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Enhancement of Plant Growth and Suppression of Damping-off of Cucumber by Low Temperature Growing *Pseudomonas fluorescens* Isolates

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저온 생장성 *Pseudomonas fluorescens* M45와 MC07을 이용한 오이의 생육촉진과 모잘록병의 방제

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ABSTRACT : Growth rates of the low temperature growing isolates, *Pseudomonas fluorescens* M45 and MC07, reached maximum stationary phase within 50 hrs at the low temperature, 4°C. But an ordinary biocontrol agent *P. putida* Pf3 did not reach logarithmic growth phase until 80 hrs. The culture filtrates of M45 and MC07 significantly inhibited the mycelial growths of *Pythium ultimum*, *Rhizoctonia solani* and *Phytophthora capsici*. Detached cotyledons of cucumber grown on Murashige and Skoog agar medium were much enhanced in their growth, compared to those without the filtrates. Population densities of M45 and MC07 in the rhizosphere at 14°C were more stable than at 27°C. When M45 and MC07 were treated into soil, the population density of MC07 continuously increased up to 9 days after treatment, and sustained the initial inoculum density up to 60 days. Cucumber damping-offs caused by *P. ultimum* and *R. solani* were significantly reduced by applying M45 as seed-inoculant and by soil treatment with MC07. The combined treatment of M45 and MC 07 provided greater effect in reducing the disease incidence than that obtained by single treatments.

Key words **: low temperature, *Pseudomonas fluorescens*, growth promotion, biocontrol agents, soil borne pathogen.**

Vegetables and ornamental crops have been cultivated in many ways to pursue maximum profit in Korea. During the winter time, many crops are cultivated in plastic film houses, and during the hot summer, some of vegetable crops are planted in alpine areas. Such culture conditions can bring one or more kinds of abiotic stress such as low temperature or inadequate oxygen supply on the roots, and the greater activities of root pathogens favored by cool and wet soil (5). Some root and seedling diseases caused by soil borne pathogens are more severe under the cool temperature conditions which reduce the root activity and make plants vulnerable to deleterious microorganisms. It was reported that resistance of watermelon to *Pythium ultimum* and *Rhizoctonia solani* was increased at soil temperatures above 20°C, and that of spinach to the same pathogens was increased below 12°C (10).

The objective of present study is to select promising biocontrol agents which suppress the soil borne diseases successfully under low temperature conditions. To attain this purpose, the attributes of low temperature growing bacterial isolates related to disease suppression and plant growth promotion were evaluated in a laboratory and a plastic film house. Attempts

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were also made to enhance the biocontrol efficiency by combined treatments of the selected isolates. In addition to these the growth patterns of the selected isolates at different temperature were studied.

MATERIALS AND METHODS

Growth rate of bacterial isolates. Through the preliminary screening test, two isolates, M45 and MC 07, which were identified as *Pseudomonas fluorescens*, were selected as the most promising biocontrol agents at low temperature conditions. Growth rates of two isolates were examined in King's B (KB) broth media at 4°C, 14°C and 27°C. A biocontrol agent, *Pseudomonas putida* Pf3 which had been previously selected in our laboratory (2), was used as a reference for the growth rate. The bacterial growth rates were determined by measuring optical densities of KB broth medium at 660 nm with Spectronic 20 (Milton Roy Co., U.S.A.).

Antagonistic activity of selected isolates to pathogenic fungi. Antibiotic activity of liquid cultures of the selected bacterial isolates was examined. Isolates M 45 and MC07 were incubated in 250 ml of KB broth medium in 500 ml Erlenmeyer flasks at 27°C for three days and at 14°C for five days. The cell cultures were filtered through sterile membrane filter (0.25 μm, Nalgene Co., U.S.A.) and mixed with molten PDA medium (1%, v/v). After the media were solidified, 1-cm-d mycelial plugs of *P. ultimum*, *R. solani* and *Phytophthora capsici* were inoculated at the center of the plates with the medium. Fungal mycelial growths were measured on the media with and without the filtrates, and compared.

Growth promotion of cucumber cotyledon by culture filtrates of M45 and MC07. The above bacterial culture filtrates were supplemented to 100 ml of Murashige and Skoog (MS) media (0.1%, v/v). Newly emerging cucumber cotyledons were aseptically detached and placed on the MS media. The fresh weights of cotyledons were compared to those on the media without amendment of the bacterial culture filtrates after days of incubation.

Colonization of the bacteria on cucumber root. Bacterial cells were harvested from 3-day cultures in KB broth medium by centrifugation at 2,600 g for 10 minutes, washed twice and resuspended in 0.1 M $MgSO_4$ solution. Bacterial suspensions were adjusted to carry 10^8 cells per ml by serial 10-fold dilution plating. Cucumber seeds (cv. Sa-yeup-Oi, Jungang Seed Co.) were soaked with the bacterial suspensions of M45 and MC07, shaked at 250 rpm for 30 minutes, and air-dried. Non-treated seeds were dipped in 0.1 M $MgSO_4$ solution only and air-dried. The root colonizing ability of the isolates was measured by the methods described by Bae (2) and Ahmad & Baker (1).

Survivability of selected isolates in soil. Spontaneous mutant isolates of M45 and MC07 which were resistant to 100 ppm rifampicin were used to monitor the population densities in natural soil. The field soil obtained from the experimental farm of Gyeongsang University was used in this experiment. Bacterial suspensions (9 ml in 0.1 M $MgSO_4$) were added to 30-mm-d test tubes containing 50 g of air-dried soil. Initial density of the bacteria was measured 10^7 cells per g of soil. The treated soils were incubated at 4°C, 14°C or 27°C. One g of the treated soil was diluted in 0.1 M $MgSO_4$ solution and spreaded on KB media containing 100 ppm rifampicin. Bacterial counts were measured at three day intervals up to 60 days after treatment with four replications.

Emergence of vegetable crops in vermiculite, autoclaved soil and natural soil. Cucumber seeds were coated with bacterial suspensions of M45 and MC07 in the same method as above. Moistened vermiculite (650 g, 40% moisture content) and autoclaved soil (900 g) were mixed and put into a pot (17×12×6.5 cm), and treated or non-treated cucumber seeds (15 seeds per pot) were planted. The pots were placed in a growth chamber at 21°C with light for 14 hrs and at 12°C without light for 10 hrs. The emergency rates of cucumber were examined at 7 days after seeding, and fresh weights of total plants were measured 30 days after seeding.

Biocontrol of cucumber damping-off. Inocula of *P. ultimum* and *R. solani* were mixed thoroughly with autoclaved soil. The cucumber seeds treated with M45 and the natural soils were amended with 10^8 cell/ml of MC07 suspension (0.1%, v/w). The treatments were applied singly or in combination. This experiment was conducted at low temperature in the growth chamber as described above and room temperature. Disease incidence was recorded up to 30 days after planting.

RESULTS

Growth rate of biocontrol agents at different temperatures. *P. fluorescens*, M45 and MC07, and the reference biocontrol agent *P. putida* Pf3 grew rapidly at room temperature (27°C). The growth rate of all

three isolates reached maximum stationary phase within 12 hrs and Pf3 grew slightly faster than M45 and MC07 at 27°C. The growth curves of the isolates at 14°C, however, were completely different from those at 27°C. The growth rate of Pf3 did not reach to the maximum stationary phase up to 32 hrs. As the temperature was lowered to 4°C, the growth of M45 and MC07 reached the maximum stationary phase within 40 hrs, whereas the growth of Pf3 was verys low and did not show logarithmic growth up to 80 hrs after inoculation (Fig. 1).

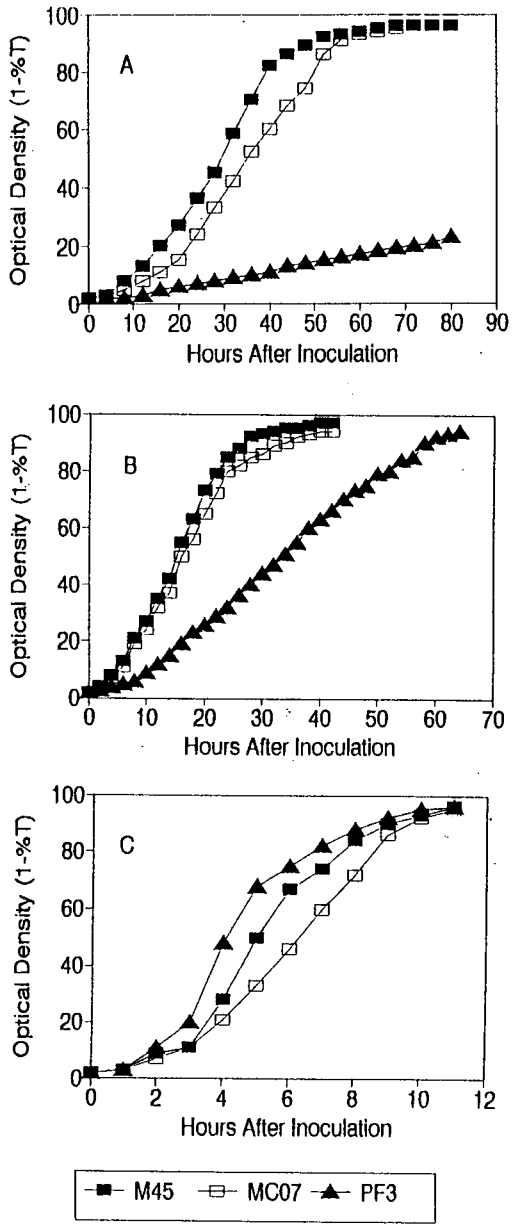

Fig. 1. Growth curves of *Pseudomonas fluorescens* M45 and MC07 and *P. putida* Pf3 in King's B broth medium at 4°C (A), 14°C (B) and 27°C (C).

Suppression of mycelial growth of plant pathogens by culture filtrates of selected isolates. Regardless of the isolates, the bacterial culture filtrates incubated at 14°C for 5 days inhibited the mycelial growths of *Pythium ultimum*, *Rhizoctonia solani* and *Phytophthora capsici* more than those incubated at 27°C for 3 days (Table 1). MC07 suppressed soil borne pathogens more effectively than M45.

Effect of culture filtrates of M45 and MC07 on the growth of cucumber cotyledon. The culture filtrates of M45 and MC07 greatly enhanced the growth of cucumber cotyledons in MS media. The culture filtrate of M45 was superior to that of MC07 at any incubation temperatures tested. The culture filtrates of M 45 and MC07 grown at 14°C enhanced the growth of cucumber cotyledons significantly greater than those grown at 27°C (Table 2).

Colonization of M45 and MC07 on cucumber root. The population density of MC07 on cucumber rhizoplane was continuously reduced either at 27°C or at 14°C, while the population density of M45 slightly increased in the first 2 days, and sustained the initial population density at 27°C (Table 3). When the seed treated with M45 were grown at 14°C, the population density of the bacterial isolates increased much up to 6 days, and decreased rapidly. However, the level of population density was slightly higher than the initial den-

Table 1. Inhibition of mycelial growth of plant pathogenic fungi by addition of 1% culture filtrates of M45 and MC07 in PDA incubated at 27°C for 3 days and 15°C for 5 days

Pathogen	Inhibition of mycelial growth[a] (%)			
	M45		MC07	
	27°C	14°C	27°C	14°C
Pythium ultimum	16x[b]	19y	21y	34z
Rhizoctonia solani	20x	30y	21x	36z
Phytophthora capsici	23x	33y	26x	40z

[a] Inhibition rate was calculated from (1 − mycelial growth of treatment/mycelial growth of control) × 100.

[b] Data in each row with diffent letters are significantly different at p=0.05 by Duncan's multiple range test.

Table 2. Effect of culture filtrates of M45 and MC07 on the growth of cucumber cotyledon in MS media

Treatment[a]	Culturing temperature	Fresh weight[b] (mg)	
		Root	Shoot
M45	14°C	170x	390x
	27°C	142y	316y
MC07	14°C	119y	344y
	27°C	85z	293y
Untreated		78z	253z

[a] The culture filtrates (1%, v/v) of the bacterial isolates were incubated at 14°C for 5 days and at 27°C for 3 days.

[b] Each number is the mean of five replications with 4 plants each. Data in each column with different letters are significantly different (p=0.05) according to Duncan's multiple range test.

Table 3. Population density of low temperature growing isolates, M45 and MC07, colonized on the root tip of cucumber at 14°C or 27°C[a]

Isolate	Log number of cfu/cm root tip							
	27°C				14°C			
	2	4	6	8 days	3	6	9	12 days
M45	3.5	3.15	3.14	3.16	4.16	4.6	3.6	4.1
MC07	3.2	2.14	1.2	1.18	3.1	2.1	1.18	1.15

[a] Population density was examined by Ahmad & Baker's method (1).

sity up to 12 days after planting (Table 3).

Survivability of M45 and MC07 in soil. To evaluate the survivability of M45 and MC07 in soil, the bacterial suspension of either M45 or MC07 was added to natural field soil. The initial concentration of bacterial cells was 10^7 per gram of dry soil. The moisture content of soil was modified to 18% (w/w) by the addition of the bacterial suspension. There were significant differences in survival between M45 and MC07 at 15°C (Fig. 2). The population density of M45 slightly increased up to 6 days after inoculation, and then reduced to 1.0×10^4 at 60 days after inoculation. But the population density of MC07 continuously increased up to 9 days, and sustained the initial inoculum density up to 60 days.

Effect of M45 and MC07 on emergence of vegetable crops in vermiculite, autoclaved soil and natural soil. The cucumber seeds inoculated with M45 or MC07 were sown in vermiculite, and the rates of

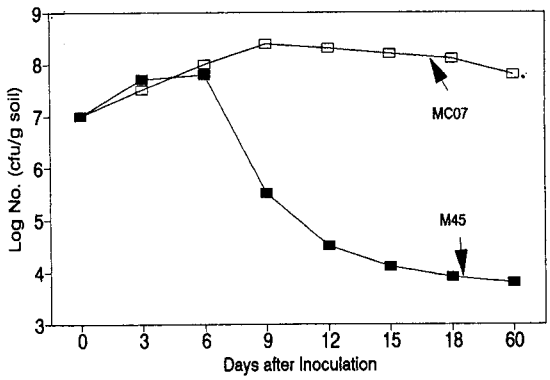

Fig. 2. Population dynamics of *P. fluorescens* M45 and MC07 surviving in natural soil at room temperature.

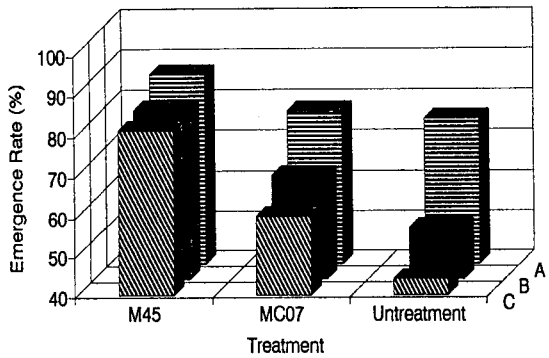

Fig. 3. Enhancement of cucumber growth by seed treatment of M45 and soil treatment of MC07 at 14°C in a growth chamber. A : total emergence rate. B : complete emergence rate. C : first foliage leat expansion rate.

emergence and first foliage leaf expansion were examined. The total emergence rate was not greatly different between the bacterial treatment and the untreated control. However, the rate of complete expansion of cotyledon and the rate of foliage leaf expansion in the treatment significantly differed from the untreated control (Fig. 3). M45 increased the rate of complete cotyledon expansion as well as the rate of first foliage leaf expansion significantly more than MC07 (Table 4). M45 was more effective than MC07 in enhancing the seedling growth (Table 4). In natural field soil, the emergence rate was different slightly, but the fresh weight was greatly different between the bacterial treatment and the untreated control. The difference in fresh weight between bacterial treatment and untreated control was more prominent in natural soil than autoclaved soil (Table 4).

Table 4. Effect of seed treatment of M45 and MC07 on the growth of cucumber in autoclaved and natural soils at low temperature[a]

Treatment[b]	Autoclaved soil		Natural soil	
	Emergence (%)	Fresh wt. (mg)	Emergence (%)	Fresh wt. (mg)
M45	95	694±33[c]	94	680±28
MC07	95	610±24	90	570±25
Untreated control	90	574±10	82	497±48

[a] Low temperature is at 21°C with light for 14 hrs and 12°C without light for 10 hrs.
[b] Seeds were soaked for 30 min in bacterial suspensions of M45 or MC07 at concentration of 1×10^8 cells/ml.
[c] Data are means±standard deviations of five replications with 15 plants each.

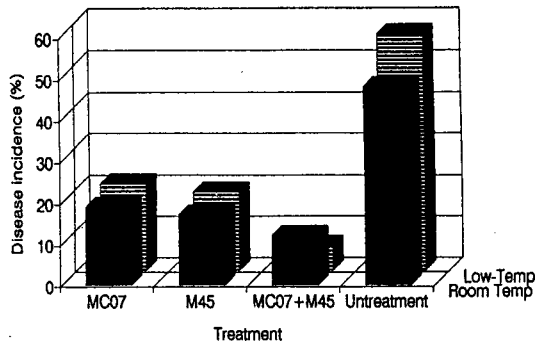

Fig. 4. Suppression of cucumber damping-off caused by *Rhizoctonia solani* by seed treatment of M45 and soil treatment of MC07. Low temperature was set at 21°C with light for 14 hrs and 12°C without light for 10 hrs. Room temperature was set at 25°C with continuous light.

Biocontrol of cucumber damping-off by M45 and MC07. Biocontrol agents, M45 and MC07, tested in this experiments effectively suppressed the damping off of cucumber. The combined treatment of M45 seed coating and MC07 soil treatment provided greater effect in reducing the disease incidence of cucumber damping-off than that obtained by either treatment alone at room temperature or at low temperature in a growth chamber (Fig. 4). The suppressive effect of M45 and MC07 to damping-off of *Rhizoctonia* or *Pythium* was greater at low temperature than at room temperature.

DISCUSSION

It is not unusual to crop plants in a plastic film house during winter or to grow in alpine areas during summer in Korea. However, unfavorable environmental conditions in the extra crop seasons, especially low temperature conditions, frequently resulted in more disease problems caused by soil borne plant pathogens, predisposing the root more vulnerable to deleterious microorganisms than in the ordinary crop seasons. Consequently, the more efficient biocontrol agents are needed to enhance the root activity and suppress soil borne diseases under low temperature conditions in the extra growing seasons (5, 13, 14).

We selected isolates M45 and MC07, as effective biocontrol agents and plant growth promoting rhizobacteria adapted in low temperature. With these isolates we confirmed that suppression of soil borne pathogens and enhancement of plant growth were more effective in lower temperature conditions than ordinary growing temperature. The suppressive effect of M45 and MC07 to *Rhizoctonia* and *Pythium* damping-offs was greater at low temperature than room temperature (Fig. 4). The reason why low temperature made the bacteria more effective in enhancing the plant growth or in suppressing the soil borne pathogens could not be elucidated in this investigation. M45 and MC07 produced more fluorescent pigment, which is assumed as antifungal substances, at low temperature conditions (data not included), suggesting that at low temperature the isolate may produce more antifungal substances.

Loper *et al.* (11) demonstrated that populations of fluorescent *Pseudomonas* estimated from the rhizosphere at 12°C were generally more stable than those at 18°C or 24°C, and suggested that the survival of bacterial cells was maximized at low temperature. Lifshitz *et al.* (10) reported that diazotrophic bacterial strains can fix nitrogen and aggressively colonize roots at low temperatures. The results obtained in our study also revealed that the biocontrol agents were more competitive on the rhizosphere at low temperature conditions. The low temperature growing biocontrol agents, M45 and MC07, developed through this investigation will contribute to suppress the soil borne diseases and to enhanced the plant growth in cropping in the plastic film houses during the winter time.

요 약

저온에서 생장하는 근권 미생물 *Pseudomonas fluorescens* M45와 MC07을 선발하여 본 실험실에서 이

미 선발한 생물적 방제균 *Pseudomonas putida* Pf3과 온도별 생장을 비교한 결과 27°C에서는 Pf3의 생장이 약간 빨랐으나. 14°C에서 Pf3은 60시간까지 정상기에 도달하지 못하였지만, M45와 MC07은 30시간 이전에 정상기에 도달하였고, 온도를 더 낮춘 4°C에서 Pf3의 생장은 아주 저조하여 80시간 이후에도 대수증식기에 이르지 못한 반면 M45와 MC07은 40시간 이전에 정상기에 도달하였다. M45와 MC07균주의 배양여액 1%(v/v)을 감자한천배지에 첨가했을 때 토양병원균 *Pythium ultimum, Rhizoctonia solani, Phytophthora capsici*의 균사생장을 현저히 저지하였으며, 특히 저온에서 배양한 MC07의 배양여액이 저지효과가 우수하였다. M45와 MC07을 27°C와 14°C에서 각각 배양하여 배양여액을 MS기본배지에 0.1%(v/v)의 농도를 첨가한 후, 오이 자엽절편의 생장을 조사해 본 결과 두 균주 모두 오이 자엽의 생장을 증가시켰다. M45와 MC07의 오이의 근권 정착능력과 토양정착 능력은 M45가 MC07보다 근권정착 밀도가 높았으며, 실온에서 보다 저온에서 근권정착 능력이 좋았다. MC07은 토양 정착 기간이 14°C에서 가장 길었으며 60일까지는 초기농도와 거의 같이 밀도를 유지할 수 있었다. M45를 종자처리하고 MC07을 토양처리 하였을 때 *Pythium*과 *Rhizoctonia*에 의한 발병율은 실온과 저온에서 방제효과가 있었으나, 저온에서 발병억제율이 높았다.

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