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Full Length Research Paper

Antifungal peptide produced by *Paenibacillus polymyxa BRF-1 isolated from soybean rhizosphere*

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A bacterial strain BRF-1 that produced antifungal peptide was isolated from soybean rhizosphere and identified as *Paenibacillus polymyxa* by combination approaches of morphological and physiological tests, Biolog identification and 16S rDNA sequencing. Its cell free metabolite can inhabit many plant pathogenic fungi growth and its activity was stable to proteinase K digestion, autoclave and lower pH treatments. The antifungal substance was separated by $(NH_4)_2SO_4$ and purified by Sephadex G-50, and acquired short peptide with molecular weight about 35.4 kDa, which has antifungal activity against *Rhizoctonia solani*.

Key words: *Paenibacillus polymyxa*, Biolog identification, 16S rDNA, antifungal activity, peptide.

INTRODUCTION

Biological control or biocontrol, the use of living organisms or its metabolite to eliminate or to control the growth of plant pathogens, has received considerable attentions over the last decade (Weid et al., 2003). Biocontrol seems to be a reliable alternative to chemical pesticides, which have raised serious concerns of food contamination, environmental pollution, and non-target effects, or development of resistance in pathogen population (Lorentz et al., 2006). Biocontrol is an eco-friendly, safe and may provide long-term protection for crop growth. Some bacterial species can serve as excellent biocontrol agents against plant pathogens, among them *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*), a gram-positive spore-forming bacterium, widely distributed in the various environments (Pirttijarvi et al., 1996), produce antimicrobial substances such as polymyxin plant hormones (Lebuhn et al., 1997; Timmusk et al., 1999) and extracellular enzymes (Meehan et al., 2001), is commonly regarded as potential biocontrol agent in many reports (Kajimura et al., 1997; Shi et al., 2009; Thomashow, 1996).

In China, soybean (*Glycine max*) is mainly distributed in northeast region, that is Heilongjiang, Jilin, and Liaoning provinces, among which Heilongjiang accounts for more than 50% of soybean planting areas. Thus the continuous cropping of soybean causes several serious problems such as the decline of seed quality, yield reduction etc. Previous researches showed that reasons for yielding reduction in continuous cropping of soybean were very complex (Liu et al. 2002). One of major reasons is soybean root rot, which is caused by many kinds of fungal pathogens such as *Fusarium oxysporium* var. *redolens*, *Fusarium avenaceum*, *Fusarium solani*, *Pythium utltimum*, *Rhizoctonia solani*, and *Phytophthora* f.sp. *glycinea* (Sun et al., 2005; Tai et al., 2004) occurred severely in the fields of continuous cropping of soybean. To solve this problem, various disease managements including biocontrol have been tested recently (Sturz et al., 2003).

Bacterial strain of BRF-1 was isolated from soybean rhizosphere (Wang et al., 2003). It has been well documented to control soybean root rot disease (Wang et al., 2004). The objective of this study was (1) to identify

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Table 1. Antifungal activity spectrum of strain BRF-1 against different pathogenic fungi in PDA plate.

Pathogenic fungi	Radius of inhibited zone (mm)
Sclerotinia sclerotorum	15.5±0.8
Rhizoctonia solani	14.0±3.7
Fusarium oxysporum	18.5±2.0
Bipolaris sorokiniana	17.2±1.2
Fusarium graminearum	13.2±1.8
Phytopathora capsici	19.5±3.6
Alternaria alternata	17.6±2.5

the strain BRF-1, and (2) to characterize and define what kind of antifungal substances produced by strain BRF-1.

MATERIALS AND METHODS

Bacterial strain isolation

Bacterial strain BRF-1 was isolated from soybean rhizosphere as described in previous report (Wang et al., 2003). The strain was maintained on PDA (Potato Dextrose Agar) slant tube at 4℃ before used.

Antifungal activity assay

Antifungal activity assay was tested by dual culture technique (Fang, 1988). Briefly, three drops of 5 µL suspension of BRF-1 (1×10^8 cfu/mL) were equidistantly placed on the margins of PDA plates and incubated at 28℃ for 24 h. A 5 mm agar disc of different fungal strains from fresh PDA medium (listed in Table 1) was placed at the center of the plate. The plates were incubated at 28℃ for 3 - 5 days. After the incubation, suppression of fungal growth was measured as the distance of the clear zone between the bacterial colony and each fungus.

Bacterial identification

Bacterial identification was based on the combination methods of morphological and physiological characteristics, Biolog identification and 16S rDNA sequencing. Morphological and physiological tests were carried out as described elsewhere (Dong and Cai, 2001). Biolog identification was carried out according to the protocols described by Biolog Identify System (Biolog, Inc., CA, USA) using a GP microplate. The 16S rDNA sequence of BRF-1 was amplified by PCR with the primers 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGC TAC CTT GTT ACG ACT T-3') (Muyzer et al., 1993), and submitted to a commercial sequencing company (Shanghai Yingjun Biotec, China) for sequencing. Phylogenetic data were obtained by alignment of the different 16S rDNA sequences retrieved from the BLAST algorithm (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]), using the software CLUSTAL W version 1.8 (Thompson et al., 1994) with the standard parameters. The alignments were visually corrected when necessary. Phylogenetic and molecular evolutionary analysis was conducted using MEGA, version 3.0 (Kumar et al., 2004).

Antifungal substance preparation and activity assay

To produce antifungal antibiotic for characterization, strain BRF-1 was cultivated in 100 mL of PD liquid medium at 28℃ in a rotary shaker at 150 rpm for 80 h. The cells were precipitated by centrifugation at 10000 rpm for 20 min and the supernatant was harvested and filtered though 0.22 µm membranes. Antifungal activity was detected by agar diffusion method by adding 100 µL of supernatant filtrate (Fang, 1988), and measured by the diameter of halo when *R. solani* used as the indicator strain.

Antifungal substance stability

The metabolite filtrate was sub-sampled (100 µL) to test its sensitivity to different treatments. For proteolytic enzymes assay, the filtrate was treated with proteinase K at 37℃ for 90 min. For thermal stability, samples of filtrate were heated to temperatures of 60℃ and 100℃ for 15 min, and 121℃, 1.4 Pa/cm^2 for 30 min (autoclave), respectively. For pH stability, samples of filtrate were adjusted to pH values of 3, 5, 7, 9 and 11 with HCl or NaOH solution, respectively. After then, a 70 µL treated sample was tested for antifungal activity against indicator *R. solani* by agar diffusion method.

Fractional precipitation of antifungal substance by ammonium sulfate

Ammonium sulfate powder was slowly added to the 100 mL filtrate to reach the salt concentration at 20% saturation. The precipitated proteins were collected by centrifugation at 10000 rpm for 20 min at 4℃. The pellet was resuspended in 1 mL of 20 mmoL/L phosphate buffer solution (PBS) (pH 6.2), and the supernatant was added ammonium sulfate to reach 40%, 60%, and 80% salt saturation, respectively. The precipitated proteins at different salt saturation were obtained as described above. Fractional precipitation of proteins were dissolved with PBS, and dialyzed with the same buffer over night. Then the fractional protein solutions and the final supernatant were used for antifungal activity assay.

Purification protein though Sephadex G-50

Crude antifungal protein was precipitated by 40% ammonium sulfate salt saturation, and its concentration was determined by Bradford method before purified by Sephadex G-50 (Bradford, 1976). Sephadex G-50 column was eluted by 20 mmoL/L PBS (pH6.2), until baseline of OD_{280}. A 0.5 mL of crude protein solution containing 17 mg protein was put into Sephadex G-50 column and eluted with the same PBS buffer. The fractions were collected according to the curve and used for antifungal activity assay.

Molecular weight determined by SDS-PAGE

The peptide molecular weight was estimated by SDS-PAGE (Laemmli, 1970). The samples were put into 4% (stacking gel) and 12% (resolving gel) polyacrylamide gels and electrophorezed at 200 V about 3 h, then the gel was dismantled and stained with 0.2% Coomassie brilliant blue R-250 in methanol-acetic acid-water (50:10:40) solution for 1 h, and destained in the same solvent.

RESULTS

In vitro antifungal spectrum of BRF-1

The growth of all fungal pathogen strains tested in this

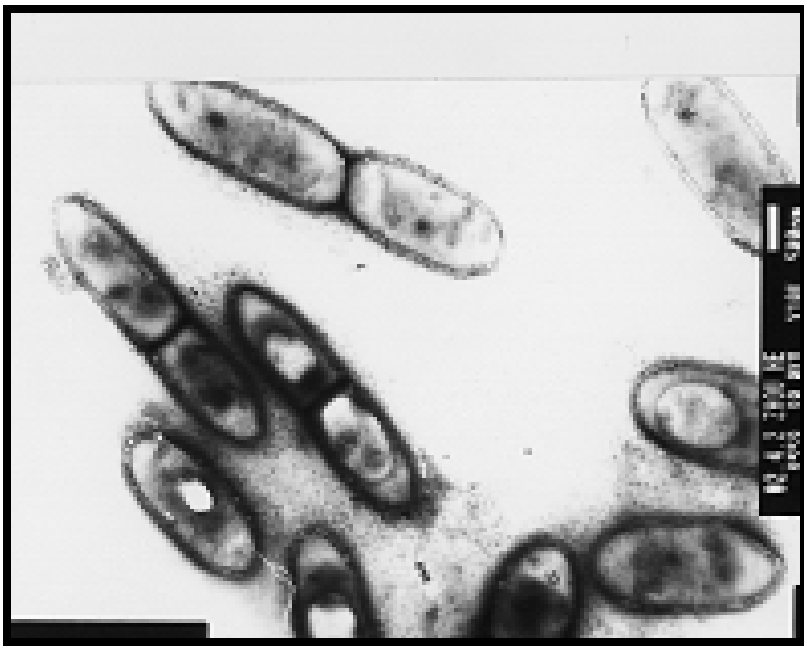

Figure 1. Electron micrograph of BRF-1 (×10000).

study was inhibited by strain BRF-1 (Table 1). An inhibited zone was clearly observed between colony of BRF-1 and fungal hyphae. The color of fungal hyphae on the edge of inhibited zone becomes dark with the incubation time. This result indicates that the metabolites of BRF-1 have high antifungal activity against many kinds of plant pathogenic fungi.

BRF-1 identification

Cells of BRF-1 were gram-positive and rod-shaped without flagellum. Cell length is 1.8 to 2.2 μm and its width is 0.6 to 0.8 μm. An ellipsoidal endospore was produced in the middle of cells (Figure 1). This bacterium was aerobic, had positive reaction of catalase, but had no activity of starch hydrolysis and nitrate reduction, had the ability to liquefy gelatin, and can survive at temperature 85℃ for 30 min, but can't grow in the presence of 7% NaCl. Biolog identification result showed that it had the similarity of 0.930 and 0.486 with *P. polymyxa* at incubation time of 6 and 18 h respectively.

Part of 16S rDNA of BRF-1 was amplified and the amplicon of 1433 bp was sequenced and submitted to the GenBank under the accession number of DQ298737. Cluster analysis obtained by phylogenetic tree showed that this strain was taxonomically very close to *P. polymyxa* (Figure 2), having more than 98% sequence similarity with *P. polymyxa*. Based on the morphological and physiological characteristics, Biolog identification and 16S rDNA sequence, strain BRF-1 was identified as *P. polymyxa*.

Antifungal substance stability

Antifungal activity test showed that the metabolite filtrate had strongly inhibited the growth of indicator pathogen *R. solani* (Figure 3). Proteolytic enzymes assay indicated that the antifungal activity of metabolite was not sensitive to proteinase K.

To evaluate the stability of antifungal substance at different pH, the supernatant filtrate was adjusted to pH values between 3 and 11, and its antifungal activity was measured against *R. solani*.

Results showed that its antifungal activity decreased in the pH range of 3.0 to 9.0, and lost activity at pH 11 (Figure 4).

The heat sensitivity of the antifungal substance was determined by measuring its filtrate activity after different temperature treatments. Results indicated that the antifungal activity was very stable to high temperature treatments, and even after the autoclaved treatment (Figure 5).

Purified antifungal peptide

Strain BRF-1 produces antifungal substance into cultural medium. In broth culture, the antifungal substance in metabolite can be precipitated by ammonium sulfate. Fractional precipitation by different saturation of ammonium sulfate showed that the antifungal activity was determined in 0% to 20% and 20% to 40% ammonium sulfate saturation, but not in 40% to 60%, 60% to 80% saturation and final supernatant. Therefore, antifungal

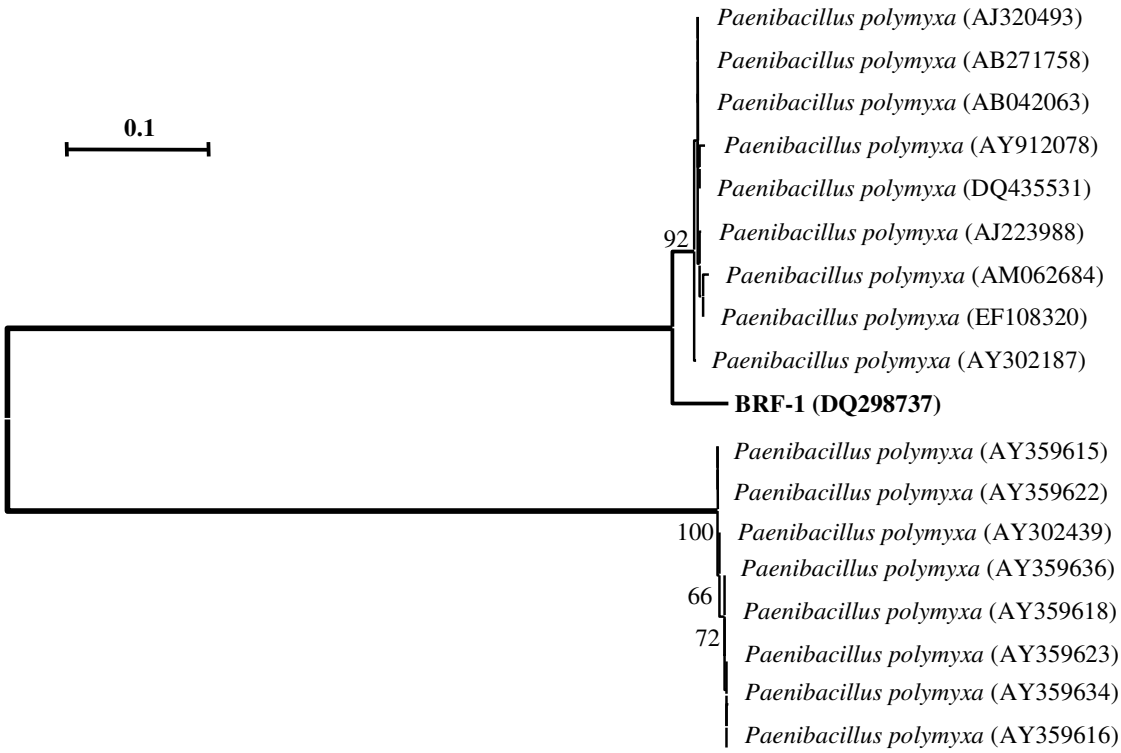

Figure 2. Phylogentic tree obtained by neighbour-joining analysis of BRF-1 based on 16S rDNA sequences. Numbers in parentheses represent the sequences accession number in GenBank. The number at each branch points is the percentage supported by bootstrap with 1000 replications.

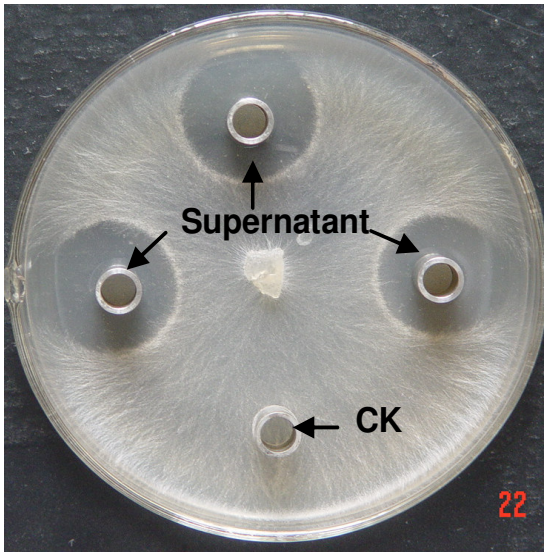

Figure 3. Antifungal activity of metabolite filtrate of strain BRF-1 against *Rhizoctonia solani*.

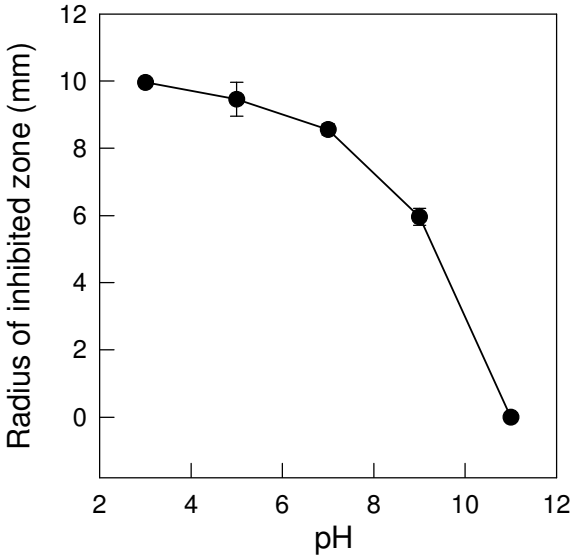

Figure 4. Effect of different pH on antifungal activity of metabolite filtrate of strain BRF-1 against *Rhizoctonia solani*.

substance produced by strain BRF-1 was assumed to be peptide. After gel filtration on Sephadex G-50 column of the crude antifungal peptide, two main components in fractions of crude extract were observed (Figure 6).

Furthermore, agar diffusion assay showed that the peak II had antifungal activity.

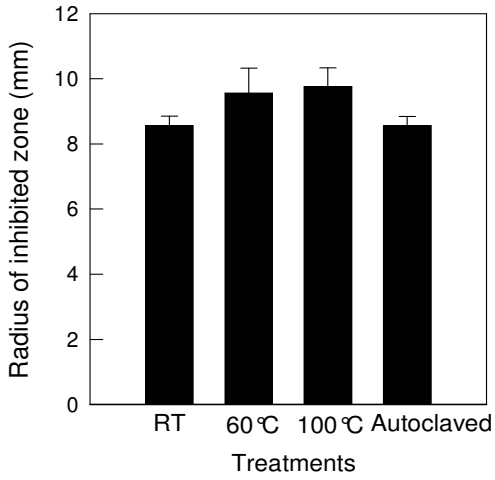

Figure 5. Effect of temperature treatments on antifungal activity of metabolite filtrate of strain BRF-1 against *Rhizoctonia solani*. RT means room temperature.

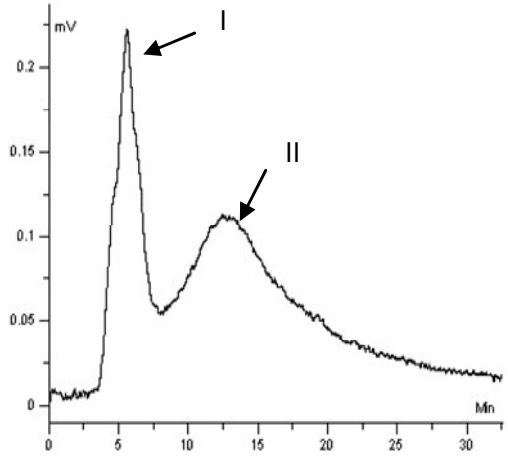

Figure 6. Chromatography of crude antifungal substances purified by Sephadex G-50.

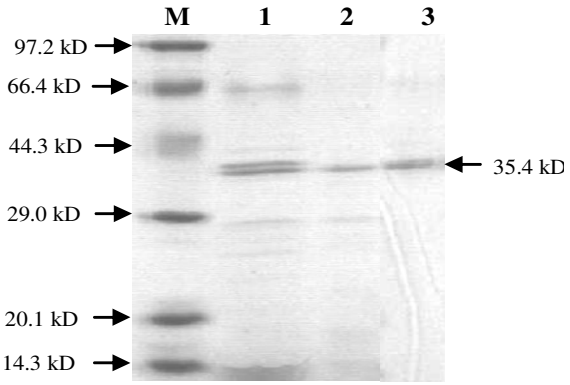

Figure 7. Gel electrophoresis of the protein substances produced by strain BRF-1. M, Protein maker; Lane 1, Metabolite filtrate; Lane 2, 40% $(NH_4)_2SO_4$ precipitated protein; Lane 3, Peak II protein.

Molecular weight

Strain BRF-1 secrets a complex profile of polypeptides with molecular weight ranging from 14 to 60 kDa into cultural medium (Figure 7 lane 1). The crude antifungal peptides obtained by 40% saturation of ammonium sulfate had at least two major distinct fractions with molecular weight 28.7 and 35.4 kDa (Figure 7 lane 2), and the active fraction purified by Sephadex G-50 peak II showed a single band with a molecular weight of 35.4 kDa (Figure 7 lane 3).

DISCUSSION

In the present investigation, bacterial strain BRF-1 isolated from soybean rhizosphere were subjected to antifungal assay, and dual culture experimental results indicated that strain BRF-1 inhibited a broad spectrum of plant pathogen fungal growth (Table 1), suggesting strain BRF-1 could be a biocontrol agent not only for control soybean root rot, and also for control other plant fungal diseases.

The genus *Paenibacillus* was created to harbour a phylogenetically coherent group of aerobic or facultatively anaerobic endospore-forming bacilli on the basis of 16S rRNA analysis (Ash et al., 1994). Some members of this genus, including *P. polymyxa*, *Paenibacillus thiaminolyticus*, and *Paenibacillus koreensis*, are known to produce antibacterial or antifungal compounds (Slepecky and Hemphill, 1991; Chung et al., 2000; Lorentz et al., 2006), and are commonly used as biocontrol agents. In this research, strain BRF-1 was identified as *P. polymyxa* based on morphology, physiology, Biolog system identification and 16S rDNA sequences analyses. Its characteristic of alive at 85℃ for 30 min indicates that it has an advantage in biocontrol agents processes such as pelleting or granulating.

Previous reports have shown *P. polymyxa* produces many kinds of antagonistic substances. Ryu et al. (2006) reported that *P. polymyxa* E681 produces several kinds of antibiotics including polymyxin, fusaric acid and polyketides. Beatty and Jensen (2002) revealed that fusaricidin-type antifungal antibiotics were produced by *P. polymyxa*. Karpunina et al. (2003) indicated that *P. polymyxa* 1460 produced lectins, which enhance cellulose degradation in the plant cell and increase β-glucosidase activity in wheat root cell wall. Although many kinds of antagonistic metabolites were produced by *P. polymyxa*, the most common antimicrobial substances are peptides. Beatty and Jensen (2002) found that *P. polymyxa* secretes different length of antimicrobial peptides with molecular weight of 883, 897, 948 and 961 Da separately.

Yao et al. (2004) isolated antifungal protein P_2 from strain *P. polymyxa* WY110 with molecular weight 26 kDa. In this study, the molecular weight of antifungal peptide produced by strain BRF-1 was about 35.4 kDa (Figure 7), which was larger than former reports. Whether the peptide of strain BRF-1 is a novel antifungal substance need further. Although only proteinase K was used to analyze the influence of proteolytic enzymes on the antifungal activity of peptide produced by strain BRF-1, no reduction of activity was observed after treatment. Previous researches showed that cyclic peptides can be resistant to hydrolysis by proteases because their cyclic structure renders them relatively inflexible, which may make cleavage sits inaccessible (Eckart, 1994; Weid et al., 2003). Therefore, the special structure of the antifungal peptide of strain BRF-1 was speculated to be a cycle form.

The antimicrobial activity of peptides resistance to pH and temperature was often found in researches on *Bacillus* or *Paenibacillus* (von Döhren, 1995). Lisboa et al. (2006) reported a peptide with molecular weight 5 kDa produced by *Bacillus amyloliquefaciens*, and its activity was stable over a wide range of temperatures, but lost activity when temperature reached 121°C for 15 min. Furthermore, its maximum activity was observed at neutral pH (6.0 to 7.0), and lost at alkaline pH.

In this study, the influence of pH on antifungal activity of peptide produced by strain BRF-1 was similar with that of *B. amyloliquefaciens* (Figure 4). But for temperature treatment, the activity of peptide produced by strain BRF-1 was more stable than that of *B. amyloliquefaciens*, its antifungal activity was not changed, even after autoclaved treatment (Figure 5). Those characteristics of peptide produced by strain BRF-1 imply that this peptide might play an important role in biocontrol of plant diseases or in food protection.

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REFERENCES

Ash C, Priest FC, Collins MD (1994). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Anto Van Leeuwenhoek, 64: 253-260.

Beatty PH, Jensen SE (2002). *Paenibacillus polymyxa* produces fusaricidin-type antifungal antibiotics active against of blackleg disease of canola. Can. J. Microbiol., 48: 159-169.

Bradford MM (1976). A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Chung YR, Kim CH, Wang IH, Chun JS (2000). *Paenibacillus koreensis* sp. Nov., a new species that produces an iturin-like antifungal compound. Int. J. Syst. Evol. Microbiol., 50: 1495-1500.

Dong XZ, Cai MY (2001). Identification manual of common bacteria. Beijing: Science and Technology Press, pp. 75-150 (in Chinese).

Eckart K (1994). Mass spectrometry of cyclic peptides. Mass Spectrom. Rev., 13: 23-55.

Fang ZD (1988). Research methods for plant disease. 3rd edition. Beijing: Chinese Agriculture Press, pp 248-249 (in Chinese).

Kajimura Y, Kaneda M (1997). Fusaricidins B, C and D, new depsipeptide antibiotics produced by *Bacillus polymyxa* KT-8: isolation, structure elucidation and biological activity. Jpn. J. Antibiot., 50: 220-228.

Karpunina LV, Mel'nikova UY, Konnova SA (2003). Biological role of lectins from the nitrogen-fixing *paenibacillus polymyxa* strain 1460 during bacterial-plant-root interactions. Curr. Microbiol., 47: 376-378.

Kumar S, Tamura K, Nei M (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform., 5: 150-163.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriaophage T4. Nature, 227: 680-685.

Lebuhn M, Heulin T, Hartmann A (1997). Production of auxin and other indolic and phenolic compounds by *Paenibacillus polymyxa* strains isolated from different proximity to plant roots. FEMS Microbiol. Ecol., 22: 325-334.

Lisboa MP, Bonatto D, Bizani D, Henriques JAP, Brandelli A (2006). Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic forest. Int. Microbiol., 9: 111-118.

Liu, XB, Herbert, SJ (2002). Fifteen years of research examining cultivation of continuous soybean in Northeast China. Field Crops Res., 79: 1-7.

Lorentz RH, Ártico S, Silveira AB, Einsfeld A, Corção G (2006). Evaluation of antimicrobial activity in *Paenibacillus* spp. strains isolated from natural environment. Lett. Appl. Microbiol., 43: 541-547.

Meehan C, Bjourson AJ, Mcmullan G (2001). *Paenibacillus azoreducens* sp. Nov., a synthetic azo dye decolorizing bacterium from industrial wastewater. Int. J. Syst. Evol. Microbiol., 51: 1681-1685.

Muyzer G, de Waal EC and Uitterlinden AC (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA. Appl. Environ. Microbiol., 59: 695-700.

Pirttijarvi TS, Graeffe TH, Salkinoja-Salonen MS (1996). Bacterial contaminants in liquid packaging boards: assessment of potential for food spoilage. J. Appl. Bacteriol., 81(4): 445-458.

Ryu CM, Kim J, Choi O, Kim SH, Park CS (2006), Improvement of biocontrol capacity of *Paenibacillus polymyxa* E681 by seed pelleting on sesame. Biol. Control., 39: 282-289.

Shi YW, Lou K, Li C, Yang L, Wang XQ, Liu WY(2009). Effects of endophytic *Paenibacillus polymyxa* S-7 on photosynthesis, yield, and quality of sugar beet, Chin. J. Appl. Ecol., 20(3): 597-602 (in Chinese).

Slepecky RA, Hemphill HE (1991). The genus Bacillus-nonmedical. In The Prokaryotes, Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder, Schleifer KH, New York: Springer, pp 1663-1696.

Sturz AV, Christie BR (2003). Beneficial microbial allelopathies in the root zone: The management of soil quality and plant disease with rhizobacteria, Soil Tillage Res., 2: 107-123.

Sun DM, Yang Q, Zhang JZ (2005). Antagonism of *Trichioderma aureoviride* mutant strain against *Fusarium* spp. the pathogen of soybean root rot. Soybean Sci., 3: 171-175 (in Chinese).

Tai LM, Xu YL (2004). Factors affecting on growth and toxin production of *Fusarium oxysporum*. Chinese J. Oil Crop Sci., 4: 71-74 (in Chinese).

Thompson JD, Hiffins DG, Gibson TJ (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22: 4673-4680.

Thomashow LS (1996). Biological control of plant root pathogens. Curr. Opin. Biotechnol., 7: 343-347.

Timmusk S, Nicander B, Granhall U, Tillberg E (1999). Cytokinin production by *Paenibacillus polymyxa*. Soil Biol. Biochem., 31: 1847-1852.

von Döhren H (1995). Peptides. Biotechnology, 28: 129-171.

Wang GH, Zhou KQ, Jin J, Pan XW, Liu XB, Luo YH (2004). Antagonism on organism BRF-1 against soybean root rot. Soybean Sci., 23: 188-191 (in Chinese).

Wang GH, Zhou KQ, Zhang QY, Wang JG (2003). *In vitro* the antifungal activity of bacterial antagonism strain BRF-1. Chin. J. Biol. Control, 19: 73-77 (in Chinese).

Weid IVD, Alviano DS, Santos AL, Soares RM, Alviano CS, Seldin L (2003). Antimicrobial activity of *Paenibacillus peoriae* strain NRRL BD-62 against a broad spectrum of phytopathogenic bacteria and fungi. J. Appl. Microbiol., 95: 1143-1151.

Yao WL, Wang YS, Han JG, Li LB, Song W (2004). Purification and cloning of an antifungal protein from the rice disease controlling bacterial strain *Paenibacillus polymyxa* WY110. Acta Genet. Sin., 31: 878-887 (in Chinese).