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Selection and Efficacy of Soil Bacteria Inducing Systemic Resistance Against *Colletotrichum orbiculare* on Cucumber

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Soil bacteria were screened for the ability to control cucumber anthracnose caused by *Colletotrichum orbiculare* through induced systemic resistance (ISR). Sixty-four bacterial strains having *in vitro* antifungal activity were used for selecting ISR-inducing strains in cucumber. Cucumber seeds (cv. Baeknokdadagi) were sown in potting mixtures incorporated with the soil bacteria, at a rate of ca. 10^8 cells per gram of the mixture. Two week-old plants were then transplanted into the steam-sterilized soil. Three leaf-stage plants were inoculated with a conidial suspension (5×10^5 conidia/ml) of *C. orbiculare*. Diseased leaf area (%) and number of lesions per cm^2 leaf were evaluated on third leaves of the plants, 5~6 days after inoculation. Among 64 strains tested, nine strains, GC-B19, GC-B35, GK-B18, MM-B22, PK-B14, RC-B41, RC-B64, RC-B65, and RC-B77 significantly ($P = 0.05$) reduced anthracnose disease compared to the untreated control. In contrast, some bacterial strains promoted susceptibility of cucumber to the disease. From the repeated experiments using the nine bacterial strains, GC-B19, MM-B22, PK-B14, and RC-B65 significantly ($P = 0.05$) reduced both diseased leaf area (%) and number of lesions per cm^2 leaf in at lease one experiment. These strains with control efficacy of 37~80% were determined to be effective ISR-inducing strains.

KEYWORDS: Anthracnose, *Colletotrichum orbiculare*, Cucumber, Induced systemic resistance, Soil bacteria

Colletotrichum orbiculare (Berk. & Mont.) Arx is the fungus causing anthracnose disease in cucumber as well as melon and squash (Wasilwa *et al.*, 1993). The fungus infects leaves, stems, and fruits of cucumber plants and severely hinders cucumber crop production (Thompson and Jenkins, 1985; Wasilwa *et al.*, 1993). Field losses caused by *C. orbiculare* have been reported to be more than 60% in the United States (Thompson and Jenkins, 1985).

Use of resistant cultivars is a well-known disease control strategy to protect cucumbers against *C. orbiculare* infection (Thompson and Jenkins, 1985). However, it is not always suitable since various races exist within populations of *C. orbiculare* (Wasilwa *et al.*, 1993). Consequently, application of agricultural chemicals has been a primarily control measure for anthracnose of cucumbers. Regulations on agricultural chemicals have resulted in the limitation and reduction of their use and development. This necessitates the development of alternative methods to control various pathogens for crop protection (Uri, 1998).

In attempts to search for alternative disease control methods, biological control has been evaluated by many researchers (Adams, 1990; Cook, 1993; Kumar, 1998; Mathre *et al.*, 1999; Moënne-Loccoz *et al.*, 1999; Smith and Goodman, 1999). Various methods of searching for microbial antagonists have been developed with *Botrytis cinerea* in cucumber and tomato (Dik *et al.*, 1999; Moline *et al.*, 1999), *Xanthomonas campestris* pv. *armoraciae* in radish (Han *et al.*, 2000), and *Rhizoctonia solani* in cotton (Kloepper, 1991). When searching for biocontrol agents for plant disease control, the safety of microorganisms, technical difficulties of use, and the costs of product development should be considered (Cook *et al.*, 1996).

Recently, induced systemic resistance (ISR) has been reported (Pieterse *et al.*, 1996; Van Loon *et al.*, 1998), which is mostly induced by pre-inoculation with plant growth-promoting rhizobacteria (PGPR) or fungi (PGPF) (Kloepper and Schroth, 1978). It has also been reported that PGPR mixtures could achieve high levels of control efficacy against foliar diseases such as angular leaf spot by *Pseudomonas syringae* pv. *lachrymans* and anthracnose by *C. orbiculare* (Raupach and Kloepper, 2000). Use of ISR expression by plants has additional benefits, i.e., the broad spectrum of protection against different pathogens including fungi, bacteria and virus, no selection of pathogen resistance, and its use is environmentally friendly. ISR is distinguished from the classical systemic acquired resistance (SAR) by the different signal pathway and resistance expression (Knoester *et al.*, 1999; Pieterse *et al.*, 1996; Press *et al.*, 1997; Van Wees *et al.*, 1997). In some cases of plants expressing ISR, PR-proteins were not accumulated (Hoffland *et al.*, 1995; Jeun *et al.* 2001; Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). In contrast to SAR, ISR can be expressed without hypersensitive reaction or necrosis in roots after treatment of PGPR (Kloep-

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per *et al.* 1980). Furthermore, ISR is independent of the accumulation of salicylic acid but ethylene or jasmonic acid plays an important role for triggering ISR (Van Loon *et al.*, 1998). Although there were some PGPR strains that effectively mediated ISR in the greenhouse, application of these strains often failed to protect against plant diseases in the field. Probably, the activity of soil bacteria inducing resistance may be influenced by the soil environment. Therefore, trials for increase of protection efficacy of the PGPR should be continuously carried out. In the other way, microorganisms effectively inducing systemic resistance should be searched in nature.

In our previous research (Chang *et al.*, 2000), we reported that 64 bacterial strains selected from 1,400 soil bacteria were antagonistic to *in vitro* mycelial growth of several fungal pathogens. With these strains, in this study, we attempted to select ISR-inducing bacteria against anthracnose fungus, *C. orbiculare* in cucumber. In addition, efficacy of ISR in the plants treated with the selected bacterial strains was examined.

Materials and Methods

Bacterial strain and treatment. Sixty-four soil bacterial strains (Table 1) inhibiting mycelial growth of eight plant pathogenic fungi: *Alternaria mali, C. gloeosporioides, C. orbiculare, Fusarium oxysporum* f. sp. *cucumerinum, F. oxysporum* f. sp. *lycopersici, Phytophthora capsici, Magnaporthe grisea,* and *Rhizoctonia solani* were used in this study (Chang *et al.*, 2000). The bacterial strains stored in the –72°C deep freezer were streaked on nutrient agar. Single colonies were inoculated into 5 m*l* of nutrient broth (NB) in 5 m*l* test tubes and incubated in a shaking incubator (150 rpm) for 24 hours. This pre-cultured bacterial suspension was poured into 500 m*l* of NB and incubated at 28°C in a shaking incubator (150 rpm) for 48 hours.

The cultured bacterial cells were harvested with 10 mM $MgSO_4$ buffer through centrifugation at 5000 g at 18°C for 10 min to eliminate culture media. After centrifugation, supernatants were discarded and bacterial cell pellets were washed twice with buffer by centrifugation. Bacterial suspensions were adjusted to ca. 10^8 cells per m*l* using a spectrophotometer. Six m*l* of the bacterial suspension were incorporated with 6 g of potting mixtures [peat moss (Acadian Peat Moss Ltd. Lamègue, New Brunswick, Canada) and TKS-2 (Floragard Product, Germany), 1 : 1.5 (v/v)]. Bacteria incorporated-potting mixtures were filled into each hole (3×3×5 cm) of a 128-hole plug tray. Ten-mM $MgSO_4$ buffer was incorporated into the mixtures as a control. Germinated cucumber (*Cucumis sativus*, cv. Baeknokdadagi) seeds were planted into the trays and then placed in a growth room with 16-h fluorescence lights (80 μmol $m^{-2}s^{-1}$) at 25°C.

Table 1. The list of 64 bacterial strains with antifungal activity, sources, and locations selected out of 1,400 strains isolated from various soils, Korea in 1998 used in this study

Bacterial strain	Source/Location	Bacterial strain	Source/Location
GC-B07	Grassland/Chunan	MW-B02	Mountain/Paju
GC-B17	Grassland/Chunan	MW-B10	Mountain/Paju
GC-B19	Grassland/Chunan	MW-B15	Mountain/Paju
GC-B23	Grassland/Chunan	MW-B18	Mountain/Paju
GC-B24	Grassland/Chunan	MW-B19	Mountain/Paju
GC-B26	Grassland/Chunan	MW-B24	Mountain/Paju
GC-B27	Grassland/Chunan	OA-B03	Orchard/Ansung
GC-B28	Grassland/Chunan	OA-B15	Orchard/Ansung
GC-B32	Grassland/Chunan	OA-B22	Orchard/Ansung
GC-B33	Grassland/Chunan	OA-B26	Orchard/Ansung
GC-B35	Grassland/Chunan	OA-B36	Orchard/Ansung
GK-B09	Grassland/Koyang	OA-B37	Orchard/Ansung
GK-B15	Grassland/Koyang	OA-B65	Orchard/Ansung
GK-B18	Grassland/Koyang	OC-B18	Orchard/Chunan
GK-B21	Grassland/Koyang	OC-B70	Orchard/Chunan
GK-B24	Grassland/Koyang	PK-B09	Pepper field/Koyang
GK-B25	Grassland/Koyang	PK-B14	Pepper field/Koyang
GK-B26	Grassland/Koyang	PK-B26	Pepper field/Koyang
GK-B28	Grassland/Koyang	RC-B33	Rice field/Chunan
GK-B29	Grassland/Koyang	RC-B37	Rice field/Chunan
LS-B01	Lake/Chunan	RC-B38	Rice field/Chunan
LS-B03	Lake/Chunan	RC-B39	Rice field/Chunan
LS-B42	Lake/Chunan	RC-B40	Rice field/Chunan
LS-B70	Lake/Chunan	RC-B41	Rice field/Chunan
LS-B80	Lake/Chunan	RC-B64	Rice field/Chunan
LS-B81	Lake/Chunan	RC-B65	Rice field/Chunan
MM-B01	Mountain/Chunan	RC-B77	Rice field/Chunan
MM-B03	Mountain/Chunan	RC-B78	Rice field/Chunan
MM-B16	Mountain/Chunan	RK-B26	Rice field/Koyang
MM-B20	Mountain/Chunan	RK-B41	Rice field/Koyang
MM-B22	Mountain/Chunan	VC-B11	Vegetable field/Koyang
MM-B25	Mountain/Chunan	VK-B14	Vegetable field/Koyang

Inoculum and Inoculation. Two weeks after sowing into the trays, cucumber seedlings were transplanted into 10 cm-diameter plastic pots containing 250 g steam-sterilized soil and grown in the growth room. Eight-11 days later after transplanting, cucumber plants at the 3-leaf stage were inoculated with 5×10^5 conidia/m*l* of *C. orbiculare*.

For inoculum, *C. orbiculare* was cultured on potato dextrose agar (Difco Laboratories, USA) in the darkness for 10 days at 28°C. Conidia of *C. orbiculare* were harvested with sterile distilled water amended with 0.03% Tween 20 (v/v) (Showa Chemicals Inc., Japan) and filtered through four layers of sterile cheesecloth to remove hyphal debris. The conidial suspensions were adjusted to 5×10^5 conidia/m*l* using a heamocytometer. The leaves of cucumber plants at 3-leaf stage, which were treated with the suspension of

bacterial strains, were inoculated uniformly with the conidial suspensions and placed into a humid chamber with 100% relative humidity at 28°C for 24-hours. The inoculated plants were placed again in the growth room adjusted to 16-hour fluorescent light (80 μmol $m^{-2}s^{-1}$) at 25°C.

Disease evaluation. Diseased leaf area (%) and number of lesions per cm^2 leaf on third leaves of cucumbers were evaluated 5~6 days after fungal inoculation using Matrox inspector version 2.2 as described by Kwack (2001). Disease protection rate was calculated by the formula, protection (%) = 100(1−x/y) in which x and y are diseased leaf area (%) or number of lesions per cm^2 leaf on the third leaves of plants treated with bacteria strains and 10 mM $MgSO_4$ buffer (control), respectively. The experiments were conducted with 10 replications.

From these screening of 64 strains, nine bacterial strains, GC-B19, GC-B35, GK-B18, MM-B22, PK-B14, RC-B41, RC-B64, RC-B65, and RC-B77 were selected and further tested. Preparations of bacteria, treatment, inoculation, and disease evaluation were done using the procedures described above. The experiments were conducted two times with 10 replicates each.

Data analysis. Statistical analysis of data was conducted using the Statistical Analysis System (SAS Institute, 1988). Percent data of diseased leaf area were transformed to arcsine before analysis. Analysis of variance was determined using the general linear model procedure and means were separated with the least significance difference at $P = 0.05$.

Results

When disease protection by 64 bacterial strains against *C. orbiculare* infection in cucumber plants was evaluated, certain strains induced systemic resistance (Fig. 1). Among 64 bacterial strains tested, 51 and 61% of the strains showed positive disease protection in the number of lesions and diseased leaf area, respectively, while others showed negative protection that indicated a promotion of anthracnose disease (Fig. 2). Among 64 strains, nine strains, GC-B19, GC-B35, GK-B18, MM-B22, PK-B14, RC-B41, RC-B64, RC-B65, and RC-B77 were capable of reducing cucumber anthracnose by *C. orbiculare*. These nine soil bacteria significantly ($P = 0.05$) reduced both diseased leaf area (%) and number of lesions per cm^2 leaf compared with untreated controls. However, strains MW-B10 and OA-B26 significantly ($P = 0.05$) increased both parameters.

The nine soil bacteria were selected and further tested in repeated trials to examine whether ISR was expressed consistently. Strains GC-B19, MM-B22, and PK-B14 significantly ($P = 0.05$) suppressed diseased leaf area (%) by the anthracnose fungus in one experiment on third leaves compared with untreated controls, while strain RC-B65

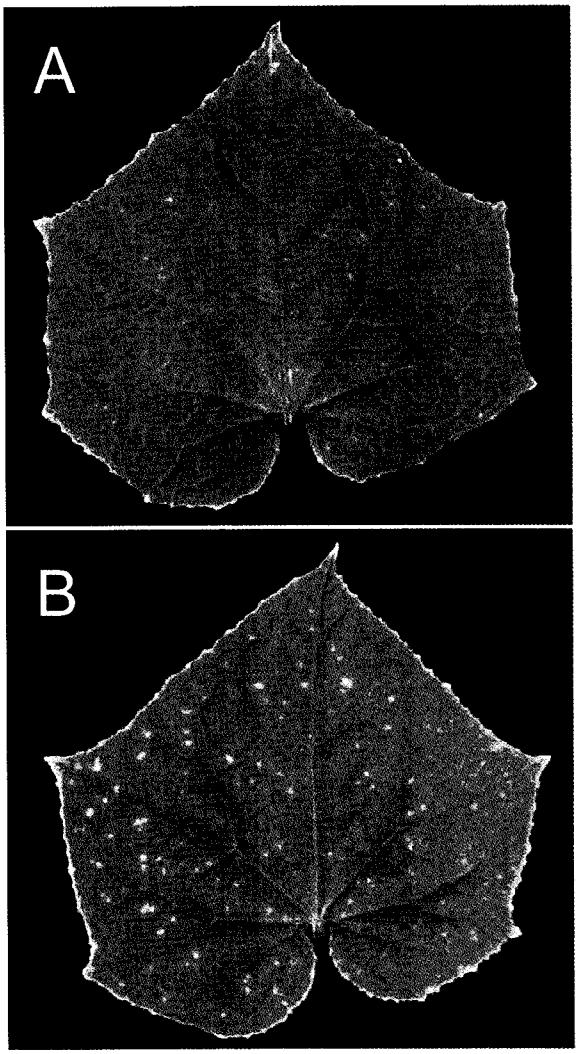

Fig. 1. Suppression of anthracnose disease in third leaves of cucumber plants plants (cv. Baeknokdadagi) treated with (A) a bacterial strain GC-B19 compared with (B) an untreated control. Bacterial cells (ca. 10^8 cfu/m*l*) were incorporated with potting mixtures at the level of 1 m*l* per gram of soil before seeding. Cucumber plants at 3-leaf stage were inoculated with 5×10^5 conidia/m*l* of *Colletotrichum orbiculare* and anthracnose lesions were appeared 5 days after inoculation. Inoculated control plants were treated with 10 mM $MgSO_4$ buffer instead of bacterial strains. Uninoculated cucumber plants did not show anthracnose symptoms.

showed consistent disease suppression in the two repeated experiments (Fig. 3A). These strains reduced anthracnose disease expressed as diseased leaf area (%) in the range of 36.8~80.4% for either experiment 1 or 2 compared with untreated controls (Table 2).

Similar results were obtained with the number of lesions per cm^2 leaf. Seven strains, GC-B19, GC-B35, MM-B22, PK-B14, RC-B41, RC-B65, and RC-B77 significantly ($P = 0.05$) suppressed disease expression by the anthracnose fungus in at least one experiments on third leaves com-

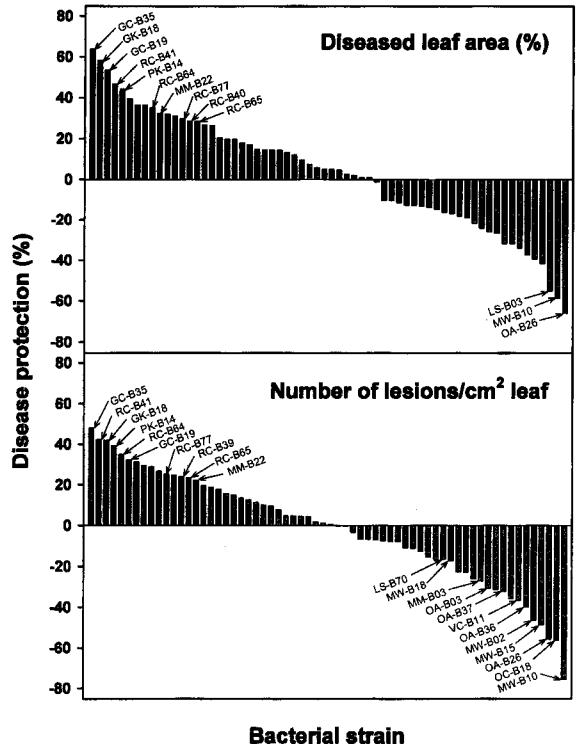

Fig. 2. Disease protection (%) of third leaves of cucumber plants against *Colletotricum orbiculare* by 64 soil bacteria based on diseased leaf area (%) and number of lesions per cm^2 leaf. Bacterial cells (ca. 10^8 cfu/ml) were incorporated with potting mixtures at the level of 1 ml per gram of soil before seeding. Cucumber plants at 3-leaf stage were inoculated with 5×10^5 conidia/ml of *C. orbiculare* and anthracnose lesions were evaluated 5~6 days after inoculation. Uninoculated cucumber plants did not show anthracnose symptoms. Bacterial strains that show significant ($P = 0.05$) disease suppression or promotion were indicated with arrows.

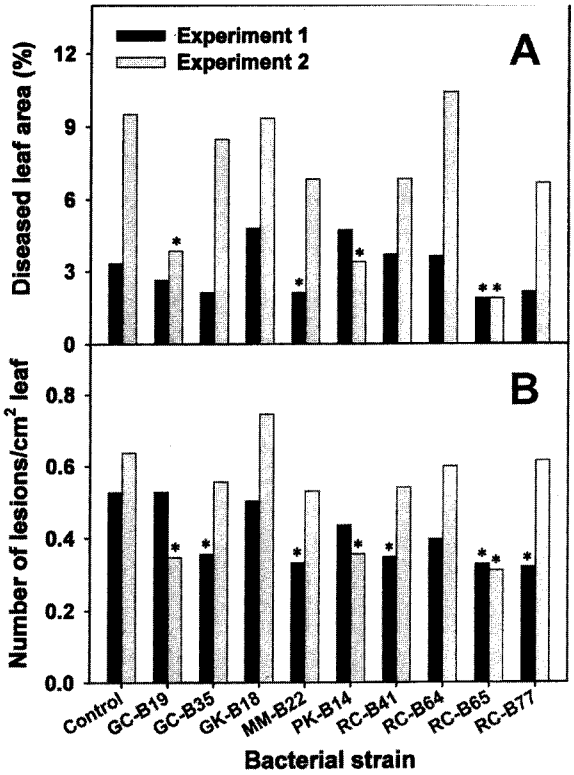

Fig. 3. (A) Diseased leaf area (%) and (B) number of lesions per cm^2 leaf on and third leaves of cucumber plants (cv. Baeknokdadagi) caused by *Colletotrichum orbiculare* after incorporation of nine soil bacteria inducing systemic resistance selected from 64 bacteria. Bacterial cells (ca 10^8 cfu/ml) were incorporated into potting mixtures at the level of 1 ml per gram of soil before seeding. Cucumber plants at 3-leaf stage were inoculated with *C. orbiculare* and anthracnose disease was evaluated 5~6 days after inoculation by using Matrox inspector version 2.2. Uninoculated cucumber plants did not show anthracnose symptoms. Data of diseased leaf area were arcsine-transformed before statistical analysis; however untransformed data are presented. Each value represents a mean of 10 replications. An asterisk indicates a significant difference compared to the control at $P = 0.05$.

pared with untreated controls (Fig. 3B). Once again, strain RC-B65 showed consistent disease suppression in the repeated experiments, as observed for diseased leaf area (%). These strains reduced anthracnose disease expressed as number of lesions per cm^2 leaf in the range of 32.4~51.1% for either experiment 1 or 2 compared with untreated controls (Table 2). In these repeated experiments, strains GC-B19, MM-B22, PK-B14, and RC-B65 effectively reduced both diseased leaf area (%) and lesion numbers per cm^2 leaf in the third leaves of cucumber compared with untreated controls (Fig. 3 and Table 2).

Discussion

Selection of rhizosphere or soil microorganisms was the first step to control cucumber anthracnose through ISR. For screening ISR-inducing soil bacteria, cucumber plants at 3-leaf stages in pots with bacterial strains having *in vitro* antifungal activity (Chang *et al.*, 2000) incorporated just before seeding were used in this study. In the prescreening, approximately 56% of the strains showed disease suppression against fungal infection, but others promoted anthracnose disease. This was also found in other plants with infections of *Drechslera teres, Microdochium nivale*, and *Tilletia caries* (Hökeberg *et al.*, 1997).

Plants usually could be physiologically changed by exogenous stimuli such as pathogens, chemicals or foreign microorganisms including soil bacteria. These changes may result in accumulation of phenylalanine ammonium lyase, pathogenesis-related proteins or active oxygen species that could lead expression of resistance against plant pathogens. However, if the resistance mechanism is not effective enough to defense the invasion of pathogens, the

Table 2. Disease protection (%) of third leaves of cucumber plants at 3-leaf stage against *Colletotrichum orbiculare* by nine soil bacteria inducing systemic resistance selected from 64 bacteria strains based on diseased leaf area (%) and number of lesions per cm^2 leaf

Bacterial Strain	Disease protection (%)[a]			
	Diseased leaf area (%)		Number of lesions/cm^2 leaf	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
GC-B19	21.0	59.7	0.0	45.4
GC-B35	36.5	10.8	32.4	12.7
GK-B18	0.0	1.8	4.5	0.0
MM-B22	36.8	28.2	36.9	16.8
PK-B14	0.0	64.6	17.3	44.1
RC-B41	0.0	28.2	33.9	15.3
RC-B64	0.0	0.0	24.6	6.0
RC-B65	44.0	80.4	37.5	51.1
RC-B77	36.0	30.0	39.0	3.5

[a]Disease protection percentage was calculated by the formula, protection (%) = 100 (1–x/y) in which x and y are diseased leaf area (%) or number of lesions per cm^2 leaf on the third leaves of plants treated with bacteria strains and 10 mM $MgSO_4$ buffer (control), respectively.

plants may fail to express resistance. Nevertheless, the stimulated plants by some soil bacteria consumed energy for the secondary metabolism and became weakly than non-stimulated plants (Kuc, 1995). The increased susceptibility of the plants by some soil bacteria to *C. orbiculare* may be explained with the deficiency of energy to defense in plants.

From this pre-screening, nine soil bacteria inducing systemic resistance to *C. orbiculare* in cucumber were selected and further tested in repeated trials since ISR sometimes did not express consistently. Liu *et el.* (1995) also observed this phenomenon. ISR expression by two ISR-inducing bacteria *Serratia marcescens* and *Pseudomonas putida* in cucumber cultivar Straight 8 against *C. orbiculare* varied in three repeated experiments. These results suggested that ISR effects by PGPR or soil bacteria were not consistent. Thus, repeated experiments are especially needed for screening effective PGPR or soil bacteria against plant pathogens. In our repeated experiments, we found that strains GC-B19, MM-B22, PK-B14, and RC-B65 reduced disease expression against *C. orbiculare* in at least one experiment on the third leaves of cucumber. Therefore, these bacterial strains were determined to be effective strains for induced systemic resistance against *C. orbiculare* in cucumber.

It is thought that the efficacy of disease protection by ISR-inducing strains may be related with root colonization. Liu *et al.* (1995) observed that population of PGPR strains decreased steadily but their ISR effects in cucumber were increased. These results may have indicated that ISR effect did not correlate with colonized bacterial population in root system or soil. However, Meera *et al.* (1995) found that persistence of ISR by PGPF against *C. orbiculare* was correlated with root colonization and expressed over 9 weeks. In our study, ISR effects by the selected soil bacteria were maintained with control efficacy of 37~80% over one month although we did not test the correlation of colonization with ISR effect. Further research with these effective strains inducing systemic resistance will be carried on the identification of the ISR-inducing bacteria, the relationship between colonization and ISR, and the mechanisms of ISR expression on cucumber with the ISR-inducing strains. Finally, the activity of resistance induction of these bacterial strains to the environment should be also examined through the field experiment.

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