.83
Botrytis cinerea	6.75 ± 0.83
Cladosporium sp.	6.00 ± 0.71
Colletotrichum gloeosporioides	6.25 ± 0.83
Fusarium oxysporum	7.25 ± 0.83
Phytophthora capcisi	7.75 ± 0.83
Pythium ultimum	6.25 ± 0.83
Rhizoctonia solani	10.75 ± 0.83
Sclerotinia sclerotiorum	8.75 ± 0.83

[a] Values are the mean of triplicates

Extraction and purification of antifungal metabolites

Paenibacillus lentimorbus strain WJ5 was grown in 1 l of PDB for 5 days on a rotary shaker (200 rev/min) at 30°C. After incubation, cells were removed by centrifugation at 10,000 rev/min for 15 min. About 500 ml of cell-free culture supernatant was used to precipitate the extra-cellular proteins by using two volumes of ice-cold acetone. The remaining culture supernatant was sequentially extracted with 500 ml of chloroform, ethyl acetate and *n*-butanol. About 100 μl of organic extract and aqueous phase from each extraction phase were used for disc diffusion assay as described previously. For purification, the *n*-butanol extract of antifungal metabolite was applied to thin layer chromatography plates and developed with chloroform: methanol (10:1, v/v). Plates were observed under ultra-violet light as well as exposed to iodine vapor

to visualize the spots. Individual spots were eluted with methanol and used for bioassay to locate the bio-active compound(s). Active metabolites were dried and redissolved in 500 µl of methanol. The TLC-purified fraction with antifungal activity was applied to a methanol-equilibrated Sephadex LH 20 column (1.5 × 35 cm) and eluted with methanol at a flow rate of 0.2 ml/min. One milliliter fractions were collected in vials and the antifungal activity was located by bioassay. Bioactive fractions were pooled and concentrated to dryness. Dried antifungal metabolite was re-dissolved in 1 ml of HPLC grade methanol.

Characterization of antifungal metabolites

Biologically active metabolites were characterized with respect to thermal and pH stability, susceptibility to protein denaturing detergents and enzymes like proteinase K and lysozyme. To analyse the thermal stability, crude *n*-butanol extract (10 mg/ml) was exposed to low temperature (−80°C for 6 h) and high temperature (60 and 121°C for 1–6 h). Then the solution was cooled immediately to room temperature and two-fold dilutions were made with sterile water. About 100 µl of each dilution was used for disc diffusion assay to determine the lowest dilution that showed antifungal activity. The same concentration of antifungal metabolite, not exposed to high temperature served as control. The loss of activity was compared in terms of lowest dilution that showed the inhibition zone. To determine the pH stability, crude *n*-butanol extract was dissolved in sterile water (10 mg/ml) with pH values adjusted to 13 and 2, incubated for 1 h. The treated samples were neutralized and then subjected to agar diffusion assay.

A solution of crude antifungal metabolite (10 mg/ml) was prepared separately with 1% solution of Tween-80, SDS and CTAB. The same concentration of antifungal metabolites was also exposed to enzymes like proteinase K (1 mg/ml) and lysozyme (1 mg/ml). Detergents (1%) without antifungal metabolite served as control. Solutions with antifungal metabolites were incubated for 6 h and then used for bioassay as described previously. In the case of enzyme treatments, the enzyme was inactivated after the incubation time by keeping it at 72°C for 15 min. For infrared spectrum analysis, bio-active metabolite was processed with potassium bromide to make pellets. Spectra of the pellets were recorded in the transmission mode between wavelengths of 4000–400 cm^{-1} using a resolution of 4 cm^{-1}/20-kHz scan speed in FT-IR spectrophotometer.

Partially purified active metabolite (20 µl) was hydrolysed with 6 M HCl at 100°C for 24 h. Hydrolysed and unhydrolysed fractions were spotted on silica TLC plates and run with solvent system containing *n*-butanol, glacial acetic acid and water (4:1:5). The plates were sprayed with ninhydrin dissolved in methanol (0.1%) and exposed to 100°C for 10 min to visualize the presence of free amino acids.

To determine the amino acid composition, antifungal metabolite was dissolved in 3 ml of 6 M HCl and hydrolysed at 121°C for 24 h in a heating block. Acid-hydrolysed sample was evaporated by using rotary evaporator at 50°C and a pressure of 40 psi. The total volume was made up to 10 ml by using sodium buffer, filtered (0.2 µm diameter) and analysed by amino acid analyser (S433-H; SYKAM). For the direct detection of antifungal activity on polyacrylamide gel, 25 µl of purified sample (1 mg/ml) was applied to 15% polyacrylamide gel and electrophoresed at 20 mA along with molecular weight markers. After the run was over, the gel was washed with sterile water to remove the SDS detergent and placed on PDA plates. Fungal spore suspension was poured onto the gel and incubated at 30°C for 4 days. Development of an inhibition zone was observed (Sarfo et al. 2003).

Results

Production, extraction and purification of antifungal metabolite(s)

The soil bacterium, *P. lentimorbus* WJ5 produced secondary metabolites with antifungal activity against several phytopathogens as listed in Table 1. After 4 days incubation of dual cultured tests, the fungal hyphae failed to reach the bacterial culture and the inhibition zone was established. Larger inhibition zones (≥10 mm) were produced against *Alternaria solani* and *Rhizoctonia solani* when compared to that of *Colletotrichum gloeosporioides*. We found that *P. lentimorbus* WJ5 produced antifungal metabolites extracellularly when it was grown in a shake culture with PDB. The production of antifungal metabolite was detected at the end of the exponential phase. At 120 h, the concentration of antifungal metabolite reached its highest level, in terms of inhibition zone size, when the

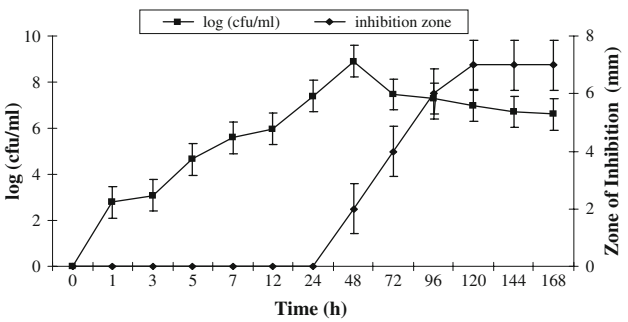

Fig. 1 Growth and antifungal metabolite production by *P. lentimorbus* WJ5 (The experiment was repeated twice ($n = 2$) with triplicates ($r = 3$))

bacterial culture was in its stationary phase (Fig. 1). Crude antifungal metabolite was extracted with *n*-butanol. Acetone-precipitated extracellular protein and other organic solvent extracts (chloroform and ethyl acetate) did not show any antifungal activity. Crude metabolites were resolved into maximum number of bands on silica gel thin layer chromatography with mobile phase of chloroform and methanol (10:1). Each band was eluted and bioassayed against *A. alternata.* The band with antifungal activity was eluted preparatively and used for size exclusion chromatography. Out of 200 fractions collected from size exclusion chromatography, only 12 fractions (from 10 to 21) showed an antagonistic activity against *A. alternata*. The active fractions were pooled, concentrated to dryness and used for further characterization.

Stability and characterization of antifungal metabolite

The stability of antifungal metabolite in the crude extract to physical and chemical stresses was examined. Activity was stable after autoclaving the crude extract at 121°C up to 3 h after which further exposure reduced the activity. No loss of activity was observed after exposure to lower temperatures ranging from −80 to 60°C for 6 h. The metabolite was found to be stable to the action of acidic and alkaline pH and other chemical agents tested (Table 2). However, cationic hexadecyltrimethylammonium bromide (CTAB) and lysozyme inactivated the metabolites. The kinds of functional groups and chemical bonds present in the antifungal metabolite were analysed with FT-IR spectroscopy. Major peaks were recorded with wave numbers of 3415, 2925, 1655 and 1547 cm^{-1} (Fig. 2). The possible functional groups or bonds assigned to the peaks at different wave numbers are listed in Table 3. Acid-hydrolysed antifungal metabolite did not inhibit the hyphal growth of *A. alternata*. It produced a brown spot on thin layer chromatography when developed with *n*-butanol–acetic acid–water (3:3:1) followed by ninhydrin spray. Amino acid analysis indicated the presence of 13 different amino acids with molar ratio as described in Table 4. The antifungal metabolite was not separated into distinct bands in SDS-PAGE analysis. It produced a streak in the polyacrylamide gel. Antifungal activity was detected on streaked portion of the gel (Fig. 3).

Table 2 Effect of physical and chemical stresses on the activity of antifungal metabolite

Agents/condition	Incubation temperature (°C)	Antagonistic activity
None added	RT	Yes
High temperature	121 for 3 h	Yes
High temperature	121 for 5 h	Reduced activity
High temperature	60 for 6 h	Yes
Low temperature	−80	Yes
pH 2 (10 mM HCl)	RT	Yes
pH 13 (100 mM NaOH)	RT	Yes
Glycerol (30%)	RT	Yes
Tween-80 (1%)	RT	Yes
SDS (1%)	RT	Yes
CTAB (1%)	RT	No
Proteinase K (1 mg/ml)	37	Yes
Lysozyme (1 mg/ml)	RT	No

RT, room temperature; experiment was repeated three times ($n = 3$) with five replications ($r = 5$)

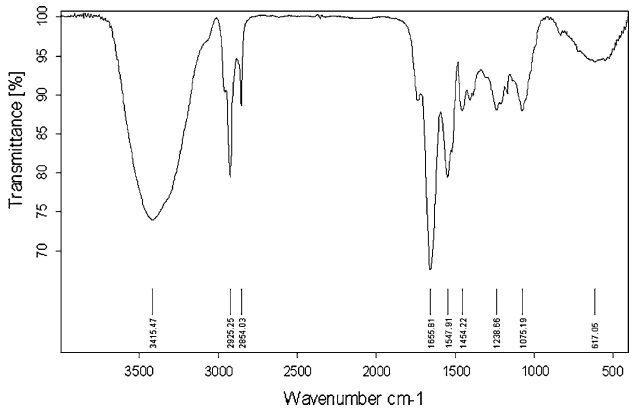

Fig. 2 Fourier transform infrared (FT-IR) spectrum of antifungal metabolites

Table 3 Fourier transform infra red (FT-IR) spectroscopy absorption bands of antifungal metabolite from *P. lentimorbus* WJ5

Frequency (cm^{-1})	Possible assignment[a]
3415	NH_2 stretching, H-bonded OH groups
2925	Aliphatic C–H stretching (fatty acids)
2854	Acyl group
1660–1535	NH_2 bending, C=O, C=N stretching
1454	C–H deformations in aliphatics
1238	Aromatic alcohols
1075	C–O, C–C stretching, C–O–H, C–O–C deformation (glycopeptide, ribose, polysaccharides)

[a] Assignment according to Bizani et al. 2005 and Motta et al. 2007

Discussion

Paenibacillus lentimorbus is well known for its insecticidal properties, however only a few reports are available on its antifungal traits. Our experimentation indicated that the antifungal activity of *P. lentimorbus* WJ5 could be recovered from the culture supernatant. The kinetics of antibiotic

Table 4 Amino acid composition of antifungal protein

Amino acid	Molar ratio (Glycine = 1.0)
Aspartic acid	9.45
Threonine	18.16
Serine	61.44
Proline	8.45
Glycine	1.0
Alanine	10.30
Valine	24.78
Methionine	1.71
Isoleucine	16.47
Leucine	31.83
Tyrosine	41.84
Histidine	14.41
Lysine	13.93

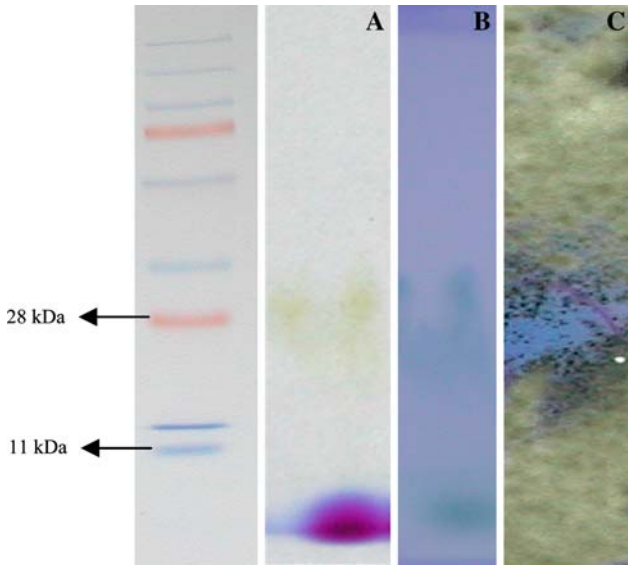

Fig. 3 Gel electrophoretic analysis of antifungal metabolite. Antifungal metabolite subjected to SDS-PAGE (**a**) followed by Coomassie blue staining (**b**) and tested for antifungal activity against *A. alternata* (**c**)

production were similar to that of secondary metabolites, as the antifungal activity was detected only during the onset of stationary phase i.e. 48 h post inoculation. No loss of activity up to 7 days in batch culture was observed (Fig. 1). Since maximum antifungal activity was detected at 120-h-grown culture supernatant, this period was considered as minimum incubation time required for maximum production of antifungal compounds under batch culture of *P. lentimorbus* WJ5. The physicochemical properties of the antifungal metabolites were studied. Antifungal metabolite was found to be highly thermostable and active over a wide range of pH value. These properties proved the biotechnological potential of antifungal metabolite from *P. lentimorbus* WJ5. Failure of protein-denaturing detergents and proteinase K to inactivate the antifungal metabolites of *Bacillus* is not unusual (Lebbadi et al. 1994; Potera 1994). The cyclic structure of the peptide with C, N terminally blocked or the presence of unusual amino acids are responsible for the resistant nature of the antibiotic peptides to proteolytic enzymes. The cyclic nature also renders them relatively inflexible and makes the cleavage site inaccessible because of steric hindrance (Eckart 1994).

Solid evidence for the presence of peptide bonds in the structure of the antifungal metabolite was recorded by FT-IR spectrum analysis. It showed the typical bands (1655 cm^{-1}) corresponding to N–H stretching of proteins and peptide bond. In addition, the bands at 2925 and 2854 cm^{-1} corresponding to aliphatic C–H stretching and acyl group respectively may also be related to the predominance of hydrophobic amino acids (Motta et al. 2007). Loss of activity during acid hydrolysis with 6 M HCl also proved the presence of amino acids in their chemical composition. Inactivation of antifungal metabolites by the cationic detergent CTAB indicated the anionic nature of metabolite or its functional groups that are essential for antifungal activity. Loss of activity due to lysozyme indicated the presence of sugar molecules that are attached to peptide through glycosidic bond. Presence of a glycosidic bond/sugar moiety was supported by the peaks at the wave number of 1075 cm^{-1} produced by FT-IR spectroscopy. The abnormal behavior of antifungal metabolites during polyacrylamide gel electrophoresis may be due to the hydrophobic nature of the metabolite (Motta et al. 2007). The hydrophobic nature of peptide antibiotics is due to the presence of hydrophobic amino acids in high proportion or the presence of small acyl chains (Beatty and Jensen 2002). In conclusion, our results suggest the peptide nature of the antifungal metabolite(s) produced by *P. lentimorbus* WJ5.

Acknowledgements This study was carried out under the Nuclear R&D Program of the Ministry of Science and Technology (MOST), Republic of Korea.

References

Beatty PH, Jensen SE (2002) *Paenibacillus polymyxa* produced fusaricidin-type antifungal antibiotics active against *Leptosphaeria maculans*, the causative agents of blackleg disease of canola. Can J Microbiol 48:159–169. doi:10.1139/w02-002

Besson F, Michel G (1990) Mycosubtilin B and C: minor antibiotics from mycosubtilin producing *Bacillus subtilis*. Microbiol 62: 93–99

Besson F, Peypoux F, Michel G, Delcombe L (1977) The structure of bacillomycin L, an antibiotic from *Bacillus subtilis*. Eur J Biochem 77:61–67. doi:10.1111/j.1432-1033.1977.tb11641.x

Bizani D, Motta AS, Morrissy JAC, Terra RMS, Souto AA, Brandelli A (2005) Antibacterial activity of cerenin 8A, a bacteriocin–like peptide produced by *Bacillus cereus*. Int Microbiol 8:125–131

Budi SW, van Tuinen D, Martinotti G, Gianinazzi S (1999) Isolation from the *Sorghum bicolor* mycorhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soil-borne fungal pathogens. Appl Environ Microbiol 65:5148–5150

Chen W, Morgan DP, Felts D, Michailides TJ (2003) Antagonism of *Paenibacillus lentimorbus* to *Botryosphaeria dothidea* and biological control of panicle and shoot blight of pistachio. Plant Dis 87:359–365. doi:10.1094/PDIS.2003.87.4.359

Dasgupta SM, Khan N, Nautiyal CS (2006) Biological control ability of plant growth promoting *Paenibacillus lentimorbus* NRRL B-30488 isolated from milk. Curr Microbiol 53:502–505. doi:10.1007/s00284-006-0261-9

Eckart K (1994) Mass spectrometry of cyclic peptides. Mass Spectrom Rev 13:23–55. doi:10.1002/mas.1280130104

Isogai AS, Takayama S, Murakoshi S, Suzuki A (1982) Structure of B-amino acids in antibiotics iturin A. Tetrahedron Lett 23:3065–3068. doi:10.1016/S0040-4039(00)87534-6

Lebbadi M, Galvez A, Maqueda M, Martinez-Bueno M, Valdivia E (1994) Fungicin M-4: a narrow spectrum peptide antibiotic from *Bacillus licheniformis* M-4. J Appl Bacteriol 77:49–53

Lee YK, Kim JS, Jang BI, Jang YS, Lee HY (2003) Biological control of *Pythium* root rot by radiation induced mutant of *Bacillus lentimorbus* WJ5a17. Korean J Environ Biol 21:276–285

Leifert C, Li H, Chidburee S, Hampson S, Workman S, Sigee D et al (1995) Antibiotic production and biocontrol activity by *Bacillus subtilis* and *Bacillus pumilus* CL45. J Appl Bacteriol 78:97–108

Lemanceau P, Alabouvette C (1991) Biological control of fusarium diseases by fluorescent *Pseudomonas* and non-pathogenic *Fusarium*. Crop Prot 10:279–286. doi:10.1016/0261-2194(91)90006-D

Majumdar SK, Bose SK (1958) Mycobacillin, a new antifungal antibiotic produced by *Bacillus subtilis*. Eur J Biochem 63:391–398

Motta AS, Lorenzini DM, Brandelli A (2007) Purification and partial characterization of an antimicrobial peptide produced by novel *Bacillus* sp. isolated from the Amazon Basin. Curr Microbiol 54:282–286. doi:10.1007/s00284-006-0414-x

Nautiyal CS, Metha S, Singh HB (2006) Biological control and plant growth-promoting *Paenibacillus* strains from milk. J Microbiol Biotechnol 16:184–192. doi:10.1159/000094830

Potera C (1994) From bacteria: a new weapon against fungal infection. Science 265:605. doi:10.1126/science.8036509

Pusey PL, Wilson CL (1984) Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. Plant Dis 68:753–756. doi:10.1094/PD-69-753

Sarfo K, Moorhead GBG, Turner RJ (2003) A novel procedure for separating small peptides on polyacrylamide gels. Lett Pept Sci 10:127–133

Selim S, Negrel J, Govaerts C, Gianinazzi S, van Tuinen D (2005) Isolation and partial characterization of antagonistic peptides produced by *Paenibacillus* sp. strain B2 isolated from the sorghum mycorhizosphere. Appl Environ Microbiol 71:6501–6507. doi:10.1128/AEM.71.11.6501-6507.2005

Smith KP, Havey MJ, Handelsman J (1993) Suppression of cottony leak of cucumber with *Bacillus cereus* strain UW85. Plant Dis 77:139–142