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Biological control of Verticillium black spot of Japanese radish using *Bacillus* spp. and genotypic differentiation of selected antifungal *Bacillus* strains with antibiotic marker

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*Bacillus*属菌によるダイコン黒点病の生物防除と抗生物質耐性マーカーを付与した分離菌の遺伝的特徴

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ABSTRACT

Bacillus species can form endospores and have broad-spectrum activity of their antibiotics. For that reason, they offer several advantages over other bacteria for protection against root pathogens. This work was intended to determine the ability of *Bacillus* spp. to inhibit *Verticillium dahliae*. First, antagonistic activities of *Bacillus* spp. were studied in greenhouse trials. These results suggest that the bacterium can control soil-borne diseases. Secondly, to investigate the situation in a culture environment, *Bacillus* spp. were examined for their activity to control Verticillium black spot of Japanese radish and plant-growth promotion rhizobacteria (PGPR) effects in a field. After 2 months, these bacteria reduced Verticillium black spot of Japanese radish. The disease severity of those Biological Control Agent (BCA) treatments was less than in the pathogen control treatment. Furthermore, the plants treated with KB-3 showed the best PGPR effect. Field conditions show that *Bacillus* spp. isolate KB-2, KB-3 is a potential BCA against *V. dahliae*.

Subsequently, to select bacteria that are able to more control disease and more colonize in the plants and which facilitate recovery from soil and plants, *Bacillus* spp. were isolated from roots and were made resistant to ampicillin using UV irradiation. In all, 17 ampicillin-resistant mutants were tested for inhibition *V. dahliae* and *F. oxysporum in vitro*. Five bacteria isolates displayed inhibitory effects on FOM. All bacteria isolates indicated high antibiotic activity on *V. dahliae*. Then, to investigate the molecular characterization of all isolated strains of *Bacillus* spp., PCR amplification of the 16S-23S rDNA intergenic transcribed spacer (ITS-PCR), restriction fragment length polymorphism of the ITS-PCR (ITS-PCR RFLP), and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) were conducted. The ITS-PCR and ITS-PCR RFLP results suggest that KB-1 and strains of isolated from KB-1 were identical. Furthermore, those of the strains of KB-2 were also identical. However, KB-3 and all strains of isolates from KB-3 were different by ITS-PCR RFLP. Results suggest that the strains of isolates from KB-3 resembled those of KB-2. Using ERIC-PCR, 20 strains of *Bacillus* spp. could be distinguished.

Keywords: *Bacillus* spp., Biological control, Verticillium black spot of Japanese radish, *Verticillium dahliae*

INTRODUCTION

Japanese radishes (*Raphanus sativus* L. var. *longipinnatus* L.H.Bailey) are important products of Hokkaido, Japan because that area's cool climate is amenable to their production. However, soil-borne diseases occur often as a result of this characteristic regional feature. Verticillium black spot of Japanese radish by *Verticillium dahliae* causes yield losses in Japanese radishes in Hokkaido, especially in its occurrence was reportedfor the first time (Kitazawa and Suzui, 1980). Once Japanese radishes are infected, external symptoms are slight, but black lesions occur in the vascular system and root qualities are reduced considerably. *V. dahliae* is a commonly reported soil-borne fungal pathogen infecting several crops (Alstrom, 2001; Mercado-Blanco et al., 2004; Garmendia et al., 2004; Tjamos et al., 2004; Tahmatsidou et al., 2005). The pathogen can survive for 14 years or more in soil as microsclerotia, which are small, multicellular and melanized structures (Soesanto et al., 2001). Microsclerotia of *V. dahliae* that develop in senescing tissues of the dead plant might persist in soil for several years. Therefore, chemical control is nearly impossible (Berg et al., 2001). For that reason, it is difficult to control this soil-borne plant pathogen.

Application of agrichemicals and use of resistant cultivars have been developed to control soil-borne pathogens.

However, harmful effects to the environment and humans

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might result from those countermeasures. For that reason, soil disinfestation by steaming or fumigation is often used to control diseases on several crops. Methyl bromide, the most widespread soil fumigant, was banned in 2005 because it was detected in high concentrations in the earth's stratosphere (Noling, 2002). Consequently, an urgent need exists to develop ecologically compatible alternative methods. Some methods, such as soil solarization, irrigation (Katan, 1996), and crop rotation (LaMondia et al., 2002) are culture methods that are used for management. In addition, biological control is the active object of study in this field (Whipps, 2001; Emmert et al., 1999).

As biological control agents (BCAs), *Bacillus* species offer great potential benefits because of their ability to form endospores and because of the broad-spectrum activity of their antibiotics. Endospores have suitable features for use as BCA for structures that are capable of surviving desiccation, heat, oxidizing agents, and UV and radiation (Setlow, 1995; Nicholson, 2002). These characteristics allow for long-term storage and easy commercialization (Brannen and Kenny, 1997). *Bacillus* spp. produce a broad range of antibiotic compounds that are inhibitory to many plant pathogens (Silo-suh et al., 1994). Because antibiotics are produced from *Bacillus*, iturin A is widely known for its strong antifungal activity. It is a valuable antifungal agent that is known for its broad spectrum and low toxicity (Grau et al., 2001); surfactin is well known as a potent surface-active agent and as an antibiotic (Hiraoka et al., 1992). Furthermore, the species of *Bacillus* as plant growth-promoting rhizobacteria (PGPR) are beneficial native soil bacteria that colonize plant roots and engender improved plant growth (Kokalis-Burelle et al., 2006). Therefore, isolates of *Bacillus* have shown the capacity to control various plant diseases, e.g., damping-off of alfalfa seedlings (Handelsman et al., 1990), sugar beet cercospora leaf spot (Collins and Jacobsen, 2003), mummy berry disease (Scherm et al., 2004), and gray mould disease (Toure et al., 2004).

The first objective of this work was to evaluate the effect of *Bacillus* spp. as BCA in the control of Verticillium black spot in the greenhouse and in the field.

A greenhouse experiment was conducted in 2005 to evaluate antagonistic activity of *Bacillus* spp. to *V. dahliae* (unpublished data). Eggplants were used as the object plant. Results of this study show that some plants were diseased, but others exhibited controlled disease. *Bacilli* that more controlled disease and more colonized plants were selected: endophytic bacteria were isolated from roots of plants that controlled disease exceptionally. This endophytic relationship between the antagonistic bacterium and fungus and the plants suggests possibilities for biological control (Wagner and Lewis, 2000; Cao et al., 2005). In many studies that were conducted, endophytic bacteria were considered as candidate BCAs. Nejad and Johnson (2000) showed that suppressing vascular wilt diseases is possible and can involve induced resistance to soil-borne pathogens.

Study of the diversity of antifungal rhizobacteria using fingerprint techniques is important not only useful for elucidating their ecological role in the rhizosphere, but also for characterization of biological control agents for (i) registration and patenting biological control strains, (ii) recognizing the strains, (iii) quality checking during production, and (iv) ecological characterization (Lemanceau et al., 1995; Marten et al., 2000). Therefore, ITS-PCR and ITS-PCR RFLP methods were used to study whether the strains of isolates were *Bacillus* spp. Furthermore, genotypic diversity among the isolates was characterized by molecular fingerprinting methods using ERIC-PCR. It has been applied for typing of various *Bacillus* strains and *B. subtilis* (Shangkuan et al., 2000).

The secondary objectives of this work were (i) to select endophytic bacteria from roots and to evaluate their antagonistic activity to soil-borne plant pathogens (ii) to study genotypic differentiation of isolated *Bacillus* spp.

MATERIALS AND METHODS

2.1 Biological control of Verticillium black spot of Japanese radish

2.1.1 Microbial cultures

Plant pathogenic *V. dahliae* Kleb. (TS-21) obtained from an infected Japanese radish at Memuro in Hokkaido were used. The pathogenic fungi were cultured in darkness at 25°C for two weeks on PDA plates. These strains were then grown in moist gardening soil and wheat bran (4:1,w/w) for two weeks.

Three strains of *Bacillus* spp. were provided by Kiyomoto Bio. Co. Ltd.: *Bacillus subtilis* (KB-1), *Bacillus subtilis* (KB-2), *Bacillus vallismortis* (KB-3). One commercial preparation was stored at potato dextrose agar (PDA) slants at 4°C. For use, the bacteria were grown in darkness at 25°C for 7 days on a No. 802 (10 g polypepton, 2 g yeast extract, 1 g $MgSO \cdot 7H_2O$, 15 g agar, per liter) plate. For application, the bacterial inocula were grown in No. 802, except agar, using a rotary incubator (37°C, 24 h, 390 rpm). Later, the bacteria were cultured in wheat bran media and 5% molasses water (1:1, w/w) for 14 days; then they were dried completely in a tray (ca. 1×10^8 CFU/ml).

2.1.2 Greenhouse experiment 1.

First, three *Bacillus* strains (KB-1, KB-2, KB-3) were evaluated for control of *V. dahliae* performing with Japanese radish (*Raphanus sativus* L. var. *longipinnatus* L.H.Bailey) cv. "TAKAMIYA", susceptible to *V. dahliae,* under greenhouse conditions. One seed was sown in a jiffy pot (6 cm diameter; Sakata Seed Co.) filled with culture soil, then five pots were placed on a plastic tray. After seven days, 1 g of each *Bacillus* spp., which was cultured as described above, was inoculated around the plant stem. Four days later, 100 g of soil containing *V. dahliae* (5%) was put in a plastic tray. No-bacteria inoculation plots and non-treated antagonist and soilborne fungi plots were prepared as controls. The experiment was repeated three

times. The disease rate, expressed as a percentage of diseased leaves over the total number of leaves per plant, was observed for 30 days after *V. dahliae* inoculation.

2.1.3 Greenhouse experiment 2.

Plants were grown until germination in jiffy pots filled with culture soil. Each isolate of *Bacillus* was inoculated as described in 2.1.2. After six days, three jiffy pots were transplanted to 21 cm pots containing soil from a polluted field at the bottom of the pot and soil containing *V. dahliae* (5%) on the top. Non-inoculated plants and soil served as controls. These plants were grown for 60 days in a greenhouse. Afterwards, disease symptoms were assigned rankings according to a six-grade evaluation system. Fresh plant weight and fresh root weight were measured to evaluate plant promotion effects.

2.1.4 Field experiments

For evaluating plant disease suppression, three bacterial strains were tested in field experiments. The method of bacterial inoculation was 1 g of each *Bacillus* put on the bottom in a jiffy pot and filled with gardening soil. Two seeds were then planted and at once transplanted in the field. After germination, buds were thinned.

The experimental design was a complete randomized block with five replications for each bacterium. A field of about 19 m × 12 m consisted of nine ridges. A ridge of about 10 m × 1 m included six plots. Each plot was 50 cm wide and 3 m long; 15 Japanese radish seedlings were planted in each jiffy pot transplanted in plots at intervals of 20 cm. Planting was done on 26 July and harvesting on 9 September 2005.

At harvest, the incidence of naturally occurring disease in the field was observed. Furthermore, the attack rate was assessed: Disease incidence index (%) = (number of disease individuals)/ (number of all individuals) × 100. Plant growth measurements including the total and root weight were taken at harvest. In addition, commercialization of Japanese radish was estimated.

2.2 Isolation of bacterial antagonists

2.2.1 Isolation of endophytic bacteria from plant

To select bacteria that are better able to colonize in plant tissues and better control diseases, *Bacillus* spp. were isolated from internal tissues of eggplant roots. The plants had been used for evaluating antagonistic activity to *V. dahliae* in another experiment the previous year (unpublished data). Plants that indicated a high control effect were selected.

The bacteria were disinfected individually by soaking each in 70% ethanol for 1–2 min, followed by immersion in 5% sodium hypochlorite for 20–30 min. They were then rinsed three times in sterile distilled water and dried in hard filter paper. Finally, they were put on PDA and No. 802 and incubated in darkness at 25°C. If a colony appeared colony, it was purified on No. 802 and judged as *Bacillus* or not by their appearance.

2.2.2 Selection of ampicillin resistant *Bacillus* strains

A key to progress in the field of biological control to protect plants against soil-borne pathogens is to understand the interaction between biological control agents and pathogens in the rhizosphere. Isolates of bacteria were varied with UV irradiation and selected an ampicillin-resistant mutants to facilitate recovery from soil and plants. Ampicillin-resistant mutants of each strain were generated using the method described by Bacon and Hinton (2002).

A collection of 20 bacterial isolates (strains of origin to KB-1 were 7, KB-2 were 9, KB-3 were 4) were cultured on No. 802, except agar, in a shaking incubator for one day. All bacterial isolates were irradiated under UV light for 30 s, 1 min, and 2 min; they were subsequently applied on No. 802 supplemented with 50 µg/l of ampicillin at 37°C for one day in an incubator. The experiment was replicated five times. Therefore, ampicillin resistant bacterium were obtained and cultured on No. 802 supplemented with 100 µg/l of antibiotic. Thereby, 17 isolates of bacterial strains were recovered. The isolates comprise 10 strains of origin to KB-1 (1-A, 1-B, 1-C, 1-D, 1-E, 1-F, 1-G, 1-H, 1-I, 1-J), 4 strains of origin to KB-2 (2-A, 2-B, 2-C, 2-D), and 3 strains of origin to KB-3 (3-A, 3-B, 3-C).

2.2.3 *In-vitro* inhibition of phytopathogenic fungal mycelial growth by *Bacillus* spp.

A collection of 17 bacterial isolates was used for evaluation of *in-vitro* antagonistic activity against *V. dahliae* and *Fusarium oxysporum* on PDA. In this study, *F. oxysporum* f. sp. *melonis* (FOM) (race 0) was used.

All *Bacillus* strains were increased on No. 802, except agar, using a shaking incubator (37°C, 24 h, 390 rpm). After incubation, a suspension of *Bacillus* spp. was dropped on PDA and agar discs of *V. dahliae* or *F. oxysporum* placed on identical plates at 3 cm intervals. Zones of inhibition were measured after one week (FOM) or three weeks (*V. dahliae*). The trial was replicated three times.

To evaluate spore germination suppression, 17 isolates of *Bacillus* spp. were dual cultured with soil-borne pathogens. *Bacillus* spp. was cultured as described above. The *V. dahliae* and *F. oxysporum* were cultured in PDB for 2 weeks and adjusted to 1×10^6 spores/ml using a hemacytometer. Then, 0.2 ml of suspension was spread on PDA and cultures of six isolates of *Bacillus* spp. were dropped in a circle. Inhibition zones were measured after 7 days' incubation at 25°C. This trial was replicated five times.

2.3 Genotypic differentiation of *Bacillus* spp.

2.3.1 DNA extraction

Three strains of *Bacillus* and 17 isolates of ampicillin-resistant mutants were cultured as described

above. Then, 1.0 ml of bacterial suspensions was centrifuged at 4000 g for 5 min and the supernatant was removed. Cell pellets were resuspended in 0.5 ml of sterile distilled water and boiled for 15 min. After centrifugation at 3500 g for 3 min, 1 µl of supernatant fluid was used as template DNA in PCR assay (Li and Mustapha, 2002).

2.3.2 16S-23S rDNA ITS-PCR

Amplification of the 16S-23S ITS region was carried out as described by Ouoba et al. (2004): 25 µl of the reaction mixture containing 1 µl of DNA template, 2.5 µl of 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 0.5 µl of dNTPs (10 mM dATP, 10 mM dCTP, 10 mM GTP, 10 mM TTP), 1 µl of the forward primer 16S-1500F (50 pmol/µl) (5'-AA G TC GTA ACA AGG AA-3'), 1 µl of the reverse primer 23S-32R (50 pmol/µl) (5'-GCC ARG GCA TGG ACC-3'), 0.25 units of Taq polymerase (Sigma Genosys Japan Inc., Japan), and 18.75 µl of autoclaved Milli-Q water were used. Negative controls (no DNA template) were included in each trial to clarify that no contamination by DNA or reagents occurred.

The cycling program was started with an initial denaturation at 94°C for 5 min followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s, and elongation at 72°C for 30 s. Then, we performed 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s. The PCR was ended with a final extension at 72°C for 7 min. The amplified product was cooled at 4°C using a thermal cycler (iCycler; Bio-Rad Laboratories Inc., CA, USA).

To visualize the DNA fingerprint, 3 µl of amplification products were separated by electrophoresis in 2.0% agarose-TBE gels in 2 h 20 min at 50 V and visualized after staining with ethidium bromide under UV.

2.3.3 16S-23S rDNA ITS-PCR-RFLP

ITS-PCR-RFLP analyses were also performed as described by Ouoba et al. (2004). Each 5 µl of PCR product was digested for 16 h at 65°C by mixing 1 µl of NE buffer for Taq (10×), 1 µl of bovine serum albumin (BSA 100×), and 0.38 µl of Taq (TaKaRa Holdings Inc., Japan). The final volume was adjusted to 10 µl using sterile water. The DNA fragments were separated by applying 4.5 µl of each PCR product with 1 µl of loading buffer to 3% agarose gel for 4 h at 35 V. A 100 b DNA ladder (Takara) was used as a molecular size marker. The gel was soaked in ethidium bromide solution and DNA was visualized under UV light.

2.3.4 ERIC-PCR

To distinguish among all strains of *Bacillus*, ERIC-PCR was carried out using the ERIC1R primer (5'-ATG TAA GCT CCT GGG GAT TCA C-3') using methods described by Versalovic et al. (1991). The reaction mixture (25 µl volume) contained 2.5U of Taq polymerase, 2.5 µl of PCR buffer (10×), 0.5 µl of dNTPs, 1 µl of single primer, and Milli-Q water. We included negative control of contaminants in the reagents and reaction mixture. Amplification was performed using a thermal cycler with temperature ramping as follows: 95°C for 3 min to denature the template; 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and final incubation at 72°C for 10 min. Finally, 3 µl of amplification products with 1 µl of loading buffer were separated using agarose gel electrophoresis in 2.0% agarose gels in 2 h 20 min, and visualized using UV light.

RESULTS

3.1 Greenhouse experiment

The first experiment in the greenhouse showed the suppressive effect of three isolates of *Bacillus* on Verticillium black spot caused by *V. dahliae*, as shown in Fig. 1. All bacteria strains exhibited restrained disease incidence; three isolates of *Bacillus* and *V. dahliae* control showed statistically significant differences. Of them, KB-3 was the most efficient, by approximately 30%, in reducing disease severity in *V. dahliae* -inoculated plants.

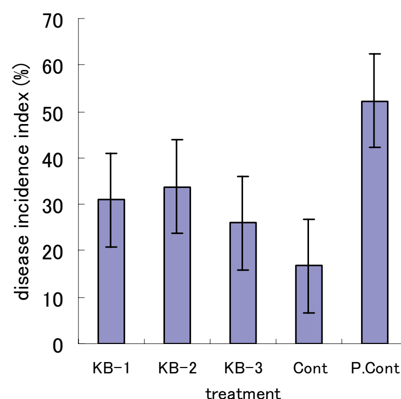

Fig. 1. Effect of *Bacillus* spp. in controlling Verticillium black spot under first greenhouse condition. Vertical bar indicates S.D.

Table 1. Comparisons of efficacy of biological control agents in control of *V. dahliae*, fresh plant weight and fresh root weight under second greenhouse conditions.

Treatment	DS (0-5)*	Fresh plant weight (g/plant)	Fresh root weight (g/plant)
KB-1	3.1a	53.6a	12.6a
KB-2	3.7a	61.6ab	19.1ab
KB-3	2.9a	59.2a	16.7a
V. dahliae	3.9a	56.8a	14.3a
control	0b	89.4b	33.3b

Means designated with the same letter are not significantly different as determined using Scheffe's F test (P<0.05).
*Scale 0–5 where: 0, healthy root; 1, about 20% of roots infected; 2, about 40% of roots infected; 3, about 60% of roots infected; 4, about 80% of roots infected; 5, 100% infected

In the second greenhouse study, three strains of *Bacillus* inoculation reduced disease symptoms in comparison with the untreated control (Table 1). Lesions on Japanese radishes treated with *Bacillus* spp. were smaller and less severe than those of Japanese radishes that had been treated

with *V. dahliae* alone. Superior disease control was obtained when KB-3 was applied. Furthermore, KB-2 and KB-3 inoculation increased plant flesh weight and plant root weight compared with the untreated control (Table 1). However, no treatments showed statistically significant differences.

3.2 Biocontrol of Verticillium black spot under field conditions

Three strains of *Bacillus* reduced disease incidence in the field experiment after 2 months (Fig. 2). Especially, KB-2 was the most effective against Verticillium black spot in three strains of bacteria. This strain showed 19% protection efficiency. Furthermore, KB-1 and KB-3 also showed suppressive effects against ***V. dahliae***. These results suggest that these strains of *Bacillus* offer the potential for use as BCAs against soil-borne plant pathogens. However, no treatments showed statistically significant differences.

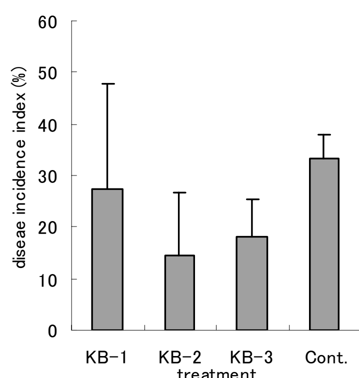

Fig. 2. Efficacy of three isolates of *Bacillus* in control of Verticillium black spot in a field experiment. Vertical bar indicated S.D.

Table 2. Efficacy of biological control agents in fresh plant weight, fresh root weight and commercialization rate.

Treatment	Fresh plant weight (g/plant)	Fresh root weight (g/plant)	Commercial-ization rate (%)
KB-1	883.3	561.8	51.4
KB-2	880.2	599.7	57.3
KB-3	946.0	649.8	64.2
Verticillium	888.8	634.5	52.0

Effects of antagonistic treatment on plant fresh weight, root fresh weight, and the commercialization rate of Japanese radish are listed in Table 2. Results of KB-3 inoculation suggest that it presents the possibility for PGPR. However, no significant difference from the control treatment was found in any treatments of *Bacillus* spp. inoculation.

3.3 PCR analysis

To classify the isolates, ITS-PCR and ITS-PCR RFLP were used. Using ITS-PCR, KB-1 and 10 strains of isolates from KB-1 were characterized by two bands of 456 and 282 bp (Fig. 3). Of those, 1-I and 1-J showed a slight difference from two additional bands, but it was not always reproducible. Three strains of isolates from KB-2 were characterized respectively by three bands of 456, 342 and 282 bp (Fig. 4). Also, KB-2 showed only a band of 282 bp, but in another experiment, other bands appeared (data not shown). Three others, KB-3, 3-A and 3-B, were characterized by three bands as well as the strains of isolates from KB-2 (Fig. 4). Also, 3-C showed only a band of 282 bp, but in another experiment, the remaining two bands appeared (data not shown). Using ITS-PCR RFLP (Fig. 5) KB-2, 2-A, 2-B and 2-D showed four bands of 339, 233, 159 and 154 bp. In addition, 3-A and 3-C showed the same bands as strains of KB-2, but KB-3 showed four bands of 275, 233, 159 and 154 bp. The strains of isolates from KB-1 showed 233, 159 and 154 bp (Fig. 6). However, another band was not clearly evident despite repeated attempts.

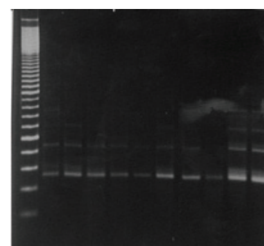

Fig. 3. Agarose gel of ITS-PCR products of strains selected from KB-1. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: KB-1, 1-A, 1-B, 1-C, 1-D, 1-E, 1-G, 1-H, 1-I, 1-J, respectively.
Lane m, 100 bp DNA ladder

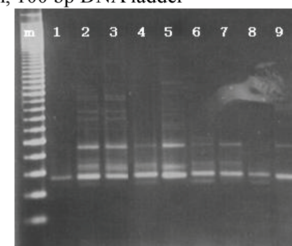

Fig. 4. Agarose gel of ITS-PCR products of isolates of *Bacillus*. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9: KB-2, 2-A, 2-B, 2-D, KB-3, 3-A, 3-B, 3-C, 1-F, respectively. Lanes m, 100 bp DNA ladder

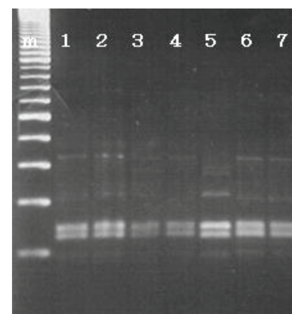

Fig. 5. Agarose gel of ITS-PCR RFLP products of strains of isolates from KB-2 and KB-3. Lanes 1, 2, 3, 4, 5, 6, 7: KB-2, 2-A, 2-B, 2-D, KB-3, 3-A, 3-C, respectively. Lanes m, DNA size marker

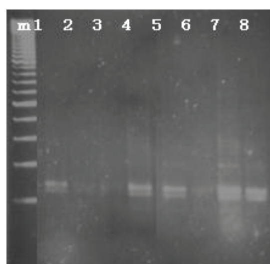

Fig. 6. Agarose gel of ITS-PCR RFLP products of strains of isolates from KB-1.
Lanes 1, 2, 3, 4, 5, 6, 7, 8: 1-A, 1-C, 1-D, 1-E, 1-G, 1-H, 1-I, 1-J, respectively.
Lanes m, 100 bp DNA ladder

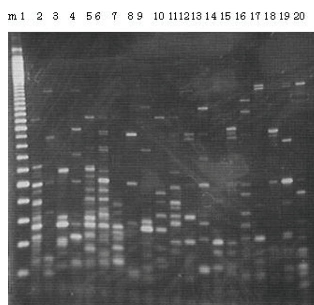

Fig. 7. ERIC-PCR genomic fingerprints of *Bacillus* spp. Lanes 1–20: KB-1, KB-2, KB-3, 1-A, 1-B, 1-C, 1-D, 1-F, 1-G, 1-H, 1-I, 1-J, 2-A, 2-B, 2-C, 2-D, 3-A, 3-B, 3-C.
Lanes m, 100 bp DNA ladder.

All the ERIC-PCR types of 20 isolated *Bacillus* spp. were unique (Fig. 7). Several DNA band patterns were observed. Sequencing of the 16S rDNA allowed the identification at KB-1 and all strains of isolates from KB-1. Furthermore, KB-2 and all strains of isolates from KB-2 were also identified. However, KB-3 and two strains of isolates from KB-3 were shown to be different and similar to KB-2 by their ITS-PCR RFLP results.

3.4 Antagonistic activity of biocontrol agent against soil-borne pathogens *in vitro*

Table 3. Inhibitions of hyphae growth in *Fusarium oxysporum* by *Bacillus* spp.

Isolates	Inhibition	Isolates	Inhibition
KB-1 A	-	KB-1 J	+
B	+	KB-2 A	-
C	+	B	-
D	+	C	-
E	-	D	-
F	+		
G	-	KB-3 A	++
H	-	B	++
I	+	C	-

Size of inhibition zone: -, no inhibition zone; +, 0-2 mm; ++, 2-4 mm; +++, 4-6 mm; ++++, more than 6 mm

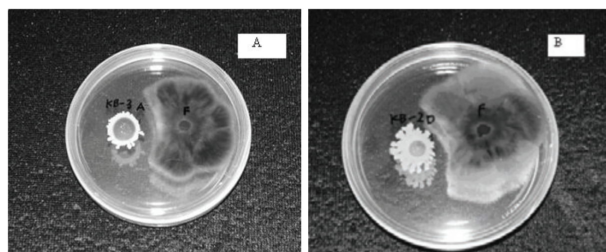

Fig. 8. Inhibition zone between *Bacillus* spp. (A, the strain of A was derived from KB-1, B, the strain of D was selected from KB-2) and *Fusarium oxysporum*.

F. oxysporum

Table 3 shows results of *in vitro* tests of antagonism toward *F. oxysporum*, a plant pathogen, using a dual culture technique on a PDA plate. The inhibition zone surrounding a colony of *Bacillus* spp. was identified clearly. The 13 isolates of 20 *Bacillus* spp. showed antifungal activity against *F. oxysporum*. The B • D strains were derived from KB-2; those of A • B • C that were derived from KB-3 inhibited the growth of FOM significantly (Fig. 8). In addition, a collection of eight bacterial strains was given the suppression of spore germination, and especially the strain of A • B derived from KB-3 displayed greatly inhibited spore germination (Table 4).

Table 4. Suppressions of *F. oxysporum* spore germination by *Bacillus* spp.

Isolates	Inhibition	Isolates	Inhibition
KB-1 A	+	KB-1 J	-
B	+	KB-2 A	-
C	+	B	+++
D	+	C	-
E	+	D	++++
F	+		
G	+	KB-3 A	++++
H	+	B	++++
I	-	C	++++

Sign meanings: -, no inhibition; +, inhibition;
++, clear inhibition

V. dahliae

All *Bacillus* strains suppressed *V. dahliae* growth significantly; inhibition zone diameters were greater than 6 mm (Table 5). Inhibition was considerable in the surrounding a colony of *Bacillus* spp. (Fig. 9). The 12 isolates of *Bacillus* spp. controlled spore germination in the pathogen. The F strain, derived from KB-1, and A, which was derived from KB-3, were more clearly suppressed to *V. dahliae* than other isolated bacteria (Table 6).

Table 5. Screening for antagonistic activity of *Bacillus* spp. to *V. dahliae*.

Isolates	Inhibition	Isolates	Inhibition
KB-1 A	++++	KB-1 J	++++
B	++++	KB-2 A	++++
C	++++	B	++++
D	++++	C	++++
E	++++	D	++++
F	++++		
G	++++	KB-3 A	++++
H	++++	B	++++
I	++++	C	++++

Size of inhibition zone: -, no inhibition; +, 0–2 mm;
++, 2–4 mm; +++, 4–6 mm; ++++, more than 6 mm

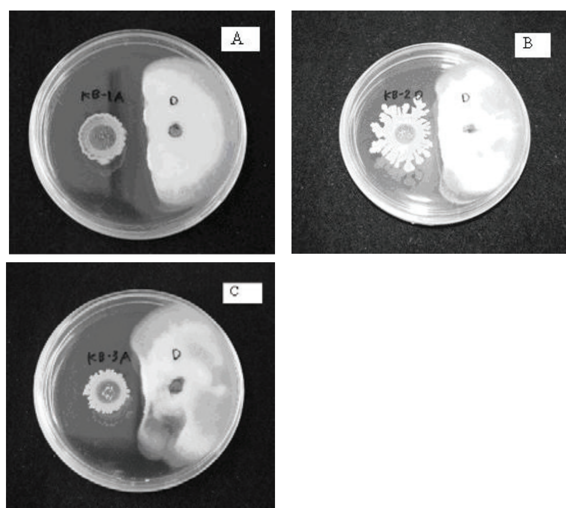

Fig. 9. Inhibition zone between *Bacillus* spp. (A, the strain of A was selected from KB-1; B, the strain of D was derived from KB-2; C, the strain of A was derived from KB-3) and *Verticillium dahliae.*

Table 6. Inhibitions of spore germination in *V. dahliae* by *Bacillus* spp.

Isolates	Inhibition	Isolates	Inhibition
KB-1 A	+	KB-1 J	+
B	+	KB-2 A	-
C	+	B	-
D	-	C	-
E	-	D	+
F	++		
G	+	KB-3 A	++
H	+	B	+
I	+	C	+

Sign meanings: -, no inhibition; +, inhibition;
++, clear inhibition

DISCUSSION

This paper reports the usefulness of three strains of *Bacillus* spp. of our laboratory for suppression of Verticillium black spot. Two greenhouse studies and a field study were conducted to analyze control effects.

In the first pot assay in a greenhouse, all isolates of *Bacillus* reduced leaf wilting significantly in comparison with *V. dahliae*-inoculated specimens. Three strains of bacteria that reduced symptom development reached 18–26% compared to treatments with no bacteria. Particularly, the *Bacillus* sp. isolate KB-3 suppressed diseases caused by all bacteria.

The second greenhouse experiment showed nearly healthy Japanese radish plants in bacterium-inoculated plots, but all plants in *V. dahliae* inoculated plots showed disease. *Bacillus* spp. showed suppression effects against *V. dahliae*, but the difference from control plants was not significant. We infer that the high density of the pathogen population used this study caused this result. Despite that situation, KB-3 better suppressed disease occurrence by about 20% than pathogen inoculation. Because the goal was to control disease symptoms in plant roots, these results suggest that *Bacillus* can colonize in roots and suppress *V. dahliae.* Several works indicate that colonization behavior of BCA is related to modes of disease suppression (Islam et al., 2005).

These results indicated that three strains of *Bacillus* spp. present the possibility for control soil-borne plant pathogens. Other works report that *Bacillus subtilis* were able to suppress wilt diseases of maize (Cavaglieri, 2005) and damping-off of tomato and cucumber (Kita et al., 2005). In addition, the ability of PGPR in growth promotion and resistance induction in various crops is well known and many studies have been conducted (Guo et al., 2004; Ji et al., 2006). Therefore, *Bacillus* spp. are adequate bacteria for commercialization as BCA. The experiment was conducted in a field that resembles that of an agricultural system to reflect this method’s potential use for commercialization.

In the field trial, reduction in symptom development of 18.6% was achieved by inoculating KB-2 and 15.3% by KB-3 compared to untreated controls under *V. dahliae* polluted soil. However not all strains of bacteria significantly suppressed soil-borne plant pathogens. Moreover, disease suppression and PGPR effects in the field were not as marked as those observed in the greenhouse. Because of environmental stresses and various populations of soil-inhabiting fungi, it might be difficult to study these phenomena in field conditions (Estevez de Jensen et al., 2002). Myriad microorganisms live in soil. Accordingly, competition occurs in the rhizosphere and at the root surface of plants, which offer suitable environments for almost all microorganisms and environmental resources (Cavaglieri et al., 2004). Therefore, competition for limited important resources and colonization in proper environments are the primary antagonism mechanisms. Two greenhouse experiments showed that *Bacillus* spp. is capable of controlling plant pathogens. Therefore, future studies should establish a

greater understanding of the dynamics of applied beneficial organisms under field conditions to optimize their application methods and timing. It is also important to understand the effects of applied biological control strains on populations of indigenous beneficial bacteria and known to suppress pathogen establishment and disease (Kokalis-Burelle et al., 2006).

Ampicillin-resistant *Bacilli* were selected using UV irradiation to examine how *Bacilli* suppress plant disease pathogens and how much are they colonize soils and plants. Therefore, this treatment would simplify re-isolation from soils and plants and facilitate more precise studies. In this study, 17 strains of ampicillin-resistant mutant of *Bacillus* were selected. *Bacillus* spp. is known to control soil-borne pathogens because they produce antibiotics that suppress the growth of competing microorganisms (Asaka and Shoda, 1996). To examine whether *Bacillus* spp. can control soil-borne pathogens or not, dual culture inhibition assays were conducted. All isolates of bacteria were examined for antibiotic activity; some strains showed excellent effects to *V. dahliae* and *F. oxysporum*. Strains of *Bacillus* derived from KB-3 showed better suppression effects to pathogens than the original bacterium (unpublished data). Ampicillin-resistant mutants are inferred to show increased ability to produce antibiotics. Antifungal activity of *Bacillus* spp. was resistant to high temperature, a wide range of pH, and the action of many hydrolytic enzymes (Souto et al., 2004). These characteristics indicate that mutants might control plant pathogens not only in a greenhouse, but also in the field, with its attendant complex conditions.

This study investigated original bacteria and strains of isolates from roots. The genotypic characterization of ampicillin-resistant mutants was investigated using ITS-PCR, ITS-PCR RFLP and ERIC-PCR. Results obtained by ITS-PCR analyses showed that all strains of mutant were identity of each of origin bacteria. Interestingly, *B. subtilis* (KB-1 and KB-2) and *B. vallismortis* (KB-3) showed identical bands. Recent taxonomic studies have revealed that *B. subtilis* is heterogeneous and should be considered as a complex of closely related species (Reva et al., 2004). Isolates that had been previously classified as *B. subtilis* are now recognized as *B. atrophaeus*, *B. mojavensis*, and *B. vallismortis*. Furthermore, *B. subtilis* in turn has been classified into two subspecies: *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii* (Nakamura et al., 1999). Because *B. subtilis* and *B. vallismortis* are phenotypically similar species, the results of PCR assay showed no differentiation among species.

Genotypic analyses of the rDNA region have been used to distinguish strains. RFLP analyses of the spacer region between the 16S and 23S rDNA gene provides a useful method for classification within species (Haque and Russell 2005). In this study, the type strain of 3-A and 3-C originates from KB-3 inoculated plants. However, the results demonstrated that these strains were assigned distinctly from KB-3 and similar to KB-2. The strains of 3-A and 3-C were inferred to be *Bacillus subtilis*, which is present in soils and colonizes roots. These strains had higher antibiotic activity to soil-borne plant pathogens than other strains. For that reason, they are new promising candidate strains as BCAs and should be examined *in vitro* and in a greenhouse.

The molecular typing technique based on the presence of repetitive DNA sequences that are dispersed throughout the genome of diverse bacterial species, such as REP-PCR and BOX-PCR, were used to discriminate various *Bacillus* species (Herman et al., 1998; Kim et al., 2001). In addition, ERIC-PCR was used as an approach based on targeting of repeated DNA sequences. It was used for characterization (Shangkuan et al., 2001). The data presented here indicate that ERIC-PCR can differentiate the isolates of strains. This result suggests that ERIC-PCR assay enabled classification of closely related strains of *Bacillus* isolate and that it can be useful in greenhouse and field studies as a molecular ecological marker.

In this study, these results suggest that *Bacillus* spp. had the ability to control *V. dahliae*. However, for practical use, *Bacillus* spp. must be more enhanced in their inhibition effects. Therefore, we hope that studies are conducted to explore mechanisms for control and the relationship between BCAs and plant pathogens in the rhizosphere and endorhizosphere. The application of ampicillin-resistant mutants will be an effective method in study. This kind of research has relevance to minimize the use of synthetic fungicides, thereby contributing to preservation of the environment.

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摘要

Bacillus 属菌は芽胞を形成することで悪条件下でも生存することができ、また広い宿主範囲をもつ抗生物質を産生する。このことから生物防除資材として適しているとされており、その可能性を探って多様な研究が行われている。そこで本実験では、キヨモト・バイオ（株）より分譲された*Bacillus*属菌３菌株（KB-1, KB-2, KB-3）を用いてダイコン黒点病に対する生物防除資材としての可能性について検討した。二回のポット試験を行った後、圃場内で発病抑制効果をみた。その結果、すべての菌接種区において病原菌単独処理区と比較して病気を抑制している傾向がみられ、特にKB-2、KB-3接種区で優れた効果がみられた。さらに土壌や植物体内から菌を分離しやすくし、より正確なデータを得るために、*Bacillus*属菌３菌株にマーカーを付与した。すなわち、UV 照射により突然変異を生じさせ、アンピシリンに対する抵抗性をもつ*Bacillus*系統を選抜した。以上の処理によって得られた合計１７菌株を用いて対峙培養を行い、*Verticillium dahliae*および*Fusarium oxysporum*に対する拮抗作用を調査した。その結果、*F. oxysporum*に対しては６菌株で、*V. dahliae* に対してはすべての菌株で優れた抑制効果がみられた。このことから変異株は病原菌の生育を抑制することが示唆された。また、ITS-PCR, ITS-PCR-RFLP, ERIC-PCRを用いてKB-1, KB-2, KB-3 とアンピシリン耐性変異株の遺伝的特徴を調べた。これにより遺伝的にもこれらの菌を識別することが可能となり、今後のポット試験・圃場試験での有効的な活用が期待される。

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