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Characteristics and Antimicrobial Effects of Novel *Burkholderia cepacia* No. 15-2 Isolated from Compost

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To develop the functional-compost containing antifungal substance by using antagonistic microorganisms, *Spinacia oleracea* L and *Rhizoctonia solani* Kuhn O-28 were used as a model plant and phytopathogen, respectively. Total 80 strains were isolated from the compost of various waste foods mixture processed for a year. Among them, No.15-2 strain was selected due to its highest antifungal activity against *R. solani* Kuhn O-28 and was identified phyno- and phylogenotypically as *Burkholderia cepacia* genomovar V, which is rare probability in pathogen, by 16S rDNA sequencing and specific primer pair PCR method. *B. cepacia* No.15-2 preferentially dominated during the compost and its cell numbers were maintained almost $\times 10^{13}$ cuf/g for 15 days. The morbidity caused by *R. solani* Kuhn O-28 in *S. oleracea* L cultivation was reduced to 40% by addition of *B. cepacia* No.15-2. In conclusion, the antifungal compost using *B. cepacia* No.15-2 could be applied to biocontrol of various crops` blights caused by fungal pathogen.

Key words: Antagonistic, biocontrol, *Burkholderia cepacia*, compost, pytopathogens

The agricultural medicines synthesized with chemicals have been widely used to prevent the blights in cultivation of vegetables and fruits in last decades because its high selectivity and long efficacy terms. In recent, however using the chemicals for cultivation of plants is recognized as a source of secondary pollution such as its accumulation in soil due to hard degradation [13]. Some microbes capable of preventing from crops blight by production of antimicrobial substances were reported as antagonistic microorganisms [8, 14]. Increasing attention of biocontrol using antagonistic microorganisms on phytopathogens is considered as an substitution for chemicals control due to its not only effectiveness with low concentration, low cost, and biodegradability but also its possibility of mass production.

Burkholderia cepacia is important in agricultural and clinical standpoint due to not only its pathogenic properties on human and plant such as cystic fibrosis but also its antagonistic activity. In recent, many studies about the taxonomic and pathogenicity of *B. cepacia* by using PCR technology have been reported [2, 10, 11]. From the results, *B. cepacia* is consisted in at least five neogentical similarity complex that is genomovar. Among the five genomovars I-V, genomovar II and III were suggested the main cause of pathogen [7, 11, 17].

Many kinds of antibacterial substances are produced in commercial scale. Nystatin and Amphotericin B that are effective on eubacteria such as *Candida* and *Cryptococcus* were known as antifungal substances. However, the use of nystatin and amphotericin B is incredulous due to their strong toxicity [21]. Therefore, the development of new antifungal substance, which is less toxic against human, is needed.

Compost has been widely used as a bioorganic fertilizer for agricultural cultivation because of its costless and environmentally friend fertilizer compared to chemicals fertilizer. However, the consumption of the compost is decreasing due to decreasing of farmland. Instead, more functional or special compost to cultivate for special crop was required by farmers.

The strategy of the study is to develop the functional-compost to prevent from various crops blights caused by fungi. In this study, to examine the pathogenic properties of *B. cepacia* No.15-2, which was isolated from the compost processed for an year. The taxonomic feature of *B. cepacia* No.15-2 and the prevention effects of antifungal compost

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on *Rhizoctonia solania* Kuhn O-28 during the cultivation of *Spinacia oleracea* L were described.

Materials and Methods

Screening

One hundred grams of compost of bark, food waste sludge, waste milk, and molasses, which was completely matured for a year, was obtained from 50 different parts of the compost plant (Kumisangyo, Hiroshima, Japan). To screen the bacteria producing antifungal substance, i) *Rhizoctonia solani* Kuhn O-28 (*R. solani* O-28), ii) *Fusarium oxysporum* f. sp. spinaciae O-27 (*F. oxysporium* O-27), iii) *Fusarium oxysporium* f. sp. ladicis lycopersici O-34 (*F. oxysporum* O-34) were used. The three fungi were inoculated on potato dextrose agar (PDA) plates and used as the target strains. To test of antifungal activity, 10% compost suspended with saline solution was centrifuged at 5000×g for 5 min and 60 il of the supernatant was poured in sterilized penicillin cup on the PDA plate inoculated target strains previously. The plates were incubated at 25°C for 3-7 days. The antifungal activity was evaluated by determination of diameter of clear zone formed.

Isolation and identification

The strains formed clear-zone in antifungal test were isolated by using PDA medium. The strains were inoculated in PD medium and cultured at 30°C for 2 days. The culture broth was also tested antifungal activity and the strain formed clear zone was isolated. Among them, the strain No. 15-2 showed the highest antifungal activity in the screening was examined the morphological, physiological, biochemical and biological characterizations [19]. To exam of sugar utilization, API20NE kit (BioMerieux, France) was used. The G+C content was quantified by DNA-GC Kit (Yamasa Shoyu Co. Ltd., ChibaKen, Japan) consists of the purified nuclease P1 preparation for degrading DNA into deoxyribonucleotides and the standard mixture of four purified deoxyribonucleotides. The hydrolysates and standard solution were applied to HPLC separately. The nucleotide complex such as dAMP, dGMP, dCMP, dTMP were used as an internal standard solution for quantification of the mole % G+C of DNA. The 16S rDNA sequence was analyzed by NCIMB Japan Co. Ltd. To check the pathogenicity by clear identification of *B. cepacia genomovars,* PCR method was used.

PCR primer used

The primers of SC-GII, BC-GV, and BC-R, which are specific for species within *B. cepacia* complex, were designed according to an alignment of the 16S rDNA gene sequences of five strains by NCIMB Japan (Table 1). The sequences of BC-GV, SC-GII and BC-R primer were obtained from 165-184, 578595 and 1005-1022 position of 16S rDNA gene sequences, respectively. PC-SSF and PC-SSR, which is primer pair, had been designed based on 16S and 23S rRNA gene sequences of ATCC 25416 (Genomovar I), respectively [12].

Genome DNA

Genome DNA was prepared using InstaGene Matrix (Bio-Red, Calf. U.S.A.) as following methods. A colony isolated was picked up and suspended it into 1 ml of sterilized water and centrifuged at 12,000×g for 1 min twice at 4°C. The supernatant was discarded and the precipitate was suspended with 200 μl of InstaGene Matrix. The mixture was incubated at 56°C for 20 min and mixed well again for 10 seconds. Then it was shocked at 100°C for 8 min. After mix it for 10 seconds, it was centrifuged at 12,000×g for 2 min at 4°C and 20 μl of the supernatant was used for PCR reaction.

PCR reaction

Fifty microliter of PCR reaction were composed of 20 μl of template DNA, 25 μl of Premix *taq* polymerase, and 2.5 μl of each Primer. The PCR reaction was denatured at 95°C for 1 min, 30 cycled at 94°C, 1 min (denature), 55°C for 1 min (aniling), 72°C for 1 min (extension), and extended at 72°C for 4 min. Agarose gel electrophoresis was performed to check the PCR product using Mini-gel electrophoresis system (Mupid EosmoBio, Tokyo, Japan) at 100V for 30 min. DNA band was visualized by ethidium bromide (Wako Chemical, Tokyo, Japan) staining and trans-illuminator (UVP NTFL-40, Funakoshi, Tokyo, Japan).

Pursuit of *B. cepacia* No.15-2 by SS-PCR

To detect of *B. cepacia* No.15-2 in the complicate sample mixed various microorganisms, *B. cepacia* JCM5964T, *Ralstonia pickettii* JCM 5669T, *Pseudomonas aeruginosa* JCM2776, *B. cepacia* No.15-2, strain No.1 and strain No. 3 recognized as *Pseudomonas* species were used. BC-GV and BC-R (Table 1) that is specific primer pair with *B. cepacia* genomovar V [3] was used. BC-GII primer

was detected at position 595 for *B. multivorans*.

To pursuit of *B. cepacia*, SS-PCR method *B. cepacia* JCM 5964T and *B. cepacia* No.15-2 were amplified but not *Ralstonia Pickettii*, *B. cepacia* and *Pseudomonas aeruginosa*. It was considered that SS-PCR method is suitable to detect for specific species from the mixture of various microorganisms. Therefore, SS-PCR method was used to explore the movement of *B. cepacia* No.15-2 during composting process.

To examine the pathogenicity of cepacia No.15-2, primer pair PCR method developed by Lipuma *et al.* [10]. The primer pair (G1)-(G2) specifically amplified the prototypic isolates of genomovars I and III for *B. substilits* [21]. These two species also shared sequences that is different from genomovars I, III and IV between positions 1005 and 1013. A reverse primer, BC-R, was designed based on these differences to specify for both *B. multivorans* and *B. vietnamiensis*. Sequence differences among genomovars I, III, and IV were insufficient to allow design of primers specific for these species. However, a primer pair, PC-SSF and PC-SSR, designed based on ATCC 25416 sequence data, amplified genomovar I, III, and IV. These primers target 16S and 23S sequences, respectively, and their use in PCR reaction results in amplification of polymorphic fragments of 16S-23S intergenic spacer region DNA (Table 1).

Antimicrobial activity

The culture broth (40 ml) of *B. cepacia* No.15-2 grown for 30°C for 3 days was centrifuged. The supernatant was dialyzed and lyophilized. The lyophilized substance was resuspensed into 1 ml of 50 mM Tris-HCl (40 times concentrated solution) and sterilized by filteration prior to use it. Eighty microliter of the solution was placed on the test plates prepared previously. The nutrient agar plates for bacteria and PDA plates for yeast and fungi were used.

Preparation of seed culture

Ten grams of compost was transferred into 100ml flask and suspended with deionized water. The pH was adjusted to 6.0-7.0. The strain No.15-2 cultured in 400 ml nutrient broth at 30°C for 24h was centrifuged at 15,000×g for 5 min at 4°C. The harvested cells were suspended into 200 ml of fresh NB medium and mixed it well with compost. The mixture was used for cultivation of *Spinacia oleracea* L and for composting of mixture of bark, food waste sludge, waste milk, and molasses at 30°C for 30 days. Ten grams of the compost was taken at 3, 9, 15, and 30 days and used as a sample for enumeration of target strain, total DNA, and PCR reaction.

Morbidity in *S. oleracea* L cultivation

Spinacia oleracea L. (Tohoku Seed, Saitama, Japan) was used as a model plant. The cultivation of *S. oleracea* L. was performed in a growth-cabinet (25-28°C, moisture 70%) in Hiroshima University. Ten kilograms of sandy roam and 3.5 L of perlite were used as a soil per one planter (15 L). The composition of fertilizer per planter was ammonium sulfate, 7.5 g (300 kgN/ha); phosphorus lime, 8.6 g (344 kgP_2O_5/ha); potassium sulfate, 1.1g (120 kg K_2O/ha); magnesia lime, 20 g (400 kg/ha). In the planter added compost, half amount of compost was replaced by ammonium sulfate. Two kinds of compost processed for 30 days (this study) and 1 year (Kumisangyo) were used.

In each planter, 15 pieces of seeds of *S. oleracea* L were sowed and cultivated for one month. The morbidity of damping-off or stem canker symptoms of *S. oleracea* L. was evaluated by counting the seeds delayed germination and non-germinated of *S. oleracea,* or break down of stem right after germination for 2 weeks.

Table 1. The sequences of primers used in genomovar determination of the *Burkholderia cepacia* complex.

Primer	Sequences (5`-3`)	Target site	Nucleotide position [a)]
PC-SSR	GCCATGGATACTCCAAAGCA	23S rDNA–*B. cepacia* genomovar I	NA
PC-SSF	GGGATTCATTTCCTTAGTAAC	16S rDNA–*B. cepacia* genomovar I	9941013
BC-GII	AGGCGGTCTGTTAAGACA	16S rDNA–*B. multivorans*	578595
BC-GV	TAATACCGCATACGATCTAT	16S rDNA–*B. vietnamiensis*	165184
BC-R	AGCACTCCCGAATCTCTT	16SrDNA–*B. multivorans & B. vietnamiensis*	10051022

[a)]Numbering corresponds to 16S rDNA sequences in GenBank whose accession numbers are provided in the text. NA, not applicable.
*Numbers correspond to position in the sequence with GenBank accession numbers X16368 for primer PC-SSR; all other numbers correspond to position in the sequence with GenBank accession number X87275.
* Primer PC-SSR is also called G1, and primer PC-SSF is also called G2, as described by Lipuma *et al.* [10].

Results and Discussion

Total 80 strains were isolated from the compost of mixture of barks, food waste sludge, molasses and waste milk etc. All strains were screened of their antifungal acitivity against *R. solani* O-28, *F. oxysporum* O-27, and *F. oxysporum* O-34 (data not shown). Among them, 27, 31, and 22 strains were showed antifungal activity against *R. solani* O-28, *F. oxysporum* O-27, and *F. oxysporum* O-34, respectively, which were known as phytopathogens in *S. oleracea,* potato, tomato and carrot. The antifungal activity of strain No. 15-2 against *R. solani* O-28 was shown in Fig. 1. The inhibition zone formed by suspension of the compost containing strain No.15-2, which is isolated from the compost of bark, food waste sludge, waste milk, and molasses completely matured for a year, was obviously bigger than control. Hence, the strain No.15-2 was identified and used to make functional-compost producing antifugal substances.

The morphological and biochemical properties of strain No.15-2 are showed in Table 2. The strain No.15-2 was suggested preliminary as non-coliform bacteria, such as *Burkholderia cepacia* or *Agrobacterium radiobacter* from the results of gram negative, short rod type, oxidase and catalase positive [2]. After API20NE test for utilization of sugars, and G+C content (63.2 mole %) analysis, the strain No.15-2 was preliminary identified as *Burkholderia cepacia*. Moreover, to ensure the identification of strain No.15-2, 16S rDNA sequencing was analyzed and its similarity with neighbor join tree was compared (Fig. 2).

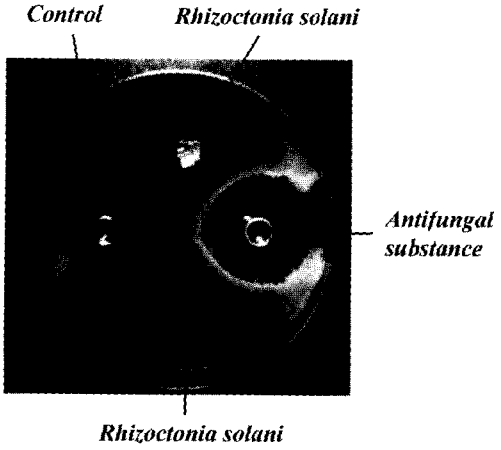

Fig. 1. Antifungal activity of strain No.15-2 against *Rhizoctonia solani* kuhn O-28.

Table 2. Morphological and biochemical characteristics of strain No. 15-2.

Items	Characteris
Shape	Short rod
Gram stain	–
Color of colony	Milk-white
Oxidase	+
Catalase	+
OF test	Oxidative
G+C content (%)	63.2
Biochemical test	
Nitrate reduction	+
Indole production	–
Arginine dihydrolase production	–
Urease production	+
Decomposition of esculin	+
Decomposition of gelatin	
β-Galactosidase production	+
Utilization of carbohydrates (API20NE)	
Glucose	+
Arabinose	+
Mannose	+
Mannitol	+
N-acetylglucosamine	+
Maltose	
Potassium gluconate	+
Caplic acid	+
Adipic acid	–
Malic acid	+
Sodium citrate	+
Phenylacetate	+

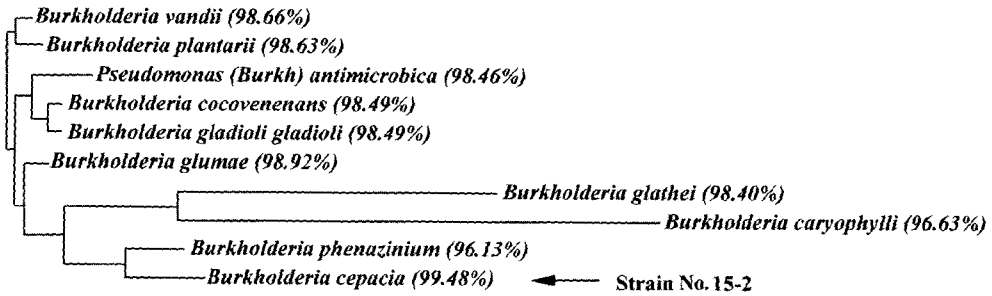

Fig. 2. Similarity of 16S rDNA sequence of *B. epacia* No.15-2 to *Burkholderia* species.

From the results, the strain No.15-2 was identified as *Burkholderia cepacia* because of its 99.48% similarity.

However, unfortunately *B. cepacia* are not easily identified and still problematic [19]. Many reports have demonstrated misidentification rates as high as 20% [1, 9]. Recently, Whitby *et al.* [22] and Lipman *et al.* [11] have developed the method with the high degree of identify of the *B. cepacia* complex using primer pair PCR method. In taxonomical, it was suggested that strain *B. cepacia* consisted of at least five different species, *i.e.* genomovars pending definitive biochemical and molecular methods to allow discrimination [20]. It is also called as *B. cepacia* complex. *B. cepacia* genomovar I-V includes *B. cepacia* genomovar I, *B. multivorans* (genomovar II), *B. cepacia* genomovar III, *B. cepacia* genomovar IV, and *B. vietnamiensis* (genomovar-V) [6, 15-17]. Among *B. cepacia* complex, *B. cepacia* genomovar III is known as a main cause of increasing of morbidity and mortality by cystic fibrosis in human.

B. cepacia No.15-2 is included in genomovar-V caused by sensitivity of specific primer amplification of genomovar-V, such as BC-GV and BC-R primer pair. Therefore, *B. cepacia* No.15-2 was identified as a *B. cepicia* genomovar V. Since the possibility of pathogen of *B. cepacia* No.15-2 is low, the compost of barks, waste food sludge, molasses used in this study can be applied to prepare functional compost.

Culture condition

To explore the optimum culture condition, some factors affecting the growth of *B. cepacia* No.15-2 were examined (data not shown). From the results, no effect of various sugars for the growth of *B. cepacia* No.15-2 was obtained. The growth of the cells in the medium containing organic acids as a carbon source was higher than that of sugars. Among organic acids, highest growth was obtained by using glutamic acid. In the medium containing glucose, fructose, sucrose and glycine, the pH was declined to 2.0-3.0. However, when nutrient broth, organic acid and glutamic acid were used, the pH was near to 9.0. Also no significant effect of some metal ions was shown. In conclusion, the culture of *B. cepacia* No.15-2 was performed with nutrient broth at initial pH 6.0-7.0, 30°C for 24 h.

Antimicrobial spectrum

Table 3 shows that no antimicrobial activity of the culture extract of *B. cepacia* No.15-2 against all tested bacteria strains such as *E. coli*., *Bacillus*, and *Staphylococcus* sp. was obtained. In contrary, the culture extract inhibited the growth of all fungi and yeasts resulted in formation of clear zone. However, the clear zone formed from *Aspegillus* and *Rhizopus* plates disappeared after 2 days. Therefore, the activity was examined by using high concentration of commercial nystatin (0.1 mg/ml), which is typical antibiotic for eubacteria, because it was suspected that the concentration was too low. From the result, however the clear zone formed by nystatin was also disappeared after 2 days. Therefore, it was suggested that the efficacy of antifungal substance produced by *B. cepacia* No.15-2 is useful for only initial growth stage of *Rhizopus* and *Aspergillus*. The substance produced by *B. cepacia* showed more effective antifugal activity by even its low concentration on yeasts than that of fungi.

Table 3. The antimicrobial spectrum of substance produced by *B. cepacia* 15-2.

Microorganisms	Clear zone (mm)
Escherichia coli IFO 3301	–
Bacillus subtilis IFO 3183	–
Staphyrococcus aeureus IFO 3007	–
Pichia membranifaciens JCM 1442	22
Candia albicans JCM 1542	24
Schizosaccharomyces octosporus JCM 1801	20
Saccharomyces cerevisiae JCM 2220	21
Aspergillus oryzae JCM 2058	22*
Rhizopus oryzae JCM 5557	26*
Rhizoctonia solani Kuhn O-28	24

Note: All values represented mean value of triplicates. –, not detected.
*The activity was disappeared soon.

B. cepacia has been considered as a good candidate to use as seed inoculants and root dips for biological control of soil-borne plant pathogens [23] because of its ability to produce several antibiotics. Most of them have antifungal activity, such as cepaciamide A, cepacidine A and xylocandin complex [5, 9, 14]. Also, Janisieewicz and Roitman [4] reported that *B. cepacia* is a ubiquitous bacterium with potential for biological control against fungal pathogens.

Preparation of antifungal compost

To achieve the compost of barks, food waste sludge and wastewater etc., its extract was used for the growth of *B. cepacia* No.15-2. The 3 days cultured *B. cepacia* No.15-2 was inoculated to compost and the numbers were measured

(data not shown). Almost $\times 10^2$ cuf/g-compost of *B. cepacia* No.15-2 was increased for 3 days and $\times 10^{13}$ cuf/g-compost cell numbers were maintained for 15 days. However, the cell numbers were decreased to below $\times 10^8$ cfu/g or no cells existed after 15 days because the experiment was not performed for the cells number of below 10^8. It was suggested that the most of *B. cepacia* No.15-2 in the compost progressed for 30 days were died because of nutrient exhaust or lack of moisture and air. It was conformed that the cells were not existed in the compost processed for 30 days. In addition, *S. oleracea* L was infected by phytopathogenic fungi during its germination for 15 days. Therefore, *B. cepacia* No.15-2 can be used for antifungal fertilizer to prevent the plant blight caused by *R. solani* Kuhn O-28 for cultivation of *S. oleracea* L.

The colonies suspected as a *B. cepacia* No.15-2 were selected randomly and determined their antimicrobial activities (data not shown). The results showed that all colonies were obtained the antimicrobial activities. *B. cepacia* No.15-2 are preferentially dominated in the compost and the prevented the growth of fungi and actinomyces.

Valuation of antifungal compost

Fig. 3 illustrates that the morbidity in planters contained composts, *R. solani* L, which is contained pathogen or *B. cepacia* No.15-2. In the planter No.2 and 5, which is containing soil and compost with *R. solani*, showed high morbidity of 70%. However, in the case of addition of compost (planter 4 and 7), the morbidity was reduced to less than 40%. In the compost, which was not contained *B. cepacia* No.15-2 and infected by pathogen, *R. solani* O-28, the morbidity was not reduced. The results indicated that the reduction of morbidity by *R. solani* O-28 in cultivation of *S. oleracea* L was caused by *B. cepacia* No. 15-2.

The results clearly demonstrated the utility of the antimicrobial compost in Fig. 4. In the planters containing compost, the growth of *Spinacia oleracea* L was higher

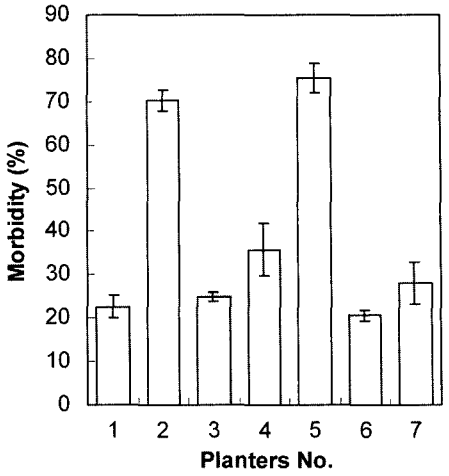

Fig. 3. Morbidity caused by *R. solani* in cultivation of *S. oleracea* L in various planters. 1, control (Soil + fertilizer); 2, Soil + fertilizer + *R. solani*; 3, Soil + fertilizer + compost included *B. cepacia* No.15-2; 4, soil + fertilizer + compost (this study) included *B. cepacia* No.15-2 + *R. solani*; 5, Soil + fertilizer + compost (this study) + *R. solani* except *B. cepacia* No.15-2. 6, Soil + fertilizer + compost (Kumisangyo); 7, Soil + fertilizer + compost (Kumisangyo) + *R. solani*.

than that of soil alone (control planter I). *R. solani* is known as a major cause for pathogen in 250 species of plants. Therefore, the compost could be used for various kinds of plants infected by *R. solani*. *B. cepacia* [23], previously known as *Pseudomonas cepacia* and originally considered only as a phytopathogen causing soft rot of onions [1], emerged in the early 1980s as a multidrug resistant species.

In conclusion, therefore, these characteristics may be exploited in the bioremediation of contaminated soil and water, or in agriculture through the biocontrol of phytopathogens.

Acknowledgement

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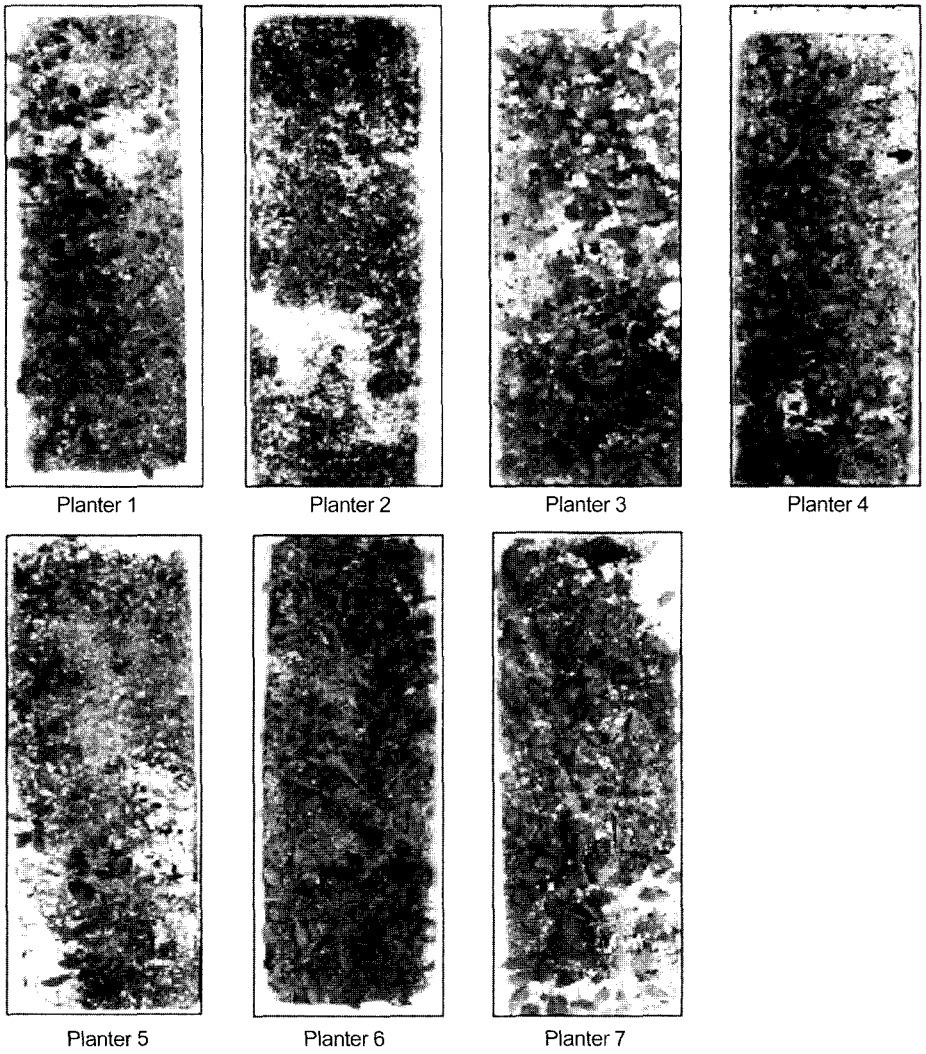

Fig. 4. Cultivation of *Spinacia oleracea* L in various planters. The content of all planters were the same as described in Fig. 4.

초 록

퇴비로부터 분리된 *Burkholderia cepacia* No.15-2의 특성과 항균 효과

윤순일

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길항미생물을 이용한 항곰팡이성 퇴비의 개발을 목적으로 공시 식물로써 *Spinacia oleracea* L과 식물 병원균 *Rhizoctonia solani* Kuhn O-28을 모델로 사용하여 수행 되었다. 다양한 음식쓰레기를 일년동안 숙성시킨 퇴비로부터 80 균주를 분리하였고, 그 중 No.15-2 균주가 *R. solani* Kuhn O-28에 대해 가장 높은 항곰팡이 활성을 보였다. 16S rDNA sequencing과 primer pair PCR에 의해 표현형과 분류학적으로 거의 독성이 없는 것으로 밝혀진 *Burkholderia cepacia* genomoval V로 분류되었다. 이 B. cepacia No. 15-2가 퇴비화 중에 우점하였으며 그 균수는 15일 동안 거의 10^{13} cfu/g을 유지하였다. *S. oleracea* L을 배양 했을 경우 *R. solani* Kuhn O-28에 의한 발병율은 *B. cepacia* No.15-2를 첨가함으로써 40% 감소하였다. 결론적으로 *B. cepacia* No.15-2는 다양한 식물의 곰팡이 유래의 병을 방제하는 데 이용 가능할 것으로 사료된다.

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