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Antifungal Activity of Root Colonizing *Pseudomonas fluorescens* MC07 is Responsible for Its Disease Suppression Ability

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근권 정착 세균 *Pseudomonas fluorescens* MC07의 항진균 활성과 병 억제 능력

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ABSTRACT: An antagonistic bacterium, *Pseudomonas fluorescens* MC07 inhibited the mycelial growth of *Rhizoctonia solani, Pythium ultimum, Fusarium oxysporum*, and *Phytophthora capsici* in on potato dextrose agan (PDA) and other media. The strain MC07 colonizes various plant roots and possesses antifungal activity. To determine the role of antifungal activity of the bacterium in disease suppression, a mutant Okm3-4 which lost its activity was isolated after screening 2,500 colonies generated by Omegon-Km insertions. The mutant Okm3-4 showed diminished growth inhibition of *R. solani, P. ultimum, F. oxysporum*, and *Ph. capsici in vitro* and had reduced suppressive effects on sesame damping-off compared to the parental strain. In soils, accumulation of the pathogens by continuous cropping, 90% of sesame plants were killed by natural infection of damping-off whereas, only 29% of plants grown from seeds treated with MC07 were killed. On the other hand, 85% of plants died when sesame seeds were treated with the Okm3-4 cells. This indicated that antifungal activity of MC07 *in vitro* is directly responsible for the suppression of damping-off disease. Emergence rates of sesame seeds in pots containing diseased soil were 33%. However, MC07 treatments on seeds significantly improved emergence rates, which had similar effects of Benomyl treatment. The mutant Okm3-4 exhibited 53% of emergence rate. This indicated that antifungal activity of MC07 also affects the emergence rate of sesame seeds.

***Key words*: antifungal activity, biocontrol, damping-off, mutagenesis, *Pseudomonas fluorescens*, root colonization.**

An antagonistic bacterium, *Pseudomonas fluorescens* MC07 was reported as a promising biocontrol agent which has a wide spectrum of growth temperature, especially in low temperature (13, 20). The bacterium suppresses effectively the damping-off of cucumber and enhances its growth significantly have a good rhizosphere competency on various crops (13, 20).

Suppression of soil-borne plant pathogens by fluorescent pseudomonads depends on complex interactions between the pseudomonads and their biotic and abiotic environments. To function effectively as biological agents, the fluorescent pseudomonads should have the ability to colonize roots and to produce antimicrobial secondary metabolites. Evidences have been obtained for the involvement of two groups of metabolites, siderophores and antibiotic compounds, in the pathogen suppression. Competition for available iron (Fe^{3+}) through the activity of siderophore-producing pseudomonads was implicated as a mechanism of biocontrol in Fusarium wilt diseases (14). *P. fluorescens* 2-79 produces phenazine-1-carboxylic acid (PCA), which accounted for 60~90% of the control of take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* (17, 18). PCA-deficient (phz^-) mutants generated by Tn5 insertions were significantly less effective in suppressing the severity of take-all than the parental strain. Other secondary metabolites, including 2,4-diacetylphloroglucinol (phl), hydrogen cyanide (HCN), pyoluteorin (plt) (3, 10, 11) and pyrrolnitrin (7) are also produced by fluorescent pseudomonads. These metabolites usually function as antibiotics and play major

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roles in suppression of various diseases caused by soil-borne plant pathogens. Of *Bacillus* spp., one well-studied example is *Bacillus cereus* strain UW85, which suppresses diseases caused by Oomycetes (5, 6, 15). Analysis of mutants of *B. cereus* showed a significant quantitative relationship between disease suppression and the production of two antibiotics, zwittermicin A and kanosamine (4, 12, 16).

The objective of this research was to determine if the antifungal activity of *P. fluorescens* MC07 *in vitro* is responsible for disease suppression in the field. We generated a mutant defective in antifungal activity and tested it for the ability of inducing suppressiveness to soil-borne diseases and root colonization.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Cells of *P. fluorescens* MC07 were grown overnight at 28°C in liquid Luria Broth (LB) medium or King's B (KB) medium under vigorous aeration. Cells also were grown on solidified LB or KB agar plates. When appropriate, the media were supplemented with rifampicin (Rf) at a final concentration of 50 μg/ml. All strains were stored in 5% glycerol at -80°C. An *E. coli* strain carrying pJFF350 which is a source of Omegon-Km (Fig. 1) was grown overnight in liquid or on solidified LB plates. To maintain pJFF350 in *E. coli*, kanamycin (Km) was added in the culture medium at a final concentration of 25 μg/ml.

Transposon mutagenesis. Mutagenesis of *Ps. fluorescens* MC07 was done as described by Fellay *et al.* (2). Equal volumes of mid-log phase cells of *Ps. fluorescens* MC07 and *E. coli* DH5α, harboring pJFF350 were mixed on the 0.2 μm membrane filters. Filters were incubated on LB plates at 30°C for 16 hr. Cells were resuspended in 0.9% NaCl solution and plated on LB plates containing Rf and Km at 50 μg/ml each. Transconjugants growing on LB containing Rf and Km were isolated and used for screening in antifungal bioassays.

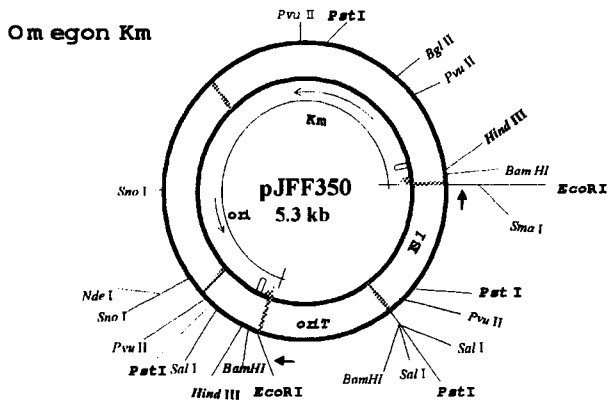

Fig. 1. Genetic map of plasmid pJFF350. Thin arrows represent the direction of transcription, heavy arrows indicate the 28-bp inverted repeats of Omegon-Km, black triangles denote the triple translation stop signals. Hairpin symbols are the T4 gene 32 transcription stop signals.

Screening for mutants defective in antifungal activity. The 2,500 colonies carrying Omegon-Km were picked and inoculated with equal spacing around mycelial disks (1 cm diameter) of *P. ultimum, R. solani, P. capsici,* and *F. oxysporum* f. sp. *cucumerinum* which were grown on Potato Dextrose Agar (PDA; 24 g Difco potato dextrose broth, 20 g agar, 1 L distilled water) and incubated at 27°C for 6 days for *F. oxysporum* f. sp. *cucumerinum* and *Ph. capsici* and 3 days for *P. ultimum* and *R. solani.* The inhibitory activities were measured for each bacterium as a width of the clear zone between the bacterial colony and fungal pathogens when the mycelial growth of each fungal pathogen reached to the point of bacterial inoculation.

Root colonization. Seeds of cucumber (*Cucumis sativas* L. cv. 'Shinpung', Hungnong Seed Co.) were disinfected with 1% NaOCl for one min and inoculated with bacterial cells. The root colonizing abilities of the test isolates were examined with DLF methods as described previously (8). The colony forming units (cfu) were determined by plating a series of 10-fold dilutions on KB agar plates containing 50 μg/ml of Rf. The number of bacterial colonies were counted after one day after incubation. Root colonization was further examined using the method describeb by Ahmad and Baker (8). After germination of the seeds, roots were cut into 1 cm segments with a sterile scalpel, and the first, middle, and last 1 cm segments were used for population analysis. Root segments were transferred into test tubes and stirred vigorously with a vortex mixer. Numbers of bacterial colonies were determined by plating a serious of 10-fold dilutions on KB agar plates containing Rf and 50 μg/ml of cycloheximide one day after incubation at 28°C.

Seed treatments and evaluation of the suppression of sesame damping-off. Bacterial cells were collected by centrifugation (8,000×g, 5 min) from the overnight culture of bacterial cells, and the pellets were resuspended in 0.1 M $MgSO_4$ solution. One hundred of sesame seeds (cultivar. Suwon 6) were soaked in the bacterial

suspension for 1 hr. The treated seeds then were air dried at room temperature for 30 min. The seeds were sown in pots (10×10×4 cm) containing soil taken from a sesame field where sesame plants were cultivated for 3 consecutive years. The pots were kept in a dark chamber at 23°C. Two days after incubation, the pots were moved to a greenhouse. The plants were examined carefully everyday for the development of damping-off, and number of infected plants and emergence. The emergence rate was recorded at 10 days after seeding and the disease rate was determined at 30 days after seeding by counting healthy stand among the total seeds sown.

RESULTS

Isolation of mutants lacking antifungal activity. Transconjugants were selected on KB agar plates containing Rf and Km. Approximately 2,500 colonies grown on selection plates were isolated for further analyses. Among 2,500 colonies tested, 4 mutants defective in antifungal activity were obtained (0.16%). All four mutants completely lost antifungal activity against *P. ultimum, R. solani, Ph. capsici*, and *F. oxysporum* f. sp. *cucumerinum* (Fig. 2, Table 1). All of these mutants did not grow on minimal medium, and their growth was recovered by supplementing with 0.2% casamino acids or 50 mM adenine (data not shown). One mutant, Okm3-4, was chosen arbitrary for further analyses.

Antifungal activity-defective mutants lost disease suppression effects. To determine if antifungal activity-defective mutants maintain biological control ability, sesame seeds were treated with the parental strain MC 07 and the mutant Okm3-4 cells, and the occurrence of damping-off was evaluated in pots. More than 90% of plants were killed by damping-off in untreated soil and 29% of them were dead when seeds were treated with MC07 (Fig. 3). However, the disease incidence of Okm3-4 treated seeds reached to 85% (Fig. 3). This indicated that antifungal activity of the strain MC07 is responsible for the ability of biological control.

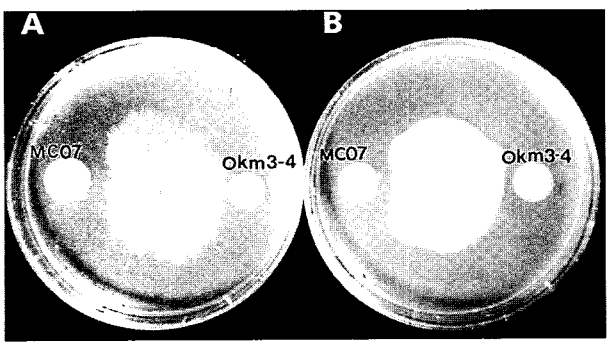

Fig. 2. *In vitro* antagonism assays. The bacterial cells and two fungi, *Rhizoctonia solani* (A) and *Phytophthora capsici* (B), were spotted on the surface of 1/2 strength of PDA containing 0.5% peptone, and then incubated at 28°C for 2 days.

Table 1. Antifungal activity of *Pseudomonas fluorescens* MC07 and antifungal defective mutant Okm3-4 against fungal root pathogens

Root pathogens	MC07	Okm3-4
Pythium ultimum	+++[a]	-
Phytophthora casici	+	-
Rhizoctonia solani	++	-
Fusarium oxysporum	+	-

[a]+: ~5 mm, ++: 5~10 mm, +++: >10 mm. The inhibitory activities of parental strain and mutant were measured as a width of the clear zone between the bacterial and fungal colonies when the mycelial growth of each fungus reached to the point of bacterial inoculation.

Antifungal activity affected the emergence rate of sesame plants. The emergence rate of sesame seeds treated with the parental strain MC07 and the mutant Okm3-4 was evaluated to find out if antifungal activity affects the emergence rate of sesame seeds. The percent of healthy stands of sesame treated with MC07 was 80% which was similar to the effects of benomyl showing 79% (Fig. 4). Only 33% and 53% of seedlings were healthy in untreated plots and the mutant

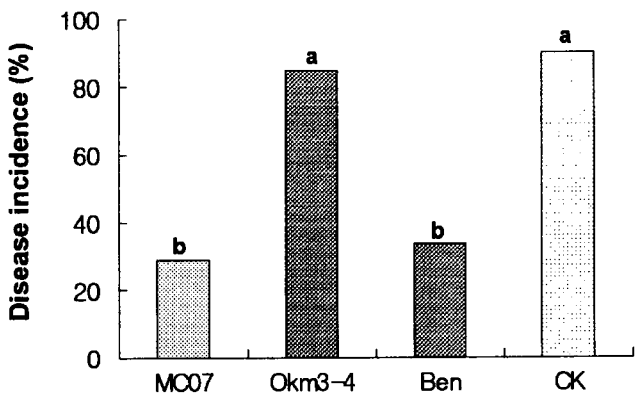

Fig. 3. Suppression of damping-off of sesame plants treated with *Pseudomonas fluorescens* MC07 and antifungal defective mutant Okm3-4 or benomyl in pathogen accumulated soil which sesame plants were cultivated for 3 consecutive years. The pots were kept in a dark chamber where the temperature was constantly maintained at 23°C. After three days of incubation, the pots were moved to a greenhouse. The letters on top of bars indicate significant differences of Duncan's multiple range test. The same letters do not differ significantly ($p=0.01$). Ben: Benomyl, CK: untreated control.

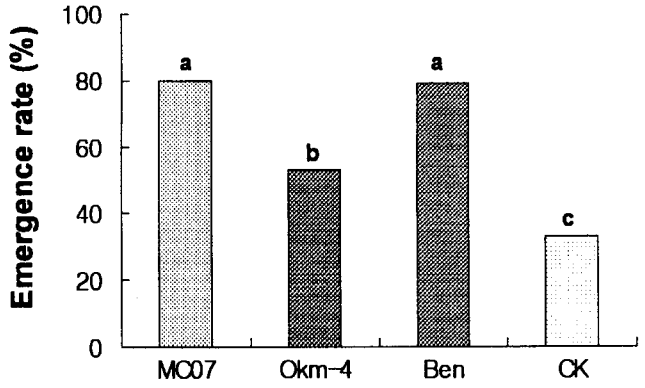

Fig. 4. Emergence rate of sesame plants treated with *Pseudomonas fluorescens* MC07 and antifungal defective mutant Okm3-4 or benomyl in pathogen accumulated soil which sesame plants were cultivated for 3 consecutive years. The pots were kept in dark chamber at 23°C. After three days of incubation, the pots were moved to a green house. The letters on top of bars indicate significant differences of Duncan's multiple range test, same letters do not differ significantly (p= 0.01). Ben: Benomyl, CK: untreated control.

Okm3-4 unstand treated plots, respectively (Fig. 4). This indicated that antifungal activity of the parental strain MC07 influences the emergence rate of sesame seeds.

Root colonization ability of the antifungal activity-defective mutant. To determine if the mutant Okm3-4 maintains the ability of root colonization, bacterial populations on cucumber roots were measured with Ahmad and Baker methods. Bacterial populations of the parent strain and the mutant Okm3-4 in the rhizosphere were statistically different (Table 2). The colonizing populations of MC07 and Okm3-4 on the first 1 cm of root segments were 2.75×10^{6} cfu root^{-1} and 9.77×10^{5} cfu root^{-1}, respectively. However, the colonizing populations of MC07 and Okm3-4 on last 1 cm of root segments were 3.71×10^{4} cfu root^{-1} and 5.70×10^{3} cfu root^{-1}, respectively (Table 2). These results indicated that the antifungal activity-defective mutant Okm3-4 colonizes less effectively in sesame roots than the parental strain.

DISCUSSION

The root colonizing bacterium *P. fluorescens* MC07 has been known as an effective biocontrol agent and a plant growth promoting rhizobacterium adapted in low temperature (20). The culture filtrate of MC07 suppressed the mycelial growth of *R. solani, P. ultimum,* and *Ph. capsici* significantly (13, 20). In this study, we concluded that the antifungal activity of MC07 *in vitro* is responsible for disease suppression in soil.

Table 2. The differences of population densities of *Pseudomonas fluorescens* MC07 and antifungal defective mutant Okm3-4 that colonized on each part of cucumber root analyzed with Ahmad and Baker methods

Isolate	Population density on root ($\times10^{4}$ cfu/cm)[a]		
	First 1 cm	Middle 1 cm	Last 1 cm
MC07	275**[b]	8.91**	3.71**
Okm3-4	97.7	4.46	0.57

[a]The bacterial populations were determined using a dilution plate method on King's B (KB) agar supplemented with 50 μg/ml rifampicin or cycloheximide.
[b]**Significantly different (p=0.01) in each column.

The continuous cropping of sesame plants results in the accumulation of *Rhizoctonia, Pythium, Phytophthora,* and *Fusarium* that cause damping-off and root diseases, and sesame plants are very sensitive to these pathogens (9). Hence sesame plants and damping-off are an ideal system for evaluating biocontrol efficiency against soil-borne disease. Using this system, we attempted to determine if the antifungal activity of the parental strain MC07 is responsible for disease suppression. Mutants lacking antifungal activity were generated by transposable element Omegon-Km. We used Omegon-Km since this transposon has a unique advantage compared to other known transposons (2). It contains an *E. coli*-specific origin of replication within Omegon-Km which allows the rapid and easy cloning of the nucleotide sequences flanking the site of the transposition event (2). Another good trait of this transposon is that transposase necessary for IS1 transposition functions resides outside of Omegon-Km, so that further transposition events do not occur and mutants generated by this transposon are very stable (2). Therefore, we exploited Omegon-Km to obtain antifungal activity defective mutants.

The mutant Okm3-4 showed no antifungal activity against *R. solani, P. ultimum, F. oxysporum,* and *Ph. capsici* in PDA plates and it did not suppress the incidence of damping-off of sesame in pot soil. Similar results were found in other cases. The strain *Pseudomonas fluorescens* CHA625, the Phl^{-} mutant, was impaired in plant protection (19). Pot experiments with natural field soil confirmed the reduced antagonistic activity of strain CHA625 against take-all of wheat. Another example similar to these results is that mutants deficient in phenazine production is significantly less effective in suppressing take-all of wheat than the wild

type strains (17, 18). The reduced suppressiveness of the Phl⁻ mutant cannot be attributed to its reduced ability to maintain effective populations in the rhizosphere since the mutant and the parental strain did not differ significantly in root colonization (19). In other biocontrol rhizobacteria, mutants deficient in antibiotic or siderophore production did not affect their root colonization ability (1, 17). However, we have obtained evidence that the deficiency in antifungal activity of Okm3-4 contribute to its root colonization in soil habitat. Since significant differences were observed between the parental strain and Okm3-4 in their densities in the rhizosphere, reduced suppression of damping-off could be due to its inability to maintain significant populations around the roots. Unlike other cases, it is plausible that nutritional requirement of the mutant Okm3-4 may have influenced the ability of root colonization. We do not have any clues about the chemical nature of antifungal compound produced by the parental strain MC07. However, the fact that antifungal activity and adenine requirement of the mutant Okm3-4 for the growth are linked suggests that biosynthesis of the antifungal compound produced by the parental strain MC07 may share the adenine biosynthetic pathway.

The seeds and seedlings of sesame are fragile to adverse condition, hence the emergence rate in the field soil is rather low. Furthermore some seed-borne diseases and pre and post emergence damping-off may kill the seedlings. Many reports indicated that seed bacterization can enhance the seed vigor and seedling emergence (5, 13, 15). In this experiment, we showed that antifungal activities of biocontrol agents can influence emergence rates of sesame in the soil. The parental strain MC07 improved the emergence rate whereas the antifungal defective mutant Okm3-4 did not. This is a direct demonstration that the antifungal activity of the parental strain MC07 is important for the emergence rate of sesame seeds.

The effectiveness of biocontrol strains or their suppression of deleterious microorganisms is largely dependent on the establishment of a large and stable population of the biocontrol strain which is adequately distributed in the rhizosphere. Hence root colonization may be related with antifungal activity of a particular biocontrol agent. In conclusion, our present results suggest that suppression of root diseases by rhizobacteria pseudomonads is multifactorial with bacterial secondary metabolites and root colonization having a key role.

요 약

Pseudomonas fluorescens MC07은 *Rhizoctonia solani, Pythium ultimum, Fusarium oxysporum*과 *Phytophthora capsici*에 의한 여러 가지 작물의 모잘록병을 방제하는 세균주로 항진균물질을 분비한다. 모균주 MC07에 Omegon-Km 삽입으로 항진균물질을 생산하지 못하는 Okm 3-4를 비롯한 4개의 변이체를 선발하였다. Wild type인 MC07과 변이체 Okm3-4를 참깨종자에 처리하여 참깨 연작지에서 채취한 이병토양을 넣은 폿트에서 모잘록병 억제 효과를 조사한 결과 MC07 처리는 29%의 병 발생율를 보여 benomyl 34%보다도 병 발생 억제효과가 우수한데 비하여 Okm3-4는 85%로서 무처리구 90%와 거의 비슷한 수준이였다. 입모율에 있어서도 MC07의 처리구는 80%, benomyl 79%인데 비하여 Okm3-4는 53%, 무처리구는 33%로 나타났다. Okm3-4는 adenine 요구형 돌연변이체로서 오이 뿌리에 정착하는 밀도도 저하되었다. MC07의 항진균물질의 분비는 병원균이 축적된 연작지 토양에서 병원균들을 저해하여 발병을 억제하고 초기 입묘율을 향상시키는 것과 직접적인 관계가 있음을 보여주었다.

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