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Effects of a Soil-Born *Paenibacillus* spp. Strain KPB3 on Suppression of Bacterial Wilt Disease Caused by *Ralstonia solanacearum*

Jung Ki Suk, Nagesh S. Ipper, Seon Hwa Lee, Anupama Shrestha, Duck Hwan Park, Jun Mo Cho[1],Jang Hyun Hur[2], Byung Sup Kim[3], and Chun Keun Lim*

Division of Bio-resources Technology, College of Agriculture and Life Sciences, Kangwon National University, Chuncheon 200-701, Korea,
[1]Pioneer Co. Ltd., Kangwon National University, Chuncheon 200-701, Korea,
[2]Division of Biological Environment, College of Agriculture and Life Sciences, Kangwon National University, Chuncheon 200-701, Korea,
[3]Department of Applied Plant Science, College of Life Science, Kangnung National University, Kangnung 210-702, Korea

Abstract : Two hundred bacterial strains were isolated from the soil around healthy tomato plants in a polyvinyl house, where most of the other plants showed bacterial wilt symptoms. The strains were screened *in vitro* for their antibacterial activity. Among them, a strain, KPB3 showed strong bactericidal activity against bacterial wilt pathogen, *Ralstonia solanacearum*. The strain KPB3 was identified using physiological and biochemical tests, and 16S rRNA analyses. Based on these tests, the strain was found to be closer to genus *Paenibacillus*. To control the bacterial wilt caused by *R. solanacearum*, greenhouse experiments were conducted to determine the effectiveness of the *Paenibacillus* strain KPB3. Drench application of this strain (4×10^8 CFU mL^{-1}) into the pots containing tomato plants, post-inoculated with the pathogen, *R. solanacearum* could drastically reduce the disease severity, compared to the non-treated plants. To evaluate effectiveness of this strain under field conditions, experiments were carried out in polyvinyl houses infested with *R. solanacearum*, during spring and autumn of the year 2006. It was observed that, during spring, bacterial wilt was more prevalent compared to the autumn. During spring, 50.9% disease incidences occurred in non-treated controls, while, *Paenibacillus* strain KPB3 treated plants showed 24.6% disease incidences. Similarly, during autumn, around 17.2% plants were infected with bacterial wilt in non- treated polyvinyl houses, compared to the *Paenibacillus* strain KPB3 treated plants, which showed 7.0% disease incidences. These results demonstrated that, *Paenibacillus* strain KPB3 is a potential biological control agent against bacterial wilt caused by *R. solanacearum*, effective under greenhouse as well as field conditions. This is the first report showing biocontrol of *R. solanacearum* using a *Paenibacillus* spp. under field conditions.
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Key words : *Ralstonia solanacearum*, bacterial wilt disease, *Paenibacillus*, biocontrol.

Introduction

Bacterial wilt, caused by *Ralstonia solanacearum* is a serious soilborne disease and a major constraint in the production of tomato, pepper, potato, tobacco, banana, eggplant and many other economically important plants in tropical, subtropical and warm temperate regions of the world (Hayward *et al.*, 1991). The bacterium infects host via wounds or natural openings through roots and colonizes stem vascular tissue, causing stunting or complete wilting resulting in poor fruit quality and yield loss. Diseased plants can be found scattered throughout the field infested with *R. solanacearum*, however it occurs most frequently in low lying areas accumulating

*Correspounding author
(Fax : +82-33-256-8254, E-mail : chunkeun@kangwon.ac.kr)

water (McGarvey *et al.*, 1999). The upper leaves show wilting symptoms prominently during hot days and recovers in the early mornings and evenings. The wilted leaves do not loose their green color during disease progresses. Under hot and humid conditions favorable for disease, plant shows complete wilting, leading to its death. The vascular tissues in the lower stem of the wilted plants often show a brown discoloration. *R. solanacearum* can be active for as many as five years without host, which makes it a difficult candidate to control. No conventional pesticides or soil fumigants like methyl bromide are effective against *R. solanacearum* (Hayward, 1991). The efficiency of conventional practices to control this disease is limited (Sung *et al.*, 2005). Various control strategies, including, Cultural practices (Vincent and Mew, 1998), integrated control (Katayama and Kimura, 1987), host resistance (Dalal *et al.*, 1999) and biological control have been developed. Biological control agents used to control bacterial wilt of tomato (*Solanum lycopersicum* L.) include avirulent mutants of *R. solanacearum* (Dong *et al.*, 1999), genetically engineered anatagonistic bacteria (Kang *et al.*, 1995), and some rhizobacteria like, *Streptomyces* spp. (el Albyad *et al.*, 1996) and *Bacillus* spp. (Sung *et al.*, 2005). The objective of this study was to evaluate *Paenibacillus* strain KPB3, showing in vitro antibacterial activity against bacterial wilt pathogen, *R. solanacearum*, for its effects on disease severity of bacterial wilt of tomato in the green house experiments. We also studied the efficiency of *Paenibacillus* strain KPB3 in controlling the bacterial wilt of tomato under field conditions during spring and autumn.

Materials and Methods

Isolation of bacteria and growth media

Paenibacillus strain KPB3 was isolated from sandy-loam soil around tomato roots that had survived in a polyvinyl house vastly infested with *R. solanacearum* in Chuncheon, Kangwon province, Republic of Korea. The collected soil was serially diluted in sterile water, and plated onto LB (Luria Burtani) agar plates, and incubated overnight at 28°C. Numerous colonies with different morphologies were picked up from the dilution plates. The colonies isolated were subjected to *in vitro* antibacterial assay against *R. solanacearum*. A few out of two-hundred bacterial colonies screened, showed antibacterial activity. One colony, which showed clear inhibitory zone against *R. solanacearum* was selected and named as KPB3. This strain was stored by freeze-drying in 10% skimmed milk for its long-term preservation, as described by Perry (1995). Liquid or solid (1.8% w/v agar) LB media was solely used for cultivation of *Paenibacillus* strain KPB3 and *R. solanacearum* in all the experiments.

Bacterial identification

In order to identify the strain KPB3, it was subjected to physiological and biochemical tests, based on Bergey's manual of systemic bacteriology, and 16S rRNA gene analyses. Briefly, inoculum of the test organism was prepared from LB plates incubated overnight at 28°C. A suspension of the bacterial cells was made in 0.85% saline and used for all the tests. The tests included production of various enzymes, utilization of different organic compounds and sugars.

16S rRNA gene analysis was carried by PCR, performed with a DNA thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Total genomic DNA was isolated using a lysozyme- dodecyl sulfate lysis procedure (Owen *et al.*, 1987), modified as described previously (Leach, *et al.*, 1990). The 16S rRNA gene was amplified using fD1 and rP2 primers (Weisburg *et al.*, 1991). The PCR product of the 16S rRNA was analyzed by electrophoretic separation in 1% (w/v) QA-agarose (Qbiogene, Irvine, CA), containing 0.5 $\mu g\ L^{-1}$ ethidium bromide. The PCR products were excised from the gel and purified using a QIAquick gel extraction kit (Qiagen Inc., Hilden, Germany). Purified DNA was ligated into the pGEM-T easy vector (Promega Co., USA). Plasmid containing the 16S rRNA region was then directly sequenced using an ALFred autocycle sequencing kit, with M13 forward and reverses primers. DNA homology searches were carried out with the NCBI databases, using the BLAST network service (Altschul *et al.*, 1990).

In vitro antibacterial assay

For antibacterial activity, bacterial wilt pathogen *R. solanacearum* was grown overnight in LB broth. It was mixed with LB agar media at 1% concentration and poured into plates. A culture suspension of overnight grown *Paenibacillus* strain KPB3 was dropped on LB plates containing *R. solanacearum* and incubated at 28°C for 2 days. The antibacterial activity was assessed by observing inhibitory zones in the background of *R. solanacearum*.

Green house experiments

Tomato (*Solanum lycopersicum* L.) cultivar Lokku-sanmaru, which is susceptible to *R. solanacearum*, was used in the experiments. One month old plants grown in nursery trays with 3.5 × 3.5 cm^2 cell size were transplanted into 10 cm^2 pots containing artificial soil. The experimental design was a randomized complete block design with two treatments and six plants in each treatment. 100 mL of *Paenibacillus* strain KPB3 culture (4×10^8 CFU mL^{-1}) prepared in LB broth was drenched into each pot under treatment. The control plants received 100 mL tap-water. One week after drench treatment, 10 mL of *R. solanacearum* suspension (1×10^8 CFU mL^{-1}) was applied to all the plant. Disease severity was observed after ten days of pathogen treatment. The experiment was repeated three times. Disease development on each plant was rated using the following scale: 5 = plant dead; 4 = 76 to 100% leaves with symptoms; 3 = 51 to 75% of leaves with symptoms; 2 = 26 to 50% of leaves with symptoms; 1 = < 25% of leaves with symptoms; and 0 = no symptoms. The disease index was calculated from the disease ratings by the formula:

Disease Index = [$\sum$ (rating no. × no. of plants in the rating) / (total no. of plants ×highest rating)] × 100

Data was analyzed using SAS program (SAS institute, Cary, NC).

Field tests

Field studies were conducted at Geodu-ri and and Goeun-ri, near Chuncheon, Kangwon province, during spring and autumn of 2006 respectively. The tomato plants (cv. Lokkusanmaru) were grown on raised beds, mulched by polyethylene cover with drip irrigation lines underneath reaching all plants in the polyvinyl houses. The tomato plants were planted at 50 cm spacing with 700 to 800 plants in each polyvinyl house.

The field trial in spring started in mid-May and ended in early July. The bacterial preparations of *Paenibacillus* strain KPB3 (3 L, 1×10^6 CFU mL^{-1}) was mixed into the water supply tank (3,000 L) and irrigated in three polyvinyl houses. The treatments were carried out three times with a time interval of ten days between each successive treatment. Another three polyvinyl houses received regular irrigation water. Ten days after last treatment, disease incidences were noted as the number of plants survived.

The second field trial started in early August and ended in mid-September of 2006. The number of polyvinyl houses and the number of plants in each polyvinyl house were same as in the first trial. The *Paenibacillus* strain KPB3 was mixed into water tank and irrigated using drip irrigation lines in the three polyvinyl houses. The treatment with *Paenibacillus* strain KPB3 was repeated three times with ten days interval. Three more polyvinyl houses were irrigated using normal irrigation water. The disease incidences were recorded as number of plants survived after ten days of the last treatment.

Results and discussions

Characterization of *Paenibacillus* strain KPB3

The *Paenibacillus* strain KPB3 was isolated from soil around tomato roots in a polyvinyl house in Chuncheon, Korea. The isolate was identified using physiological and biochemical tests as well as 16S rRNA gene analyses. The *Paenibacillus* strain KPB3 was Gram-positive, rod-shaped, showing, gelatin and starch hydrolysis. It was found that the carbon source utilization, enzyme production and organic substances utilization pattern was similar to the genus *Paenibacillus* (Table 1).

16S rRNA gene sequence of *Paenibacillus* strain KPB3, obtained by PCR using fD1 and rP2 primers was compared with that available in the GenBank. It showed 98% similarity with *Paenibacillus* spp., while for other Gram positive bacteria, it ranged between 80 to 90%. Based on biochemical and physiological characteristics

Table 1. Physiological and Biochemical characteristics of *Paenibacillus* strain KPB3

Characteristics	Strain KPB3	*Paenibacillus* spp.	*Bacillus* spp.	*Listeria* spp.	*Clostridium* spp.
L-arabinose	+[a]	+	-[b]	-	-
Amygdalin	+	+	+	nd[d]	-
Cellobiose	+	+	+	nd	-
D-xylose	+	+	-	-	-
D-fructose	+	+	+	nd	
Glycogen	+	+	+	-	-
Inositol	-	-	-	nd	-
Inulin	-	-	v[c]	nd	-
Lactose	+	+	-	v	+
Galactose	+	+	-	v	+
Mannitol	+	+	v	-	-
D-melibiose	+	+	-	-	v
D-melezitose	-	+	-	nd	-
L-rhamnose	-	-	-	+	-
D-arabitol	-	-	-	+	nd
D-glucose	+	+	+	-	v
Glycerol	+	+	v	nd	v
Gelatin hydrolysis	+	+	+	nd	nd
Indole produced	-	-	-	nd	-
Starch hydrolysis	+	+	+	v	-
Nitrate reduction	+	-	v	-	+
Vages-Proskauer	-	+	v	+	nd

[T]Type strain, ATCC, American type culture collection, [a]+, 80% or more strains positive; [b]-, 80% or more strains negative; [c]v, variable; and [d]nd, not determined.

and 16S rRNA gene analysis, it can be inferred that the KPB3 strain belongs to genus *Paenibacillus*.

Antibacterial activity of the *Paenibacillus* strain KPB3 was investigated *in vitro* against bacterial wilt pathogen *R. solanacearum*. The *Paenibacillus* strain KPB3 exhibited a clear inhibitory zone around *R. solanacearum* on LB agar plates as shown in Fig. 1. This indicates that the *Paenibacillus* strain KPB3 was capable of producing diffusible antibacterial products, which inhibited growth of *R. solanacearum*.

Ability of *Paenibacillus* strain KPB3 to suppress *R. solanacearum* in tomato plants under greenhouse and field conditions

Bacterial wilt pathogen, *R. solanacearum* can survive in soil without host for long time, which act as inoculum source for subsequently grown crops in the field. Since none of the chemical agents satisfactorily control *R. solanacearum*, biological control using antagonistic soil-born saprophytes is a subject of increasing interest. During the screening of antibacterial strains, isolated from soil, we found that some strains

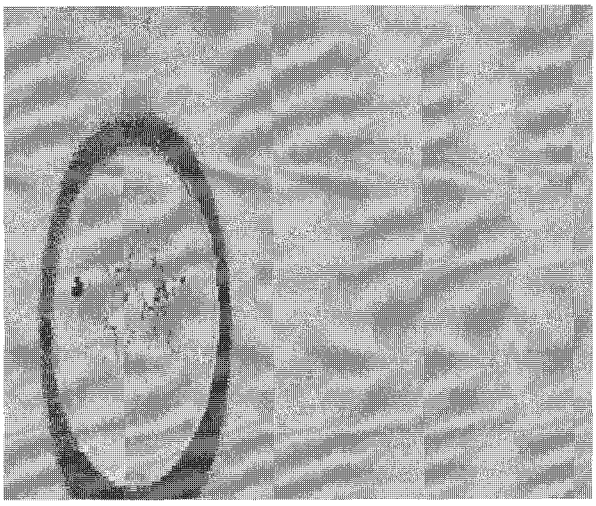

Fig. 1. *Paenibacillus* strain KPB3 showing inhibitory zone against bacterial wilt pathogen *R. solanacearum*. The inhibitory zones were observed two days after inoculation of the bacterium on medium containing *R. solanacearum*

Table 2. 16S rRNA sequence similarity between *Paenibacillus* strain KPB3 and other Gram positive bacteria

16S rRNA	Identity (%)
Paenibacillus spp.	98
Bacillus spp.	87.3
Listeria spp.	84.4
Clostridium spp.	79.7

with water (Table 3).

The mean disease severity for the three experiments was 4.66 for non-treated disease controls and 1.55 for the *Paenibacillus* strain KPB3 treated plants. The number of dead plants over a period of time was generally less in all experiments in tomato plants treated with *Paenibacillus* strain KPB3 than non-treated disease controls (Fig. 2). Thus, *Paenibacillus* strain KPB3 could significantly reducethe disease severity in case of tomato

Table 3. Effect of drench treatment with bacterial preparation of *Paenibacillus* strain KPB3 on bacterial wilt (caused by *R. solanacearum*) of tomato cultivar Lokkusanmaru under greenhouse conditions

Treatment[x]	Disease severity index[y]			
	Exp. 1	Exp. 2	Exp. 3	Mean[z]
Strain KPB3	0.67	2.67	1.33	1.55a
Water	4	5	5	4.66b

[x]Each treatment consisted of six plants, inoculated with *R. solanacearum* culture.
[y]Disease severity index was visually assessed on 10th day after the application of bacterial wilt pathogen, *R. solanacearum*, using= [$\sum$ (rating no. × no. of plants in the rating) / (total no. of plants × highest rating)] × 100.
[z]Means with different letters are significantly different at P = 0.05, average of disease index in three experiments. $LSD_{0.05}$ = 1.7304.

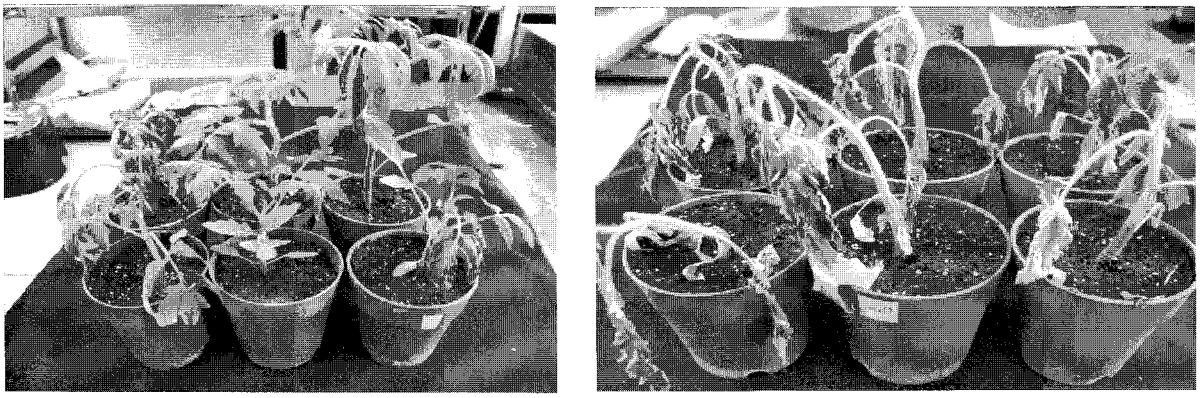

Fig. 2. Symptoms of bacterial wilt on tomato cultivar Lokkusanmaru, drench inoculated with *Paenibacillus* strain KPB3, followed by *R. solanacearum* treatment (left), and non-treated, *R. solanacearum* inoculated plants (right).

showed strong antibacterial activity *in vitro* however they did not control bacterial wilt in greenhouse experiments (data not shown). *Paenibacillus* strain KPB3, obtained from a field infested with bacterial wilt pathogen, was able to control *R. solanacearum*, both *in vitro* as well as *in vivo*. It appears to easily colonize the plant rhizosphere and produce bactericidal substances. Analysis of the data from three replicates of each pot experiment with tomato indicated that the number of diseased plants were significantly lower after treatment with *Paenibacillus* strain KPB3 than controls treated plants infested with *R. solanacearum*.

The polyvinyl houses growing tomatoes were observed to have numerous wilted plants. The cause of wilt was confirmed to be *R. solanacearum* by visual observations, looking for bacterial ooze and isolating the bacterium from the wilted plants. The disease was just started spreading when the experiment began, both, during spring and autumn of 2006. The disease incidence was high in the spring, wilting more than 50% of tomato plants at the time of harvest. It was due to high temperature and humidity conditions, favoring pathogen

Table 4. Effect of irrigation with *Paenibacillus* strain KPB3 containing water on bacterial wilt incidences caused by *R. solanacearum* under field conditions, in the year 2006, Chuncheon, Korea

Treatment[w]	Plants wilted (%)							
	Spring, 2006				Autumn, 2006			
	Exp. 1	Exp. 2	Exp. 3	Mean[x, y]	Exp. 1	Exp. 2	Exp. 3	Mean[x, z]
Strain KPB3	27.3	18.5	29	24.9a	6.7	11.9	2.5	7.0a
Water	46.49	50.71	55.5	50.9b	14.4	21.3	16	17.2b

[w]The treatment was done by adding 3 L *Paenibacillus* strain KPB3 culture into 3000 L irrigation water into each polyvinyl house by drip irrigation system. Controls were treated with normal irrigation water.
[x]Means with different letters are significantly different at P = 0.05.
[y]Mean percent of plants wilted in the polyvinyl houses in spring. $LSD_{0.05}$ = 16.21.
[z]Mean percent of plants wilted in the polyvinyl houses in autumn. $LSD_{0.05}$ = 7.40.

proliferation during spring, compared to the autumn. In the spring of 2006, treatment with *Paenibacillus* strain KPB3 provided effective protection against bacterial wilt with only 24.9% plants wilted, which is significantly lower than the untreated controls, where 50.9% plants wilted. Similarly, during autumn of 2006, wilting incidences occurred in just 7% of the tomato plants treated with *Paenibacillus* strain KPB3 as compared to the non-treated plants showing disease incidence in 17% plants (Table 4). The ability of *Paenibacillus* strain KPB3 to control bacterial wilt up to 60% is significant in absence of an efficient control strategy. The *Paenibacillus* strain KPB3 treatment was able to control bacterial wilt disease caused by *R. solanacearum* during both tomato growing seasons of the year 2006, when applied at 1000 times dilution through drip irrigation system. Thus, it can be effectively used as a novel biological control agent in managing bacterial wilt caused by *R. solanacearum*.

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토양에서 분리한 *Paenibacillus* spp. KPB3의 *Ralstonia solanacearum*에 의한 세균성 풋마름병 억제 효과

석정기, Nagesh S. Ipper, 이선화, Anupama Shrestha, 박덕환, 조준모[1] , 허장현[2], 김병섭[3], 임춘근*
(강원대학교 농업생명과학대학 생물자원공학부, [1]파이오니아㈜, [2]강원대학교 농업생명과학대학 생물환경학부, [3]강릉대학교 생명과학대학 식물응용학과)

요약 : 강원도 춘천시 신북읍의 토마토 풋마름병이 다발생한 포장으로부터 건전한 토마토 뿌리를 채취하여 약 200개의 미생물을 선발하였다. 선발된 미생물들에 대한 저지원 테스트를 실시한 결과, 풋마름병원균(*Ralstonia solanacearum*)에 항균 효과를 나타냈으며 그 중 가장 우수한 항균 효과를 나타낸 KPB3 균주를 선발하여 생리 생화학적 특성조사 와 16S rRNA 유전자 분석을 실시하여 *Paenibacillus* spp. 균으로 동정하였다. KPB3균주의 활성적 우수성을 확인하기 위하여, 먼저 포트 실험에서 KPB3 균주를 토양 관주처리 결과 무처리구에 비하여 처리구의 방제효과가 66.7% 뛰어난 것을 확인하였다. 또한 토마토 포장에서의 방제효과 시험은 2006년 봄과 가을에 2회 실시하였으며, 전반적으로 풋마름병 발생은 가을보다 봄에 높은 발병율을 나타내었다. 그 결과, KPB3 균주 처리구에서 봄과 가을 각각 50%와 60% 이상 방제 효과를 나타내어 KPB3 균주는 토마토 풋마름병 억제용 생물 제제로서의 개발 가능성이 있음을 확인하였다.

색인어 : 풋마름병, 생물학적 방제, *Ralstonia solanacearum, Paenibacillus*

*연락저자 (Fax : +82-33-256-8254, E-mail : chunkeun@kangwon.ac.kr)