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A Rapid Radicle Assay for Prescreening Antagonistic Bacteria Against *Phytophthora capsici* on Pepper

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A rapid radicle assay for prescreening antagonistic bacteria to *Phytophthora capsici*, causal agent of Phytophthora blight of pepper was developed. Sixty-four bacterial strains with *in vitro* antifungal activity selected out of 1,400 strains isolated from soils of Ansung, Chunan, Koyang, and Paju, Korea in 1998 were used for development of the bioassay. Uniformly germinated pepper seeds dipped in bacterial cells for 3 hours were placed near the edges of growing mycelia of *P. capsici* on water agar containing 0.02% glucose. Five-week-old pepper plants (cv. Nockwang) were inoculated to compare with results of the radicle assay developed in this study. For plant inoculation, pepper seeds were sown in potting mixtures incorporated with the bacterial strains, then transplanted into steam-sterilized soils 3 weeks later. Plants were hole-inoculated with zoospores of *P. capsici* 2 weeks after transplanting. Disease incidence and severity were determined in radicle and plant assessments, respectively. In radicle assay, six strains, GK-B15, GK-B25, OA-B26, OA-B36, PK-B09, and VK-B14 consistently showed the significant (*P*=0.05) disease reduction against radicle infection by the fungus, four of which also did in plant assessments. Strains OA-B36 and GK-B15 consistently reduced the fungal infection in both the radicle assay and the plant assessment. Therefore, prescreening strains using the radicle assay developed in this study followed by plant assay could reduce time and labor, and improved the possibility of selecting antagonistic bacteria for control of Phytophthora blight of peppers.

KEYWORDS: **Assay, Biological control, Pepper, *Phytophthora capsici*, Screening method**

Phytophthora blight of pepper (*Capsicum annuum* L.) has been one of the most important plant diseases worldwide (Hwang and Kim, 1995; Ristaino and Johnston, 1999). Agricultural chemicals and cultural practices have been applied to control this economically important disease in the fields (Ristaino and Johnston, 1999). Metalaxyl, a systemic phenylamide, has been the most widely used fungicide on pepper to control the disease, and its effectiveness has been largely reported (Lee *et al.*, 1991; Matheron and Matejka, 1988; Papavizas and Bowers, 1981). However, emergence of resistant isolates of *Phytophthora capsici* to metalaxyl (Ham *et al.*, 1991; Oh and Kim, 1992) makes its application difficult in the fields. An alternative management strategy for control of Phytophthora root rot of pepper has been developed in which a nonionic surfactant was used in a hydroponic system (Stanghellini *et al.*, 1996).

Current concerns about negative effects of chemical applications in agricultural practices resulted in the search for other methods including biological controls. For this biocontrol strategy, selection of antagonistic agents is the most important, first step for successful development of biological control methods (Alabouvette *et al.*, 1993). Simple screening for biocontrol agents is desirable to reduce time and efforts; thus various assay methods have been developed by many researchers (Kloepper *et al.*, 1985; Randhawa and Schaad, 1985; Rhodes *et al.*, 1987; Scher *et al.*, 1984). However, the screening methods are not always sufficient to find effective biocontrol agents (Weller, 1988). Therefore, it has been suggested that plant tests with target pathogens should be conducted after screening antagonistic microorganisms using bioassays (Alabouvette *et al.*, 1993).

Biological control of *Phytophthora* spp. has been attempted because of its economic importance in agriculture (Mao *et al.*, 1998; Stanghellini *et al.*, 1996; Utkhede, 1984; Valois *et al.*, 1996). However, such trials were not always successful and one of reasons for this would be the lack of prescreening methods to find effective biocontrol agents against *Phytophthora* spp. Thus, we attempted to develop a new method to select biologically effective antagonists against *P. capsici.* The aim for this research was to develop a quick and simple bioassay, a radicle assay, to search for effective bacterial strains for controlling Phytophthora blight of pepper. In addition, results from this assay were compared with those of plant assessments to show if the developed assay in this study would be adequate for selecting antagonistic bacteria to the fungal pathogen.

Materials and Methods

Bacterial strain. Sixty-four bacterial strains (Table 1) with *in vitro* antifungal activity against eight plant pathogenic fungi, *Alternaria mali*, *Colletotrichum gloeosporioides*, *C. orbiculare*, *Fusarium oxysporum* f. sp. *cucumerinum*,

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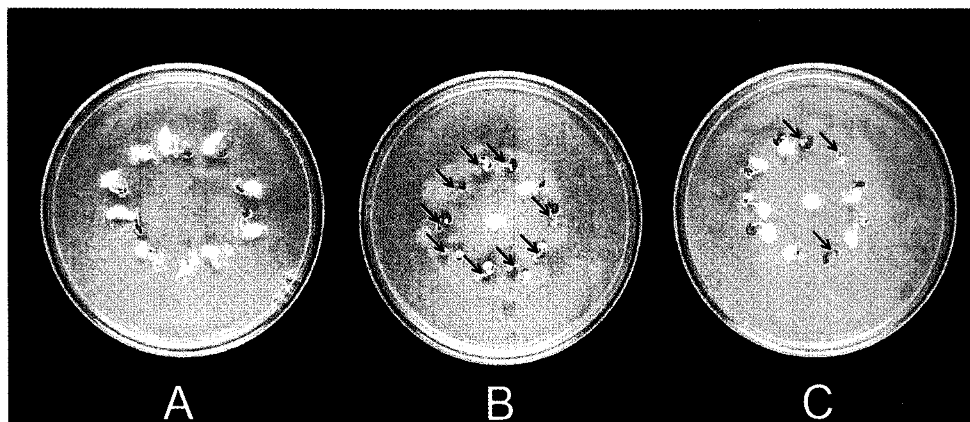

Fig. 1. Effect of bacterial strains on development of brownish symptoms (indicated by arrows) caused by *Phytophthora capsici* on radicles of pepper (cv. Nockwang) seeds treated with a bacteria strain, PK-B09 or sterile water 72 hours after treatments. A, non-inoculated control treated with sterile water. B, Inoculated seeds treated with sterile water. C, Inoculated pepper seeds treated with bacteria strains, PK-B09. Germinated pepper seeds treated with sterile tap water or bacterial suspensions for 3 hours were placed onto the margin of actively growing mycelia of *P. capsici* on water agar containing 0.02% glucose for 5 days.

F. oxysporum f. sp. *lycopersici*, *P. capsici*, *Magnaporthe grisea*, and *Rhizoctonia solani* were used in this study. The strains were selected from 1,400 soil bacteria isolated from various soils of Ansung, Chunan, Koyang, and Paju, Korea in 1998 as described in our previous research (Chang *et al.*, 2000). These bacterial strains were stored in nutrient broth (NB, Difco, Detroit, MI, U.S.A.) with 20% glycerol at -70°C until use. For treatments of radicles and plants, bacterial strains were cultured in NB with shaking at 120 rpm at 28°C for 48 hours. The cultures were centrifuged 4,000 g at 18°C for 10 minutes to separate bacteria from media. After centrifugation, supernatants were discarded and pellets (bacterial cells) were washed twice with sterile tap water by centrifugation. Bacterial cells were adjusted to A_{600}=0.5 (ca. 10^8 bacterial cells/ml) with a spectrophotometer (Du® 650, Beckman Coulter, Inc., Fullerton, CA).

Radicle assay. Seeds of pepper cv. Nockwang susceptible to Phytophthora blight were germinated in petri dishes with moist filter papers for 3~4 days 28°C in darkness. Uniformly germinated seeds were soaked in the bacterial suspensions prepared as described above for 3 hours and blotted on sterile filter papers. The seeds were then placed on the margin of actively growing mycelia of a virulent isolate S197 of *P. capsici* grown on water agar amended with 0.02% glucose at 28°C for 5 days. These treated plates were incubated at 28°C under 16-hour fluorescent light per day until disease expression. Seeds treated with sterile tap water served as untreated controls. Disease incidence was evaluated when over 90% of the seed radicles in untreated controls were infected by *P. capsici*, 48 or 72 hours after treatments. This experiment was conducted with three replications with 10 seeds each.

Plant assessment. Seeds of pepper cv. Nockwang were germinated on petri dishes containing moist filter papers at 28°C for 3~4 days. The prepared bacterial suspensions were mixed with potting mixtures [peat moss (Acadian Peat Moss Ltd., Lamègue, New Brunswick, Canada) and TKS2 (Floragard Product, Germany), 1:1.5, v/v] at the level of 1 ml of bacterial suspensions in 1 g of potting mixtures. Water served as untreated controls. Germinated seeds were sown in each hole (3x3x5 cm) of a 128-hole plug tray filled with the potting mixtures incorporated with bacterial cells. These trays were placed in a growth room with 16-hours fluorescent light (80 μmol $m^{-2}s^{-1}$) at 25°C. After 3 weeks, the seedlings were transplanted into 10-cm-diameter pots with saucers containing steam-sterilized soil.

For preparation of fungal inoculum, isolate S197 of *P. capsici* was grown on oatmeal agar for 7 days at 28°C. These cultures were then flooded with 20 ml of sterile distilled water and incubated under continuous fluorescent light for additional 7 days at 28°C for induction of sporangia. For release of zoospores from sporangia, the cultures were flooded with chilled sterile water, and placed at 4°C for 30 minutes followed by room temperature for 30 min. Mycelia and sporangial debris were removed from zoospores by filtration through four layers of sterile cheesecloth. One ml of zoospore suspensions was vortexed vigorously for 30 seconds to make zoospores encyst before counting zoospores with a haemocytometer. Two weeks after transplanting, plants were inoculated with zoospores of *P. capsici* (25 zoospores/g soil). For inoculation, zoospore suspensions were injected into three holes (1 cm diameter x 1 cm deep) around each plant. Sterile water was used as uninoculated controls. After inoculation, plants were continuously watered through saucers to avoid from drying of soil for 2~3 days, then, watered as needed. Dis-

Table 1. Disease incidence caused by *Phytophthora capsici* on radicles of pepper seeds (cv. Nockwang) treated with bacteria strains in radicle assays

Test and bacterial strain	Disease incidence (%)[a]	Test and bacterial strain	Disease incidence (%)	Test and bacterial strain	Disease incidence (%)	Test and bacterial strain	Disease incidence (%)
Test I		**Test III**		**Test V**		**Test VI**	
GC-B19	83*[b]	GK-B28	89	GK-B18	97	RC-B33	76*
GK-B15	83*	LS-B03	97	GK-B24	100	RC-B37	100
LS-B01	90*	LS-B80	97	GK-B25	72*	RC-B39	56*
LS-B42	100	LS-B81	97	GK-B26	90	RC-B40	50*
OA-B65	100	MM-B16	93	GK-B29	93	RC-B41	100
PK-B09	83*	MW-B19	83	OA-B03	97	RC-B64	83
PK-B26	97	OC-B70	89	OA-B26	59*	RC-B65	75*
RK-B41	97	PK-B14	97	OA-B37	90	RC-B77	93
Control	100	Control	100	Control	97	Control	100
Test II		**Test IV**		**Test VII**		**Test VIII**	
MM-B25	80	GC-B07	93	GK-B21	100	GC-B17	80
MW-B02	80	GC-B23	90	LS-B70	90	GC-B33	100
MW-B10	97	GC-B24	97	MW-B18	100	GK-B09	90
MW-B15	80	GC-B26	100	MW-B24	85	MM-B01	93
OA-B22	80	GC-B27	80	OA-B15	56*	MM-B03	100
OA-B36	43*	GC-B28	93	RC-B38	100	MM-B20	90
OC-B18	95	GC-B32	100	RK-B26	100	MM-B22	100
VC-B11	97	GC-B35	97	VK-B14	35*	RC-B78	100
Control	90	Control	100	Control	93	Control	93

[a]Disease incidence was determined 72 hours after placing seeds treated with bacterial strains on *P. capsici*-grown water agar.
[b]Means followed by asterisks are significantly different compared with untreated controls at P=0.05. Arcsine-transformed data of three replications with 10 seeds each were used for statistic analysis; however, non-transformed data were presented. This experiment was conducted with three replications with 10 seeds each.

ease severity was evaluated 11~21 days after inoculation based on a scale of 0 (no symptom) ~ 5 (plant dead) described by Sunwoo *et al.* (1996). A complete randomized block design with three replicates with 10 plants each was used in this experiment.

Data analysis. The percent data for disease incidence were statistically analyzed after arcsine square-root transformation. Statistical analysis of data was conducted using the Statistical Analysis Systems Institute Inc (SAS Institute, NC, USA, 1990). Analysis of variance was determined using the general linear model procedures and means were separated with the least significant difference.

Results

Radicle assay. When germinated pepper seeds treated with bacteria or water were placed on the margin of actively growing mycelia of *P. capsici* on water agar containing 0.02% glucose, brownish symptoms on radicles of seeds usually appeared 48~72 hours after treatment (Fig. 1). Using this bioassay, 64 bacterial strains (Table 1) were evaluated for protection of radicles against *P. capsici* infection. Thirteen strains, GC-B19, GK-B15, GK-B25, LS-B01, OA-B15, OA-B26, OA-B36, PK-B09, RC-B33, RC-B39, RC-B40, RC-B65, and VK-B14 showed significant ($P = 0.05$) reduction in disease incidence compared

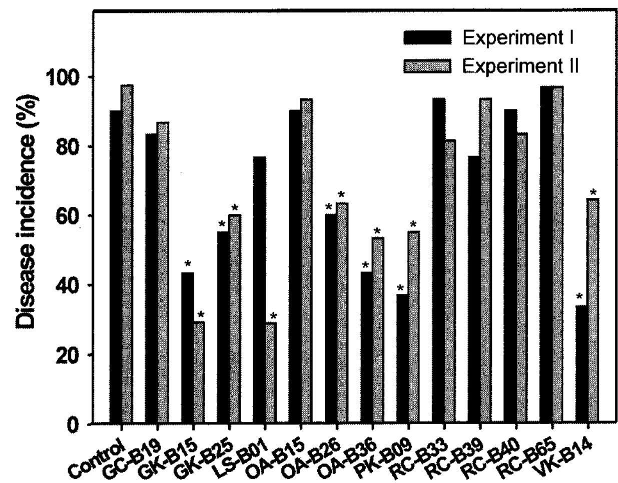

Fig. 2. Disease incidence caused by *Phytophthora capsici* on radicles of pepper (cv. Nockwang) seeds treated with 13 selected bacterial strains or sterie water 48 hours after treatments. Germinated pepper seeds treated with sterile tap water or bacterial suspensions for 3 hours were placed onto the margin of actively growing mycelia of *P. capsici* on water agar containing 0.02% glucose for 5 days. Asterisks on bars are significantly different compared with untreated controls at P=0.05. This experiment was conducted with three replications of 10 seeds each. Arcsine-transformed data were used for data analysis; however, non-transformed data are presented.

Table 2. Disease severity caused by *Phytophthora capsici* on pepper cultivar Nockwang treated by bacterial strains in plant tests

Test and bacterial strain	Disease severity[a]	Test and bacterial strain	Disease severity	Test and bacterial strain	Disease severity	Test and bacterial strain	Disease severity
Test I		**Test III**		**Test V**		**Test VII**	
GC-B19	3.0[b]	GK-B28	3.2	GK-B18	3.1	GK-B21	2.5
GK-B15	2.9*	LS-B03	3.4	GK-B24	3.2	LS-B70	3.0
LS-B01	4.5	LS-B80	3.4	GK-B25	2.8*	MW-B18	1.9
LS-B42	3.7	LS-B81	3.7	GK-B26	3.7	MW-B24	1.9
OA-B65	3.1	MM-B16	4.1	GK-B29	3.3	OA-B15	2.6
PK-B09	3.3	MW-B19	3.5	OA-B03	3.7	RC-B38	2.4
PK-B26	3.8	OC-B70	3.7	OA-B26	2.6*	RK-B26	1.9
RK-B41	3.4	PK-B14	3.6	OA-B37	1.5*	VK-B14	1.9
Control	4.0	Control	3.6	Control	3.6	Control	2.7
Test II		**Test IV**		**Test VI**		**Test VIII**	
MM-B25	3.4	GC-B07	2.4	RC-B33	3.3	GC-B17	2.9
MW-B02	1.9*	GC-B23	1.5	RC-B37	3.0	GC-B33	4.1
MW-B10	3.0	GC-B24	2.2	RC-B39	2.7	GK-B09	3.8
MW-B15	2.6	GC-B26	2.6	RC-B40	3.8	MM-B01	3.3
OA-B22	3.4	GC-B27	2.9	RC-B41	4.1	MM-B03	3.9
OA-B36	2.1*	GC-B28	2.3	RC-B64	4.3	MM-B20	3.4
OC-B18	2.4	GC-B32	3.2	RC-B65	3.5	MM-B22	3.2
VC-B11	3.3	GC-B35	1.5	RC-B77	2.9	RC-B78	4.0
Control	3.4	Control	1.9	Control	3.5	Control	2.9

[a]Final disease severity was evaluated in a scale of 0 (no symptom) ~ 5 (plant dead) 16~20 days after inoculation.
[b]Means followed by an asterisk are significantly different compared with untreated controls at P=0.05. Three replications with 10 plants each were used in this tests.

with untreated controls (Table 1).

These strains were tested again twice to select effective strains for inhibiting radicle infection by *P. capsici*. From these repeated experiments, six strains, GK-B15, GK-B25, OA-B26, OA-B36, PK-B09, and VK-B14 consistently inhibited radicle infection by the fungus (Fig. 2). Therefore, these strains were selected as potential biocontrol agents. The other seven strains failed to consistently inhibit the fungal infection of seed radicles (Fig. 2). Uninoculated control radicles of pepper seeds showed no visual disease symptom in any tests.

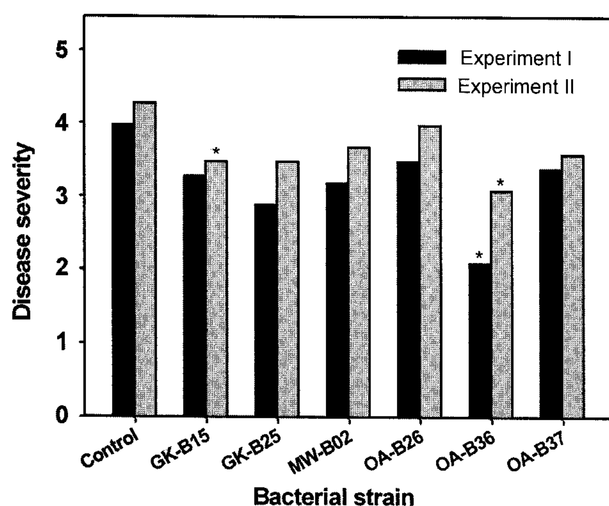

Fig. 3. Disease severity caused by *Phytophthora capsici* on pepper cv. Nockwang treated with six selected bacterial strains 16 and 11 days after inoculation for experiments 1 and 2, respectively. Disease severity was evaluated in a scale of 0 (no symptom) ~ 5 (plant dead). Asterisks on bars are significantly different compared with untreated controls at P=0.05. This experiment was conducted with three replications of 10 plants each.

Plant assessment. The same bacterial strains (Table 1) were tested for protection in 5-week-old plants against infection of *P. capsici*. Six bacterial strains, GK-B15, GK-B25, MW-B02, OA-B26, OA-B36, and OA-B37 showed significant (P=0.05) reduction in disease severity compared with inoculated controls (Table 2). These strains were tested again twice to select consistently effective strains inhibiting fungal infection to pepper plants. From these repeated experiments, strain OA-B36 consistently inhibited fungal infection on pepper plants compared with inoculated controls from both experiments, while strain GK-B15 did in one of two experiments (Fig. 3). The other strains failed to protect the fungal infection consistently (Fig. 3). Uninoculated control plants did not show any disease symptom in all tests.

Discussion

The potential for biological control of diseases has been

explored in consideration of the importance of the environment and human health. Moreover, consumers have been more willing to buy chemical-free products with even higher costs. However, chemical application is still the most effective disease management strategy in modern agricultural system. Biological control is a promising alternative to control plant pathogens instead of use of chemicals, therefore much research for biological control agents has been conducted (Handelsman and Stabb, 1996). In spite of numerous researches for biological control, the study for biocontrol of Phytophthora blight of peppers has been accomplished insufficiently. Though biological control of the disease of peppers in Korea has been attempted, favorable success in fields has not been obtained (Hwang and Kim, 1995; Jee *et al.*, 1988). The situation in field, where inoculum is dispersed within water and rapid outbreaks of epidemics occur, makes it difficult for researchers to apply biological control agents. Along with these situations, absence of an adequate bioassay for selection of promising biocontrol agents to *P. capsici* would be another reason for little success of biological control of Phytophthora blight of pepper.

Many bioassays for screening antagonists have been developed in various plant-pathogen systems (Broadbent *et al.*, 1971; Han *et al.*, 2000; Kloepper, 1991; Randhawa and Schaad, 1985; Rhodes *et al.*, 1987). We also attempted to develop the radicle assay for improving the selection of antagonistic bacteria to control Phytophthora blight of pepper. In this bioassay with germinated pepper seeds, we cultured *P. capsici* in water agar containing low amount of glucose that was used as a nutrient source for fungal pathogens to avoid bacterial contamination, possibly originated from seeds. However, nutrient rich media and cotton-seed exudates were used in other systems (Randhawa and Schaad, 1985; Kloepper, 1991). In this assay, six strains consistently inhibited radicle infection by *P. capsici* from repeated tests, four of which also reduced plant infection by the fungus in the first plant test. Especially, strains OA-B36 and GK-B15 consistently suppressed disease infection by the fungus in both radicle assay and plant assessments from repeated tests. Thus, the radicle assay developed in this study could be used for prescreening antagonistic bacteria.

Since severe brownish symptoms usually occurred 48 or 72 hours after treatments in this bioassay, the screening procedure usually needs no more than 10 days for preparation of pathogens and bacteria, and bioassay. However, evaluation of bacterial strains in pepper plants grown in pots requires labor and much more time (more than 8 weeks) although the plant assessments are desirable. Therefore, prescreening strains from the radicle assay followed by plant assessments could reduce time and labor and consequently, improve the possibility of selecting promising antagonistic bacteria for control of Phytophthora blight of peppers. These candidate strains for biological control screened from the radicle assay and the plant assessment could be evaluated in field trials for the possibility of commercial products in agricultural practices.

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