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In Vitro Antagonistic Effects of Bacilli Isolates against Four Soilborne Plant Pathogenic Fungi

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Twenty isolates of *Bacillus* spp. obtained from livestock manure composts and cotton-waste composts were tested for *in vitro* antagonistic effects against soilborne plant pathogenic fungi, *Fusarium oxysporum*, *Phytophthora capsici*, *Rhizoctonia solani* AG-4, and *Sclerotinia sclerotiorum*. Seven isolates of *Bacillus* spp. had antagonistic effects on mycelial growth of all the isolates of *F. oxysporum* tested. The bacterial isolate RM43 was the most effective to inhibit the mycelial growth of the fungal isolates. Twelve isolates of *Bacillus* spp. had antagonistic effects on mycelial growth of all the isolates of *P. capsici* tested. The bacterial isolates M34 and M47 were very effective to inhibit the mycelial growth of the fungal isolates. Thirteen isolates of *Bacillus* spp. had antagonistic effects on mycelial growth of all the isolates of *R. solani* AG-4 tested. The bacterial isolates M27 and M75 were very effective to inhibit the mycelial growth of the fungal isolates. Fourteen isolates of *Bacillus* sp. had antagonistic effects on mycelial growth of all the isolates of *S. sclerotiorum* tested. The bacterial isolates M49 and M75 were very effective to inhibit the mycelial growth of the fungal isolates. The antagonistic effects of most *Bacillus* spp. isolates against the isolates of the four fungi differed depending on the fungal species and the isolates of each fungus. The bacterial isolates M27 and M75 were the most effective to inhibit the mycelial growth of all four fungi.

Keywords : antagonistic effect, *Bacillus* species, inhibition zone, soilborne fungi

Soilborne plant pathogenic fungi exist in the soil and attack plants during the growing seasons. Many of them can survive for many years without their hosts (Domsch et al., 1993; Parker et al., 1985). Plant diseases caused by soilborne fungi are commonly leathal to the host plants because infection usually occurs on underground or soil surface parts of the host plants, and the infected plants show the symptoms after much development of lesions. Soilborne fungal diseases of plants are very difficult to control using agrochemicals or other cultural methods because the pathogens are resistant to the applied chemicals and soil environment.

People's anxiety for environmental pollution and food safety due to agrochemicals has led to demanding alternatives to chemical control of plant diseases. Subsequently, biological control using antagonistic organisms has been applied as one of alternative methods for environment-friendly control and integrated management of plant diseases. Biological control of plant diseases was defined as the reduction in the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man (Cook and Baker, 1983). A biological control agent is selected through screening antagonistic effects of an organism against a target pathogen. Antagonism is a relationship between two species of organisms in which each adversely affects the other (Ulloa and Hanlin, 2000). Three types of interspecies antagonisms leading to biological control of plant pathogens were described (Pal and Gardener, 2006).

Some of bacteria such as *Bacillus* spp. and *Pseudomonas* spp. have been selected and used for biological control of plant diseases (Baker and Cook, 1974; Cook and Baker, 1983; Hornby, 1990; Gnanamanickam, 2002; Kheten, 2001; Parker et al., 1985). Effective biological control of plant diseases relies on the successful selection of antagonists against the pathogens. Thus, this study was conducted to select prospective antagonists for the use of biological control of plant diseases caused by major soilborne fungi.

Materials and Methods

Isolation of bacteria. Livestock manure composts and cotton-waste composts were collected from compost facilities and mushroom cultivation houses. The compost samples were serially diluted using 0.85% saline solution. The diluted solution was spread on trypticase soy agar (TSA, Difco). After three days of incubation at 30°C, single colonies were purified by transferring them onto new TSA plates. The isolates were maintained in 15% glycerol at

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−70°C.

Identification of bacterial isolates. 16S rRNA gene sequencing and a BLAST search of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) were used for identification of the bacterial isolates. Sequencing of the 16S rRNA gene was performed as described previously (Kwon et al., 2003). The 16S rRNA gene sequences of the bacterial isolates were matched with those from NCBI BLAST search.

Soilborne plant pathogenic fungi. *Fusarium oxysporum* Schlecht. emend. Sny. & Hans., *Phytophthora capsici* Leonian, *Rhizoctonia solani* Kühn AG-4, and *Sclerotinia sclerotiorum* (Lib.) de Bary obtained from lesions of vegetables and conserved in the NIAST were used for the test of antagonistic effects of bacterial isolates. Three isolates from each of the pathogen were tested for antagonism of the bacterial isolates.

Test of antagonistic effects. A 5 mm-mycelial mat of each soilborne fungus was placed on one side of a potato dextrose agar (PDA), and each bacterial isolate was streaked on the other side of the medium. The paired PDA plate was cultured at 25°C for 5 to 15 days. During the cultivation, antagonistic effects of the bacterial isolates against the fungal isolates were examined by measuring the nearest width of inhibition zones formed between the bacterial isolates and the fungal isolates. The dual culture test was performed in three replicates.

Results

Twenty isolates of *Bacillus* spp. were obtained from livestock manure composts and cotton-waste composts (Table 1). All the isolates were tested for antagonistic effects *in vitro* against soilborne plant pathogenic fungi, *F. oxysporum*, *P. capsici*, *R. solani* AG-4, and *S. sclerotiorum*. Antagonistic effects were rated as width of inhibition zones formed between the *Bacillus* spp. isolates and the fungal isolates (Fig. 1).

Seven isolates of *Bacillus* spp. had antagonistic effects on mycelial growth of all the isolates of *F. oxysporum* tested (Table 2). The antagonistic effects of most *Bacillus* spp. isolates differed depending on the opposite isolates of *F. oxysporum*. The bacterial isolate RM43 was the most effective to show the large inhibition zones against the fungal isolates.

Twelve isolates of *Bacillus* spp. had antagonistic effects on mycelial growth of all the isolates of *P. capsici* tested

Table 1. Identification of *Bacillus* sp. isolates from livestock manure composts and cotton-waste composts

Isolate No.	Source[a] isolated	Location	Nearest relative[b]	Accession No. of nearest relative[b]	Similarity (%)
M15	CWC	Gimpo	*B. subtilis* NCDO 6769^T	AF074970	100.0
M16	CWC	Gimpo	*B. subtilis* NCDO 6769^T	X60646	99.8
M18	CWC	Suwon	*B. subtilis* NCDO 6769^T	X60646	99.4
M27	CWC	Gimpo	*B. vallismortis* DSM 11031^T	AB021198	98.9
M34	CWC	Suwon	*B. subtilis* NCDO 6769^T	X60646	99.6
M47	CWC	Incheon	*B. velezensis* CR-502^T	AY603658	99.9
M49	CWC	Wonju	*B. mojavensis* IFO 15718^T	AB021191	99.8
M75	LMC	Icheon	*B. velezensis* CR-502^T	AY603658	99.8
M78	LMC	Icheon	*B. vallismortis* DSM 11031^T	AB021198	99.4
M79	LMC	Icheon	*B. velezensis* CR-502^T	AY603658	99.1
RM29	CWC	Suwon	*B. subtilis* NCDO 6769^T	AF074970	99.5
RM43	CWC	Suwon	*B. velezensis* CR-502^T	AY603658	99.9
RT03	CWC	Suwon	*B. lautus* NCIMB 12780^T	X60621	98.7
4T01	CWC	Suwon	*B. licheniformis* DSM 13^T	X68416	99.5
4T47	CWC	Suwon	*B. lautus* NCIMB 12780^T	X60621	98.3
4T49	CWC	Suwon	*B. licheniformis* DSM 13^T	X68416	99.3
5M35	CWC	Suwon	*B. subtilis* NCDO 6769^T	AF074970	99.7
5M50	CWC	Suwon	*B. subtilis* NCDO 6769^T	X60646	99.7
5T32	CWC	Suwon	*B. lautus* NCIMB 12780^T	X60621	98.7
5T33	CWC	Suwon	*B. licheniformis* DSM 13^T	X68416	99.4

[a]CWC, cotton-waste compost; LMC, livestock manure compost.
[b]Based upon a Blast search of the NCBI database.

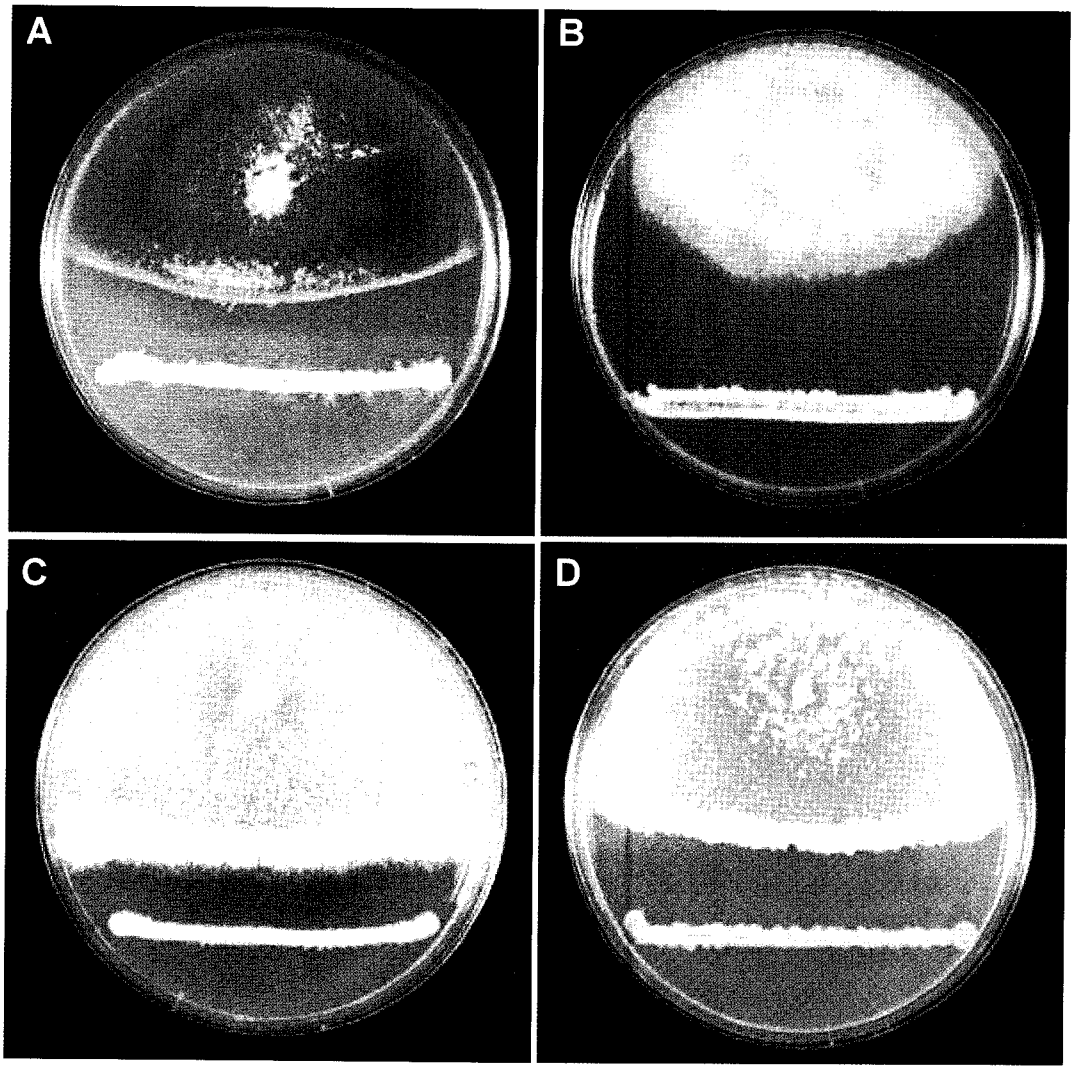

Fig. 1. Dual culture tests for antagonistic evaluation of *Bacillus* spp. isolates against mycelial growth of four soilborne plant pathogenic fungi on PDA. **A**, an inhibition zone of mycelial growth of *F. oxysporum* F6005 by *Bacillus* sp. RM43; **B**, an inhibition zone of mycelial growth of *P. capsici* P9910 by *Bacillus* sp. M47; **C**, an inhibition zone of mycelial growth of *R. solani* AG-4 940313 by *Bacillus* sp. M27; **D**, an inhibition zone of mycelial growth of *S. sclerotiorum* S97-106 by *Bacillus* sp. M49.

(Table 3). The antagonistic effects of most *Bacillus* spp. isolates differed depending on the opposite isolates of *P. capsici*. The bacterial isolates M34 and M47 were highly effective to reveal the great inhibition zone against the fungal isolates.

Thirteen isolates of *Bacillus* spp. had antagonistic effects on mycelial growth of all the isolates of *R. solani* AG-4 tested (Table 4). The antagonistic effects of most *Bacillus* spp. isolates differed depending on the opposite isolates of *R. solani* AG-4. Isolates M27 and M75 were highly effective to inhibit the mycelial growth of the fungal isolates.

Fourteen isolates of *Bacillus* spp. had antagonistic effects on mycelial growth of all the isolates of *S. sclerotiorum* tested (Table 5). The antagonistic effects of most *Bacillus* sp. isolates differed depending on the opposite isolates of *S. sclerotiorum*. The bacterial isolates M49 and M75 were highly effective to inhibit the mycelial growth of the fungal isolates.

Ten isolates of *Bacillus* sp. had antagonistic effects on mycelial growth of the isolates of four fungi tested (Table 6). The antagonistic effects of most *Bacillus* sp. isolates against the isolates of the four fungi differed depending on the fungal species. The bacterial isolates M27 and M75 were highly effective to inhibit the mycelial growth of the fungal isolates.

Discussion

Mechanisms of biological control of plant diseases gener-

Table 2. Antagonistic effect of *Bacillus* isolates against mycelial growth of *F. oxysporum* isolates

Isolate No. of *Bacillus* sp.	Inhibition zones (mm)[a] of mycelial growth of *F. oxysporum* isolates			
	F6001	F6005	F6006	Average
M15	–	2.3	0.7	1.0±1.2[b]
M16	–	–	–	–
M18	–	1.7	–	0.6±1.0
M27	4.3	7.3	8.7	6.8±2.2
M34	–	3.0	0.7	1.2±1.6
M47	2.3	4.7	4.3	3.8±1.3
M49	1.3	1.7	1.3	1.4±0.2
M75	4.7	9.0	10.7	8.1±3.1
M78	2.0	6.0	7.3	5.1±2.8
M79	5.3	7.7	6.3	6.4±1.2
RM29	–	–	–	–
RM43	6.3	12.7	11.7	10.2±3.4
RT03	–	–	–	–
4T01	–	–	–	–
4T47	–	2.7	–	0.9±1.6
4T49	–	–	–	–
5M35	–	–	–	–
5M50	–	–	–	–
5T32	–	–	–	–
5T33	–	0.3	–	0.1±0.2

[a]Inhibition zones were measured after 15 days of incubation at 25°C. –, no inhibition.
[b]The data represents the average of three replicates±standard deviation.

Table 3. Antagonistic effect of *Bacillus* isolates against mycelial growth of *P. capsici* isolates

Isolate No. of *Bacillus* sp.	Inhibition zones (mm)[a] of mycelial growth of *P. capsici* isolates			
	P9723	P9831	P9910	Average
M15	–	–	2.0	0.7±1.2[b]
M16	10.7	11.3	12.3	11.4±0.8
M18	10.7	13.3	16.7	13.6±3.0
M27	4.7	0.7	7.3	4.2±3.3
M34	12.7	14.3	17.3	14.8±2.3
M47	16.3	8.0	20.3	14.9±6.3
M49	–	1.3	3.7	1.7±1.9
M75	6.0	1.7	10.3	6.0±4.3
M78	4.7	–	7.3	4.0±3.7
M79	–	–	1.7	0.6±1.0
RM29	11.3	13.3	16.7	13.8±2.7
RM43	2.7	–	5.3	2.7±2.7
RT03	3.3	2.7	4.0	3.3±0.7
4T01	1.3	0.7	1.7	1.2±0.5
4T47	2.3	0.7	3.7	2.2±1.5
4T49	1.0	–	2.7	1.2±1.4
5M35	–	–	5.0	1.7±2.9
5M50	–	–	2.3	0.8±1.3
5T32	4.3	3.3	4.3	4.0±0.6
5T33	2.3	0.7	4.0	2.3±1.7

[a]Inhibition zones were measured after ten days of incubation at 25°C. –, no inhibition.
[b]The data represents the average of three replicates±standard deviation.

ally include antibiosis, parasitism, competition, induced resistance, and production of hydrolases. *Bacillus*-based biological control agents have modes of action that include antibiosis, parasitism, and induced systemic resistance (Jacobsen et al., 2004). Antibiosis is generally the mode of antagonism observed with *Bacillus* spp. (Edwards et al., 1994). Most *Bacillus* spp. produce many kinds of antibiotics such as bacillomycin, fengycin, mycosubtilin, and zwittermicin, which are effective at suppressing growth of target pathogens *in vitro* and/or *in situ* (Pal and Gardener, 2006). A dual culture test is extensively used as one of *in vitro* tests for preliminary screening of biological control agents (Desai et al., 2002). In the present study, dual culture tests showed that many *Bacillus* isolates from livestock manure composts and cotton-waste composts have antagonistic effects against the isolates of soilborne fungi, *F. oxysporum*, *P. capsici*, *R. solani* AG-4, and *S. sclerotiorum*.

Bacillus spp. are known to survive in soil and other substrates for long periods because they form endospores in the cells (Claus and Berkeley, 1986). The bacterial species have been used as biological control agents of plant diseases (Boland and Kuykendall, 1998; Gnanamanickam, 2002; Jacobsen et al., 2004; Tjamos et al., 1991). Screening and field trials using *Bacillus* spp. have been conducted for control of several soilborne fungal diseases of crops (Cavaglieri et al., 2005; Fernando et al., 2007; Hornby, 1990; Omar et al., 2006). Subsequently, commercialized materials of several *Bacillus* spp. have been registered as biopesticides for control of soilborne fungal diseases of crops caused by *Fusarium* spp., *Rhizoctonia* spp., and *Sclerotinia* spp. (Copping, 2004; Fravel, 2005; Schisler et al., 2004).

The present study showed that the antagonistic effects of most *Bacillus* sp. isolates against isolates of the soilborne fungi differed depending on the isolates of each fungal species as well as the fungal species. The data indicates that control effects of the antagonists could be variable depending on the strains of target fungal species in the field test. Environmental conditions also could affect the antagonistic effects of the selected isolates in the field. Therefore, it is desirable that *in vitro* tests for selection of prospective isolates for the biological control should be accomplished with several isolates of target pathogens prior to field tests.

Out of 20 *Bacillus* isolates tested, RM43 was selected as

Table 4. Antagonistic effect of *Bacillus* isolates against mycelial growth of *R. solani* AG-4 isolates

Isolate No. of *Bacillus* sp.	Inhibition zone (mm)[a] of mycelial growth of *R. solani* AG-4 isolates			
	940301	940313	941211	Average
M15	2.3	10.3	2.7	5.1±4.5[b]
M16	1.0	7.7	3.0	3.9±3.4
M18	2.7	4.0	2.7	3.1±0.8
M27	5.7	11.0	6.7	7.8±2.8
M34	–	4.0	0.7	1.6±2.1
M47	4.0	10.3	4.3	6.2±3.6
M49	4.0	10.3	4.0	6.1±3.6
M75	6.0	10.7	5.7	7.5±2.8
M78	3.3	7.3	4.0	4.9±2.1
M79	2.3	8.3	3.3	4.6±3.2
RM29	1.3	3.3	2.0	2.2±1.0
RM43	3.3	10.7	5.3	6.4±3.8
RT03	–	–	–	–
4T01	–	–	–	–
4T47	–	–	–	–
4T49	–	–	–	–
5M35	1.5	6.7	1.0	3.1±3.2
5M50	1.0	4.7	2.7	2.8±1.9
5T32	–	–	–	–
5T33	–	–	–	–

[a]Inhibition zones were measured after five days of incubation at 25°C. –, no inhibition.
[b]The data represents the average of three replicates±standard deviation.

Table 5. Antagonistic effect of *Bacillus* isolates against mycelial growth of *S. sclerotiorum* isolates

Isolate No. of *Bacillus* sp.	Inhibition zone (mm)[a] of mycelial growth of *S. sclerotiorum* isolates			
	S96-121	S97-064	S97-106	Average
M15	8.7	11.7	7.7	9.4±2.1[b]
M16	10.3	10.3	10.7	10.4±0.2
M18	4.0	4.3	6.0	4.8±1.1
M27	10.7	13.0	12.7	12.1±1.3
M34	8.7	8.0	12.3	9.7±2.3
M47	10.0	9.0	10.0	9.7±0.6
M49	13.7	13.7	14.0	13.8±0.2
M75	16.0	10.3	12.7	13.0±2.9
M78	8.7	10.7	11.7	10.4±1.5
M79	7.7	9.7	10.7	9.4±1.5
RM29	10.0	13.7	12.7	12.1±1.9
RM43	10.0	12.0	11.7	11.2±1.1
RT03	–	–	1.7	0.6±1.0
4T01	–	1.7	2.7	1.5±1.4
4T47	–	2.3	3.7	2.0±1.9
4T49	–	–	1.7	0.6±1.0
5M35	2.7	6.3	5.0	4.7±1.8
5M50	10.3	11.0	10.3	10.5±0.4
5T32	–	–	1.0	0.3±0.6
5T33	–	1.0	2.3	1.1±1.2

[a]Inhibition zones were measured after five days of incubation at 25°C. –: no inhibition.
[b]The data represents the average of three replicates±standard deviation.

Table 6. Comparison in antagonistic effects of *Bacillus* isolates against mycelial growth of four soilborne fungi

Isolate No. of *Bacillus* sp.	Average inhibition zone (mm)[a] of mycelial growth of four soilborne fungi			
	F. oxysporum	*P. capsici*	*R. solani* AG-4	*S. sclerotiorum*
M15	1.0±1.2[b]	0.7±1.2	5.1±4.5	9.4±2.1
M16	–	11.4±0.8	3.9±3.4	10.4±0.2
M18	0.6±1.0	13.6±3.0	3.1±0.8	4.8±1.1
M27	6.8±2.2	4.2±3.3	7.8±2.8	12.1±1.3
M34	1.2±1.6	14.8±2.3	1.6±2.1	9.7±2.3
M47	3.8±1.3	14.9±6.3	6.2±3.6	9.7±0.6
M49	1.4±0.2	1.7±1.9	6.1±3.6	13.8±0.2
M75	8.1±3.1	6.0±4.3	7.5±2.8	13.0±2.9
M78	5.1±2.8	4.0±3.7	4.9±2.1	10.4±1.5
M79	6.4±1.2	0.6±1.0	4.6±3.2	9.4±1.5
RM29	–	13.8±2.7	2.2±1.0	12.1±1.9
RM43	10.2±3.4	2.7±2.7	6.4±3.8	11.2±1.1
RT03	–	3.3±0.7	–	0.6±1.0
4T01	–	1.2±0.5	–	1.5±1.4
4T47	0.9±1.6	2.2±1.5	–	2.0±1.9
4T49	–	1.2±1.4	–	0.6±1.0
5M35	–	1.7±2.9	3.1±3.2	4.7±1.8
5M50	–	0.8±1.3	2.8±1.9	10.5±0.4
5T32	–	4.0±0.6	–	0.3±0.6
5T33	0.1±0.2	2.3±1.7	–	1.1±1.2

[a]Three isolates of each pathogen were tested. –, no inhibition.
[b]The data represents the average of three replicates±standard deviation.

a prospective antagonist against *F. oxysporum*. M34 and M47 were selected as prospective antagonists against *P. capsici*, M27 and M75 against *R. solani* AG-4, and M49 and M75 against *S. sclerotiorum*. M27 and M75 were also selected as prospective antagonists against all the fungal pathogens tested. It is probable that the antagonists could be used for control of plant diseases caused by the fungal pathogens. However, several researchers have reported no correlation between *in vitro* inhibition tests and field performance of biocontrol agents (Fravel, 2005). Further study with field trials using the prospective antagonists is needed to be accomplished.

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