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Isolation and Characterization of Endophytic Actinomycetes from Chinese Cabbage Roots as Antagonists to *Plasmodiophora brassicae*

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This study was conducted to select endophytic actinomycetes as biocontrol agents against Chinese cabbage clubroot caused by *Plasmodiophora brassicae*. A total of 81 endophytic actinomycetes were isolated from surface-sterilized roots of Chinese cabbage that was grown on paddy field and upland soils collected from various locations in Korea. By using 16S ribosomal DNA (rDNA) gene sequencing, they were classified to 8 actinobacterial genera. The genus *Microbispora* (67%) was most frequently isolated, followed by *Streptomyces* (12%) and *Micromonospora* (11%). Three of the 81 isolates, when inoculated in germinated Chinese cabbage seeds and then transplanted to pots, effectively suppressed the occurrence of a post-inoculated strain of *P. brassicae* in the pots. They showed control values of 58% for strain A004, 33% for strain A011, and 42% for strain A018. Based on cell wall components, morphological characteristics, and phylogenetic analyses, the three antagonistic isolates were identified as *Microbispora rosea* subsp. *rosea* (A004 and A011) and *Streptomyces olivochromogenes* (A018). Further researches on the field efficacy and action modes of the three actinomycetes are in progress.

Keywords: Clubroot, endophytic actinomycete, *Microbispora rosea* subsp. *rosea*, *Plasmodiophora brassicae*, *Streptomyces olivochromogenes*

Chinese cabbage is one of the main crops in Korea. Clubroot disease caused by *Plasmodiophora brassicae* Wor. is regarded as one of the destructive diseases of Chinese cabbage production. *P. brassicae* is a soilborne fungal pathogen and survives in soils for a long time in the absence of hosts as resting spores [17]. The disease is characterized by the proliferation of galls on infected roots, which causes wilting and seriously reduces yield. Young plants are killed by the disease within a short period of infection, whereas older plants may remain alive but fail to produce marketable heads, resulting in severe economic losses. Researches on the development of biocontrol agents against clubroot using root colonizing fungi and soilborne bacteria have been previously reported by Cheah and Page [5], Elsherif and Grossmann [10], Kim *et al.* [13], and Narisawa *et al.* [18–20].

Actinomycetes as potential biocontrol agents were reported in the protection of the plant host against pathogens and the influence of their metabolic products on plant growth and physiology [2, 8, 13, 15, 16, 30]. They have been isolated from various plants as endophytes that can live in the tissues of plants causing no apparent damage to the host plant [1, 26]. *In vitro* and *in vivo* antagonistic activities of endophytic actinomycetes against plant pathogens have been reported [3, 4, 27, 29]. The introduction of endophytic actinomycetes into plants with the ability to colonize the internal tissue would further enhance the stability and increase their potential effectiveness as biocontrol agents [6]. However, there is no study on endophytic actinomycetes as biocontrol agents against clubroot disease.

In this study, the endophytic actinomycetes were isolated from surface-sterilized roots of Chinese cabbage and then their suppressive effects against clubroot caused by *P. brassicae* were assessed *in vivo*. The effective strains selected as potential biocontrol agents were identified to the species level by cell wall components, morphological characteristics, and phylogenetic analyses, and its endophytic inhibitant was investigated in Chinese cabbage roots.

Isolation and Classification of Endophytic Actinomycetes from Chinese Cabbage Roots

For isolation of actinomycetes, Chinese cabbage (*Brassica campestris* L.) cv. Sam-Bok (Seminis Korea Inc.) was

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grown for 8 weeks in a greenhouse on paddy field and upland soils collected from various locations in Korea. Roots were excised, washed to remove any soil particles, and then sterilized by a 60 sec wash in 99% ethanol, followed by a 6 min wash in 3.1% NaOCl, a 30 sec wash in 99% ethanol, and a final rinse of three times in sterile water. Surface-sterilized root samples were aseptically divided into 1-cm fragments and plated on humic acid vitamin agar (HVA) [12] and corn meal agar (CMA) containing 25 g/l of corn meal (Difco, Detroit, MI, U.S.A.) and 20 g/l of Bacto agar (Difco), and incubated at 30°C for 3 weeks. Both cycloheximide (50 μg/ml) and nalidixic acid (10 μg/ml) were added to avoid fungal contamination in the medium. Bennett's agar and Bennett's broth were used for preservation and liquid culture of the isolated actinomycete strains, respectively. They contained 10 g of glucose, 1 g of yeast extract, 1 g of beef extract, 2 g of N-Z amine A, and 20 g of agar (or without agar) in 1 l of distilled water (adjusted to pH 7.2 before sterilization). After 3 days of incubation, endophytic actinomycetes began to grow out of root tissues. A total of 81 endophytic actinomycetes were isolated.

For the determination of the 16S ribosomal DNA (rDNA) sequences of each isolate, genomic DNA was extracted using a method of Pospiech and Neumann [22]. The universal 16S primers were designed to amplify the region between positions 27 and 1,541 of the 16S rDNA. The primers were designated 9f (5'-GAGTTTGATCCTG-GCTCAG) and 1541r (5'-AAGGAGGTGATCCAGCC). The sample was subjected to the following temperature cycling profile: 98°C for 4 min; followed by 30 cycles of 98°C for 10 sec, 55°C for 30 sec, 72°C for 1 min; and finally 72°C for 4 min. The PCR products were purified using a Wizard PCR Prep DNA purification kit (Promega, Madison, WI, U.S.A.) and then sequenced on an AB1310 DNA sequencer (PE Applied Biosystems, Foster City, CA, U.S.A.) using a Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, U.S.A.) with 9f and 1541r primers. The determined 16S rDNA sequences were compared with the DDBJ databases by using the BLASTN search program.

The 16S rDNA sequence comparisons were made with their closest neighbors found in DDBJ and exhibited 95% to 99% sequence similarity compared with the deposited sequences. The endophytic actinomycetes were classified to 8 actinobacterial genera, and *Microbispora* spp. (67%) were the most common isolates, followed by *Streptomyces* spp. (12%) and *Micromonospora* spp. (11%) (Table 1).

Isolation of endophytic *Microbispora* spp. was reported previously from roots and leaves of other plants [7, 21, 27]. It is interesting that the predominance of *Microbispora* spp. among the endophytic actinomycetes of Chinese cabbage that was observed in the present study was also noted by de Araujo *et al.* [9] on maize, where the genus *Microbispora*, followed by *Streptomyces* and *Micromonospora*, were most commonly isolated. However, the actinobacterial endoflora of Chinese cabbage is different from those reported by Coombs and Franco [7] on wheat and Sardi *et al.* [23] on field crops and Italian native plants, as a result of the molecular characterizations of the bacterial population within potatoes [24]. Among endophytic actinomycetes from wheat, 88% of the isolates were classified as the genus *Streptomyces*, and 12% were of genera such as *Micromonospora* (7%), *Nocardiodes* (3.4%), and *Microbispora* (1.7%).

Table 1. Identification of endophytic actinomycetes isolated from Chinese cabbage roots by 16S rDNA sequence analyses.

Taxa[a]	Number of isolates	Isolate frequency (%)[a]
Microbispora	54	67
Streptomyces	10	12
Micromonospora	9	11
Nocardia	2	2.5
Verrucosispora	2	2.5
Nonomuraea	2	2.5
Actinomadura	1	1.2
Thermomonospora	1	1.2

[a]Nearest matches.

Evaluation of *In Vivo* Biocontrol Activity Against Chinese Cabbage Clubroot

To select effective biocontrol agents against Chinese cabbage clubroot, *in vivo* biocontrol activities of the isolates were tested in a greenhouse. To treat Chinese cabbage seeds with the actinomycete spores, each of the isolated actinomycetes was grown on CMA medium at 30°C for 1 week. Chinese cabbage seeds, washed three times with sterile water to remove surface-coated microbicides, were placed on each colony grown on CMA medium and then incubated at 25°C for 3 days in the dark. After transplanting to non-autoclaved soil, the germinated seeds were grown for 3 weeks in a greenhouse and then transplanted to non-autoclaved soil that was inoculated with resting spores of *P. brassicae* at the concentration of 1.0×10^7 spores per gram of dried soil. Disease severity was assessed after three weeks of inoculation with a pathogen using the disease index (DI) previously reported by Narisawa *et al.* [19]. Clubroot severity was visually separated into four classes: 0, no swelling visible; 1, very slight swelling, usually confined to lateral roots; 2, moderate swelling on lateral or tap roots; and 3, severe swelling on lateral or tap roots. Disease severity was converted into a percentage of control, based on the severity ratings described above and calculated using the following equation:

$$\% \text{ Control}=100[(A-B)/A]$$

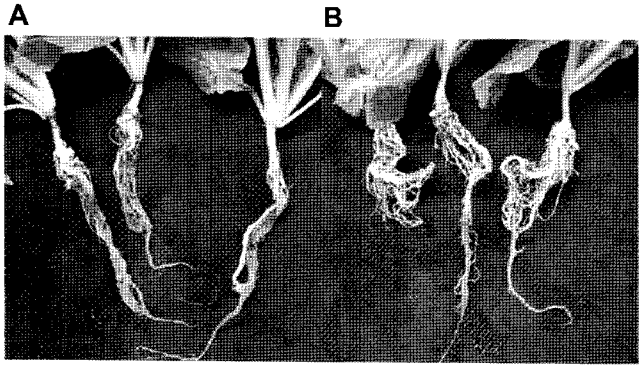

Fig. 1. Antagonistic effect of A004 on Chinese cabbage clubroot in a greenhouse.
A. Chinese cabbage plants inoculated with an actinomycete strain A004 at the time of seed germination and then post-inoculated with *Plasmodiophora brassicae*. **B**. Chinese cabbage plants inoculated with the pathogen alone.

where A and B are the disease severities in control and test treatments, respectively. Five plants in each treatment were assessed and the entire experiment was conducted twice.

Among the 81 isolates tested, 3 strains were selected as potential biocontrol agents. Most of the treated plants were severely diseased, whereas the strain A004 showed the highest control value of 58% compared with the non-treated control plant (Fig. 1). Two strains, A018 and A011, were also effective in controlling Chinese cabbage clubroot, with control values of 42% and 33%, respectively.

In order to examine if A004 is able to inhabit the internal tissues of Chinese cabbage plants, the re-isolation of actinomycetes was conducted using the roots of Chinese cabbage plants treated with A004 as described above. On Bennett's agar medium, pink colonies similar to strain A004 were isolated (Fig. 2). Among the colonies, three were selected and their 16S rDNA sequences were analyzed. All of the sequences of the three colonies were completely matched with the sequence of strain A004 (data not shown). This suggests that the three strains selected in this study could colonize and adapt to Chinese cabbage root.

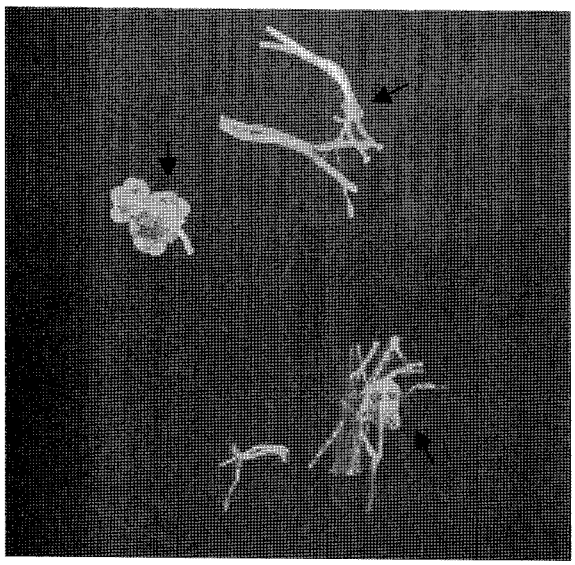

Fig. 2. Growth of *Microbispora rosea* subsp. *rosea* A004 in surface-sterilized roots of Chinese cabbage plants, which were inoculated with the actinomycete strain at the time of seed germination.
Arrows indicate the colonies of *M. rosea* subsp. *rosea* growing on roots.

Many biocontrol agents showed only limited success in the field test. These results may have come from the low level of pathogen-suppressing activity or poor competition when biocontrol agents were introduced and interacting with the indigenous microbial community of the host plant system [25]. Endophytic biocontrol agents introduced into plants, with their ability to colonize the internal tissue, would further enhance the stability and increase their potential effectiveness as biocontrol agents [26]. In fact, some strains of endophytic actinomycetes were able to reduce take-all disease in wheat up to 70% in assays done both in steamed-soil environment and in field soil [6].

Identification of Potential Biocontrol Agents A004, A011, and A018

Further characterizations of the selected actinomycetes A004, A011, and A018 were performed according to traditional morphological criteria, including colony characterization on plates, spore morphology, pigment production, and diaminopimelic acid isomers in cell wall hydrolysates [11]. Spores and mycelia of isolates were observed by using a scanning electron microscope.

Both A004 and A011 strains produced powdery colonies, oval and smooth-surfaced spores, and the spore chains of bispora type under a scanning electron microscope (Fig. 3). They contained *meso*-diaminopimelic acid in the cell wall. The cell wall components and morphological characteristics of strains A004 and A011 were consistent with those of the genera *Microbispora* and *Thermobispora*. A018 exhibited phenotypic characteristics that are consistent with those of the genus *Streptomyces*. The isolate produced unbranched aerial hyphae and spiral chains of cylindrical spores with smooth surface. LL-diaminopimelic acid was contained in the A018 cell wall. The cultural characteristics of A004, A011, and A018 on various ISP media are listed in Table 2. Among the three strains, only A018 produced melanin pigment.

For phylogenetic analyses, 16S rDNA sequences of isolates A004, A011, and A018 were aligned with reference sequences obtained from the nucleotide sequence database in DDBJ by using CLUSTALW [28]. A phylogenetic tree was constructed with the MEGA software package by using a neighbor-joining method with the Kimura two-parameter model [14]. Gaps were treated by pairwise

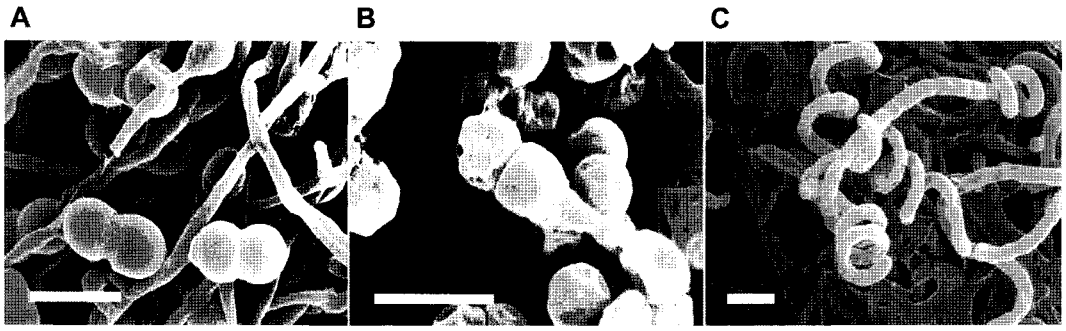

Fig. 3. Scanning electron micrograph of the spore morphology of antagonistic strains A004 (**A**), A011 (**B**), and A018 (**C**) incubated for 7 days on inorganic salt starch agar.
Bar=2 μm.

deletion. Bootstrap analysis was done by using 1,000 pseudoreplications.

Both A004 (1,386 bp) and A011 (1,400 bp) strains had 98% similarity, respectively, with type strains *Microbispora rosea* subsp. *rosea* strain IFO14044 (D86936) and *M. rosea* subsp. *aerata* strain ATCC15448 (U48984) (Fig. 4). Strains A004 and A011 were finally identified as *M. rosea* subsp. *rosea.* Strain A018 (1,417 bp) showed the highest sequence similarity (99%) with type strain *Streptomyces olivochromogenes* strain NBRC13069 (AB184289). Therefore, strain A018 was identified as *S. olivochromogenes.* The nucleotide sequences of the selected three strains determined in this study have been deposited in the DDBJ database under accession numbers AB369119 to AB369121.

Table 2. Cultural characteristics of potential biocontrol agent strains A004, A011, and A018.

Medium	A004			
	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
ISP No. 2[a]	Poor	Whitish gray	Pale yellow	–[b]
ISP No. 3	Good	Dark red	Red	–
ISP No. 4	Poor	None	Pink	–
ISP No. 5	Poor	None	Pink	–
ISP No. 6	Poor	Pink	Pink	–
ISP No. 7	Moderate	Pink	Pink	–
Bennett's agar	Good	White	Pink	–
Medium	A011			
ISP No. 2	Poor	Whitish gray	Pale yellow	–
ISP No. 3	Good	White	Pink	–
ISP No. 4	Good	White	Pale brown	–
ISP No. 5	Poor	White	Pale yellow	–
ISP No. 6	Moderate	White	Pale red	–
ISP No. 7	Moderate	White	Pink	–
Bennett's agar	Good	White	Pink	–
Medium	A018			
ISP No. 2	Good	Whitish gray	Pale yellow	–
ISP No. 3	Good	Gray	Pale brown	–
ISP No. 4	Moderate	White	Pale brown	–
ISP No. 5	Good	Gray	Pale yellow	–
ISP No. 6	Moderate	None	Brown	Dark brown
ISP No. 7	Moderate	Whitish gray	Brown	Reddish brown
Bennett's agar	Good	Gray	Pale yellow	–

[a]ISP No. 2, yeast extract-malt extract agar; ISP No. 3, oatmeal agar; ISP No. 4, inorganic salts-starch agar; ISP No. 5, glycerol-asparagine agar; ISP No. 6, peptone-yeast extract-iron agar; ISP No. 7, tyrosine agar.
[b]–, Not detected.

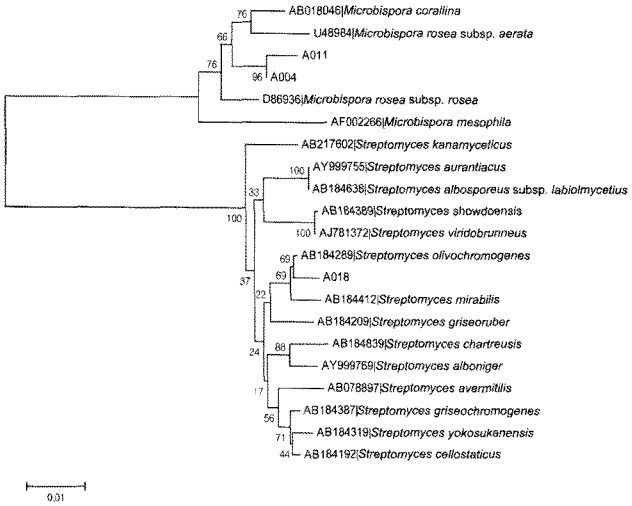

Fig. 4. NJ phylogenetic tree of putative biocontrol agent strains A004, A011, and A018 based on the 16S rRNA gene sequences. The GenBank accession numbers for the sequences are as follows: A004= AB369119, A011=AB369120, A018=AB369121.

To our knowledge, this is the first report that has demonstrated the antagonistic activity of endophytic actinomycetes against Chinese cabbage clubroot. Previously, Narisawa *et al.* [18, 20] reported the endophytic fungus *Heteroconium chaetospira* as a potential biocontrol agent for clubroot in Chinese cabbage in greenhouse and field tests. The fungus is an effective biocontrol agent against clubroot in Chinese cabbage at a low to moderate soil moisture range and a pathogen resting spore density of 10^5 or lower resting spores per gram of soil in field [19]. To succeed in biocontrol of clubroot with *H. chaetospira*-treated plants, Narisawa *et al.* [19] suggested that the resting spore density in soil should be estimated prior to transplanting and suitable draining conditions. The effects of environmental conditions, including soil moisture, soil pH, and the pathogen resting spore density, should be further assessed for high control efficiency using the antagonistic actinomycete strains selected in this study.

Seed treatment with biocontrol agents is one of the most suitable methods for the biocontrol of soilborne pathogens in the rhizosphere of field crops. Chinese cabbage seeds were incubated for 3 days on the colonies of actinomycetes in this study, but this seed treatment method took a long time and might have low application rates in large scales. An efficient method to inoculate a target actinomycete biocontrol agent into the host plant by seed treatment should be developed before it can become an agricultural practice.

This study described the isolation and selection of the endophytic actinomycetes as potential biocontrol agents against Chinese cabbage clubroot. However, the mechanism of enhanced disease resistance of Chinese cabbage inoculated with the selected strains is unknown. The study on the interaction between treated strains and Chinese cabbage is in progress. In addition, further studies are needed on the optimization of the conditions for root colonization and control efficacy in fields.

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