Antifungal Activity of *Paenibacillus kribbensis* Strain T-9 Isolated from Soils against Several Plant Pathogenic Fungi

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The bacterial strain T-9, which shows strong antifungal activity, is isolated from the soils of Samcheok, Gangwon-do and identified as *Paenibacillus kribbensis* according to morphological and taxonomic characteristics and 16S rRNA gene sequence analysis. The *P. kribbensis* strain T-9 strongly inhibits the growth of various phytopathogenic fungi including *Botrytis cinerea*, *Colletotricum acutatum*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Magnaporthe oryzae*, *Phytophthora capsici*, *Rhizoctonia solani*, and *Sclerotium cepivorum* *in vitro*. Also, the *P. kribbensis* strain T-9 exhibited similar or better control effects to plant diseases than in fungicide treatment through *in vivo* assays. In the 2-year greenhouse experiments, *P. kribbensis* strain T-9 was highly effective against clubroot. In the 2-year field trials, the *P. kribbensis* strain T-9 was less effective than the fungicide, but reduced clubroot on Chinese cabbage when compared to the control. The above-described results indicate that the strain T-9 may have the potential as an antagonist to control various phytopathogenic fungi.

Keywords : antifungal ability, *in vivo* assay, *Paenibacillus kribbensis*, plant pathogenic fungi, 16S rRNA gene

Recently, the use of synthetic fertilizers and pesticides have resulted in deterioration of soil structure and increase in soil-borne phytopathogens (Bailey and Lazarovits, 2003). As a result, along with many phytopathogenic fungi such as *B. cinerea*, *F. oxysporum*, *P. capsici*, *R. solani*, and *S. cepivorum* etc. having a wide range of host plants, it has been very difficult to control the spread of the plant disease. Although applying fungicide has been one of

the conventional methods for controlling fungal diseases, there is increasing international concern over the use of fungicides on crops because of their harmful effects on human health and the emergence of pathogen resistance to fungicides. To cope with this problem, various disease managements including biological control, have been applied.

Biological control by microorganisms (Huang et al., 2012; Kim et al., 2012; Li et al., 2012) has received considerable attention as a reliable substitute for the use of various hazardous chemical fungicides, which have raised serious concerns of food and environmental contamination (Fernando et al., 2005). Microorganisms also play an essential role in several life support functions as they enable soil to recycle nutrients, suppress plant pathogens (Mendes et al., 2011), and serve as a suitable substrate for plant growth (Kim et al., 2011). Among the myriads of bacteria in the plant rhizosphere, a few spore-forming plant growth promoting rhizobacteria (PGPR) attract special attention due to their stable habitancy in soil (Emmert and Handelsman, 1999; Jin et al., 2006).

Soil samples were collected from the Chinese cabbage, garlic, and paddy fields in 30 regions of Samcheok, Gangwon province, South Korea during November 2011. Each sample was air-dried at ambient temperature and then passed through an 850 µm mesh sieve to remove plant debris. Soil samples were preserved in polyethylene bags at room temperature before use. Soil suspensions were prepared in sterile water and cultured on the surface of potato dextrose agar medium (PDA, Difco, USA) and tryptic soy agar medium (TSA, Difco, USA). The plates were incubated at 25°C for 10 days. Afterwards, colonies with different morphological appearances were chosen from the countable plates and streaked on fresh similar medium to obtain pure cultures.

Bacterium was identified by morphological and taxonomic characterization and 16S rRNA gene sequencing assay. Morphological characteristics were examined on

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Fig. 1. Colony shape on PDA medium and spore morphological characteristics by SEM of strain T-9. Bar size indicates 5 μ m.

an SEM at an accelerating voltage of 10 kV. Taxonomic characterization of the strain was performed on the basis of test for oxidase, catalase, H_2S production, gelatin hydrolysis, starch hydrolysis, nitrate reduction, urase hydrolysis, casein digestion, arginine dihydrolase, ornithine decarboxylase, esculin hydrolysis and utilization of carbon such as glucose, sucrose, dulcitol, salicin, D-mannitol, D-adonitol, L-arabinose, D-xylose, and D-trehalose (Takagi et al., 1993). The results of these tests were scored as either positive or negative.

The total genomic DNA was extracted as described by Nishiguchi et al. (2002). The 16S rRNA gene was amplified by polymerase chain reaction using universal primers 27F and 1492R (Bioneer, Korea). The amplified DNA was purified using a gel purification kit (Bioneer, Korea) and was sequenced at Bioneer. Similarity searches of the sequence obtained was performed using the BLAST function of GenBank.

The predominant strain obtained from soil samples of Samcheok was named as T-9 and found to be a Gram-variable, rod-shaped, and facultative anaerobic bacterium. It produced flat colonies and unsmooth-surfaced spores (Fig. 1) and catalase (Table 1) although it did not produce H_2S . Methyl red reaction was positive while Voges-Proskauer reaction was negative. An array of carbon sources were utilized like glucose, lactose, sucrose, salicin, L-arabinose, raffinose, maltose, and cellobiose, but not dulcitol, D-adonitol, myo-inositol, and L-rhamnose. The 16S rRNA gene sequence analysis indicated that the closest relative (99%) of the strain T-9 (1465 bp) was *P. kribbensis* AM49 (AF391123) (Fig. 2). The 16S rRNA gene sequence of strain T-9 was deposited in the GenBank database with an accession number KF019740. From the results

of morphologic and biological characteristics, and 16S rRNA gene sequence analysis, the predominant strain T-9 obtained from soil samples was identified as *P. kribbensis* and thus named *P. kribbensis* strain T-9.

The genus *Paenibacillus*, originally included within the *Bacillus* and reclassified as a separate genus (Ash et al., 1993), is prevalently distributed in different soils and plant rhizospheres (Berge et al., 2002; von der Weid et al., 2002; Garbeva et al., 2003; Yoon et al., 2003). Different species of *Paenibacillus* have been demonstrated as having potential antifungal activities (Fortes et al., 2008; He et al., 2007; Liu et al., 2008; Raza et al. 2008; von der Weid et al., 2003; von der Weid et al., 2005). The *P. kribbensis* is recently described as a new *Paenibacillus* species isolated from soil (Yoon et al., 2003). Some strains produced antimicrobial substances, suggesting an important role in the soil. However, no biological role of this species in the rhizosphere have been reported.

The antifungal activity of *P. kribbensis* strain T-9 against the phytopathogenic fungi was performed by dual culture. Briefly, a mycelial column (6.5 mm in diameter) was cut off from the white perimeter portion of the colony, which had been cultured on PDA plates at 25°C for 7 days, and placed at a distance 1.5 cm away from the rim of a fresh PDA plate. Afterwards, the *P. kribbensis* strain T-9 growing in PDA was sowed in a hole with a sterilized toothpick on the PDA plate (1.5 cm away from the other rim). Simultaneously, control plates inoculated in different mycelial columns were prepared without *P. kribbensis* strain T-9. The antagonistic effect of the control plates was evaluated by measuring the size of the inhibition zone on the PDA plates following culturing at 25°C for 14 days.

The *P. kribbensis* strain T-9 showed strong antagonistic

Table 1. Differential characteristics of antagonistic strain T-9 and *Paenibacillus kribbensis*

Characteristic	Strain T-9	<i>P. kribbensis</i> ^a
Cell shape	rod	rod
Cell size (μm)	1.3–1.7×4.2–6.8	1.3–1.8×4.0–7.0
Oxygen requirement	facultative anaerobic	facultative anaerobic
Endospore formation	+ ^b	+
Gram stain	+/-	+/-
Nitrate reduction	+	+
Gelatin hydrolysis	+	+
Starch hydrolysis	+	+
Urea hydrolysis	–	–
Casein hydrolysis	+	+
Oxidase	–	–
Sole carbon and energy sources:		
L-Arabinose	+	+
D-Cellobiose	+	+
D-Glucose	+	+
Lactose	+	+
D-Mannose	+	+
Melibiose	+	+
D-Raffinose	+	+
D-Sorbitol	–	–
L-Rhamnose	–	+
D-Xylose	+	+
D-Trehalose	+	+
D-Mannitol	+	+
Adonitol	–	–
Maltose	+	+
Sucrose	+	+

^aData for *P. kribbensis* are from Yoon et al. (2003).^b+, Positive; –, Negative; +/-, Gram-variable.

activity against a wide range of phytopathogenic fungi that cause disease on different crops (Table 2). It produced the highest antifungal activity against *P. capsici*, which was cosmopolitan in distribution. Furthermore, there was a moderate antifungal activity against *B. cinerea*, *C. acutatum* and *M. oryzae*. Other test phytopathogenic fungi were also inhibited, but the antifungal activity was relatively less.

The *in vivo* efficiency of *P. kribbensis* strain T-9 for control of anthracnose in pepper fruits, Phytophthora blight in pepper plant, blast, sheath blight in rice plant and

gray mold in tomato plant, was evaluated in plastic sieve systems or under greenhouse conditions (Table 3). Pepper (*Capsicum annuum*) cv. Super manitta and Hongjinjoo, rice (*Oryza sativa*) cv. Nakdongbyeon and tomato (*Solanum lycopersicum*) cv. Super dotaerang were sown in horticultural soil in seedling plate. The plants were kept in the greenhouse for one month. To obtain culture broth, *P. kribbensis* strain T-9 was cultured in potato dextrose broth medium (PDB, Difco, USA) for three days, which included incubation in a rotary shaker at 200 rpm and 25°C. After incubation, *P. kribbensis* strain T-9 suspension was diluted in distilled water to 10⁸ cfu/ml (OD₆₂₀=0.1). Each seedling and fruit was treated with growth broth one day before inoculation with *B. cinerea*, *C. acutatum*, *M. oryzae*, *P. capsici*, and *R. solani*. For pepper anthracnose, each fruit was soaked in culture broth, water, or propineb for 30 min. For pepper Phytophthora blight, each seedling was dip-treated with culture broth, water, or dimethomorph for 10 min. Also culture broth, water, or fungicides were sprayed on each seedling for other plant diseases. Chemical fungicides were used as the positive control and water was used as the negative control. The inoculated plants were placed in a moist chamber to maintain approximately 90% humidity at 25°C for 24 h, and then transferred to the growth room for further incubation. There were three replicates for each treatment.

The *P. kribbensis* strain T-9 for control of Phytophthora blight in pepper plant, blast, sheath blight in rice plant and gray mold in tomato plant, was highly effective, reducing disease severity by 92.9%, 78.5%, 100%, and 89.8% relative to the inoculated control, respectively. The chemical fungicides were used as reference, which reduced disease severity by 99.3%, 74.3%, 100% and 93.2%, respectively. The *in vivo* efficacy of *P. kribbensis* strain T-9 against anthracnose in pepper fruits was evaluated in plastic sieve systems. The *P. kribbensis* strain T-9 was highly effective, which showed a control efficiency of 96.7%. The synthetic fungicide was used as reference, which showed a 92.3% control value.

The suppressive effects of *P. kribbensis* strain T-9 against *Plasmodiophora brassicae* in the greenhouse and the field were assessed in 2011 and 2012, respectively (Fig. 3). Chinese cabbage (*Brassica campestris*) cv. Gaeulmas, a highly susceptible to clubroot, was sown in horticultural soil in seedling plate. The plants were kept in the greenhouse for one month. To obtain culture broth, the method was same in upper. Frozen galls were soaked in distilled water for a while to soften the tissue, and then they were macerated in a homogenizer with distilled water at high speed. For inoculation, inoculate suspension was mixed

Fig. 2. Phylogenetic relationship between strain T-9 and representative species based on partial 16S rRNA gene sequence developed with the ClustalW program in MEGA 5.10 and constructed using the Neighbor-joining method with 1,000 bootstrap replicates. The values indicate the percentage of clustering matches. The scale bar indicates the number of differences in base composition among sequences.

Table 2. Antagonistic activity of strain T-9 against various phytopathogenic fungi in dual culture assay

Pathogen	KACC accession No. ^a	Inhibition zone (mm) ^b
<i>Botrytis cinerea</i>	KACC 40574	12.09 ± 1.74
<i>Colletotrichum acutatum</i>	KACC 43123	12.05 ± 0.63
<i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i>	KACC 40031	5.61 ± 0.63
<i>Magnaporthe oryzae</i>	KACC 40441	11.78 ± 0.61
<i>Phytophthora capsici</i>	KACC 40483	19.04 ± 2.71
<i>Rhizoctonia solani</i> (Chinese cabbage bottom rot)	KACC 40113	8.00 ± 0.29
<i>Rhizoctonia solani</i> (Rice sheath blight)	KACC 40103	6.48 ± 0.30
<i>Sclerotium cepivorum</i>	KACC 41234	8.97 ± 1.14

^aKACC: Korean Agricultural Culture Collection.

^bValues are mean ± SD of three independent observations.

into horticultural soil. In 2011 and 2012, Chinese cabbage was transplanted in a plastic pot containing the clubroot-infested horticultural soil. Each seedling was dipped into culture broth. Cyazofamid was used as the positive control and water as the negative control. The seedlings were watered regularly to keep constant soil moisture during cultivation in the greenhouse. After forty days, each root was rinsed with tap water and assessed for clubroot severity based on a 0–4 scale, where 0 indicated no infection; 1 indicated 1–25% of roots infected; 2 indicated 26–50% of roots infected; 3 indicated 51–75% of roots infected; and 4 indicated more than 75% of roots infected. Statistical analyses were conducted with the Statistical Analysis System for personal computers (Ver. 9.2, SAS Institute Inc.). Fisher's protected least significant difference

(LSD) at $P \leq 0.05$ was applied to determine whether or not differences between treatments were significant.

In 2011 and 2012, a study using Chinese cabbage was conducted to assess the efficacy of microbes in the field, based on the efficacy observed in a previous experiment. Inoculate suspension was mixed into field soil using a rotary cultivator. Each seedling was dipped in culture broth for 10 min. Seedlings were transplanted in the infected field and cyazofamid was used as the positive control and water as the negative control. The trials were arranged in a randomized complete block design with three replicates. The seedlings were watered regularly to keep constant soil moisture. After seventy days, each root was rinsed with tap water and assessed for clubroot severity based on the 0–4 scale mentioned above.

Table 3. The control effect of the strain T-9 against several plant pathogenic fungi in susceptible host plants

Plant diseases ^a	Treatments ^b	Disease severity (%) or disease spot extended length (mm) ^c	Control value (%)
Pepper anthracnose	Strain T-9	2.3 ± 1.8 b ^d	96.7 ± 1.8 A
	Propineb	5.3 ± 2.6 b	92.3 ± 2.5 A
	Control	68.3 ± 22.9 a	–
Pepper phytophthora blight	Strain T-9	4.0 ± 2.7 b	92.9 ± 6.4 A
	Dimethomorph	0.4 ± 0.3 b	99.3 ± 0.6 A
	Control	56.4 ± 28.5 a	–
Rice blast	Strain T-9	10.8 ± 3.8 b	78.5 ± 9.4 A
	Tricyclazole	13.3 ± 1.4 b	74.3 ± 4.2 A
	Control	51.7 ± 5.2 a	–
Rice sheath blight	Strain T-9	0.0 ± 0.0 b	100.0 ± 0.0 A
	Validamycin A	0.0 ± 0.0 b	100.0 ± 0.0 A
	Control	137.4 ± 19.5 a	–
Tomato gray mold	Strain T-9	6.7 ± 2.0 b	89.8 ± 2.0 A
	Fludioxonil	4.6 ± 3.6 b	93.2 ± 5.1 A
	Control	65.9 ± 5.1 a	–

^aFruits of pepper were inoculated by dropping 20 µl of *C. acutatum* conidia suspension (more than 10⁵ conidia/ml). Pepper seedlings were inoculated by drenching 20 ml of *P. capsici* zoospores suspension (more than 10⁴ sporangia/ml, chilling) near the seedling. Rice seedlings were inoculated by spraying *M. oryzae* spores suspension (more than 10⁵ spores/ml, 1% Tween 20) on foliage. Rice seedlings were inoculated by sticking PDA disk with *R. solani* on stem base. Tomato seedlings were inoculated by spraying *B. cinerea* spores suspension (more than 10⁵ spores/ml, PDB) on foliage.

^bFor pepper anthracnose, each fruit which surface was slightly scratched by a sandpaper was soaked in culture broth, water (control), or propineb for 30 min. For pepper phytophthora blight, each seedling was dip-treated with culture broth, water (control), or dimethomorph for 10 min. Culture broth, water (control), or fungicides were sprayed on each seedling for other plant diseases.

^cDisease spot extended length was calculated for rice sheath blight. A disease severity was calculated over all of the plants for the treatment of other plant diseases.

^dMeans and standard error based on three replicates sharing a common capital letter (control value) or lower case letter (disease severity or disease spot extended length) were not significantly different based on the protected LSD test at $P \leq 0.05$.

For greenhouse experiment, the *P. kribbensis* strain T-9 was highly effective against clubroot, which showed a control efficiency of 89.3% and 99.2% in 2011 and 2012, respectively. Cyazofamid was used as a reference, which showed 86.3% and 100% control values. For field trials, *P. kribbensis* strain T-9 had no substantial impact on clubroot severity, with 79.6% and 82.2% disease severity on the susceptible cultivar of Chinese cabbage, which reduced the disease severity by 11.7% and 12.6% in 2011 and 2012, respectively. The cyazofamid treatment reduced the disease severity by 24.9% and 85.8%.

The stratagem of controlling the biological properties of the *P. brassicae* has become an important approach for creating a long-lasting effect on the facilitation of sustainable agriculture; however, only a few antagonistic microorganisms have been reported, including *Micromonospora rosea* subsp. *rosea* and *Streptomyces* spp. (Kim et al., 2004; Lee et al., 2008; Wang et al., 2012), *Streptoverticillium olivoreticuli*

spp. (Kim et al., 2002), *Cellulosimicrobium cellulans*, and *Paenibacillus* spp. (Choi et al., 2007). The progress and applicable fields of biological control for clubroot are limited compared to other soil-borne plant diseases, and there are no commercial biological control products available. The continuous search for antagonistic agents against clubroot is necessary. In this study, the suppressive effects of *P. kribbensis* strain T-9 against *P. brassicae* were assessed in the greenhouse and the field in 2011 and 2012. We found the efficacy observed from the field trial to be lower than that of the greenhouse experiment. For the greenhouse experiment, *P. kribbensis* strain T-9 showed a good control efficiency of 89.3% and 99.2% in 2011 and 2012, respectively. However, for field trials, there was no substantial impact, but the treatment reduced the clubroot on the susceptible cultivar of Chinese cabbage, with disease severity, by 79.6% and 82.2% in 2011 and 2012, respectively. Basic environmental conditions, such

Fig. 3. Disease severity (A) and control value (B) of greenhouse experiment and field trails by strain T-9 and fungicide treatment to control clubroot caused by *P. brassicae* in 2011 and 2012. Plant roots were dip-treated with culture broth, water (control), or cyazofamid for 10 min. Means and standard error based on three replicates sharing a common letter were not significantly different based on the protected LSD test at $P \leq 0.05$.

as temperature, moisture, and soil physical and chemical characteristics, can greatly affect the interactions among plant, pathogen, and biocontrol agents in various ways, and all of these effects may influence the efficacy of biological control (Larkin and Fravel, 2002). On the other hand, enormous microorganisms exist in the rhizosphere of plants, which may influence the efficacy of biological control. Finally, endospore formation and longer generation times influence them to rapidly colonize roots, which they directly protect against soil-borne pathogens while they adapt their fitness to that of rhizosphere (Ryu et al., 2005).

In summary, this study is the first antagonistic activity assay of *P. kribbensis* strain T-9 against various soil-borne phytopathogenic fungi. It was found that the *P. kribbensis* strain T-9 has the potential to offer protection to plants against different soil-borne phytopathogenic fungi, in addition to its antagonistic activity, suggesting that further studies should be considered for using *P. kribbensis* strain T-9 as a biological control agent and enhancer of control efficiency in field trials.

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