한국식물병리학회지 13(3) : 160-166(1997)
Korean J. Plant Pathol. 13(3) : 160-166(1997)

Colonizing Pattern of Fluorescent Pseudomonads on the Cucumber Seed and Rhizoplane

Ji Hyo Kang and Chang Suk Park*
Department of Agricultural Biology, Gyeongsang National University, # 900 Gazwa-Dong, Chinju 660-701, Korea

형광성 *Pseudomonas*屬 균주의 오이 종자처리 후 근권에서의 밀도와 정착양상

강지효 · 박창석*
경상대학교 농과대학 농생물학과

ABSTRACT : Number of bacterial isolates were collected from high mountainous areas at various locations in the whole country to select promising biocontrol agents. Most of selected isolates belonged to fluorescent pseudomonads. Population densities of fluorescent pseudomonads were examined by DLF method. All selected isolates rapidly multiplied on spermosphere after seed inoculation upto 24, and then the population abruptly declined. When seeds were germinated fully, bacteria moved to newly emerging radicle. The good root colonizing isolates, B16 and V13 proliferated on the growing root and moved down to the root tip and lateral roots. But the poor root colonizing isolates, MC07 and X01 moved much slower. Scanning electron microscopic observations showed that the cells of the good colonizing isolates were arranged linearly on the growing root and parallel to growing root axis and continuously existed on the root tip, Whereas the cells of poorly colonizing isolates were gathered and scattered randomly on the root surface.

Key words : **Root colonization, fluorescent pseudomonds, seed treatment.**

Application of biocontrol agents to the seed is an attractive approach to introduce a biocontrol agent into soil instead of supplying greater amounts of bio-material to a large area. Applied biocontrol agents were proliferated on the seed, then transferred and colonized the subterraneous plant parts, e.g., roots to cause enhanced plant growth and also results in disease suppression by a rhizosphere-competent organism (1, 3, 5, 8, 9, 12, 14, 17, 18). Therefore colonization is a significant relationship between population size and the degree of disease suppression (15, 16).

Pseudomonas spp. are receiving much attention with regard to their biocontrol properties because of their potential to colonize the rhizosphere after seed inoculation and suppress a wide range of plant pathogens (4, 15, 16).

Root colonization by bacterial strain involves traits not only related to cell dispersal, but also to attachment, growth, competition and survival. The intrinsic growth rate or generation time of given bacteria on the host roots is considered one of the key factors for root colonization.

Knowledges of the colonization abilities of biocontrol agents and scientific information on the population dynamics in the rhizosphere are essential to understand microbial interactions and mechanisms for disease suppression by specific strains against specific pathogens. Throughout this study, we tried to investigate the process of root colonization via seed treatment and to elucidate factors which might be involved in root colonization abilities of fluorescent pseudomonads selected as biocontrol agents

MATERIALS AND METHODS

Screening of root colonizing isolates of fluores-

*Corresponding author.

cent pseudomonads. A total of 422 bacterial isolates were collected from the alpine and high mountainous areas at various locations in the whole country, mainly from rhizosphere of monocotyledonous plants. Out of these isolates, more than 100 bacterial isolates were selected as biocontrol agents under low temperature condition during 1994.

Selection of *Pseudomonas fluorescens* isolates. Four isolates of *P. fluorescens* were selected to investigate the factors involved in root colonizing abilities. The isolates, B16, V13, MC07 and X01 showed no difference in colony type, physiological characters and ability to produce antibiotics *in vitro*. However, in preliminary experiments, B16 and V13 were shown to be the good root colonizers, while MC07 and X01 were the poor root colonizers.

Growth rate of selected isolates. Growth rate of the selected isolates were examined in King's B broth at 27℃. Bacterial cells grown on King's B medium were harvested and resuspended in 0.1 M $MgSO_4$ solution. The cell densities of the suspension were adjusted to 25% transmittance at 660 nm with spectrophotometer (Spectronic 20, Milton Roy Co.). This optical value is equivalent to 1×10^8 (cell/m*l*). 2.5 m*l* of bacterial suspension was added to 250 m*l* of King's B broth in 500 m*l* Erlenmeyer flasks and incubated in rotary incubator (KHF-0830, Kum Hwa Industrial Co.) with 150 rpm at 27℃. The optical densities of the cell suspension at 660 nm were measured every hour at 27 ℃. The measurement was recorded until the bacterial growth of each isolate reached to the maximum stationary growth phase.

Seed treatment. Cucumber (*Cucumis sativus* L. cv 'Shinpung' Hungnong Seed Co.) seeds used throughout the study. Surface disinfection was made with 1% NaOCl solution for 10 min, washed with sterile distilled water and air-dried. The seeds were then soaked in each of the bacterial suspension for 15 min and air-dried at the room temperature. Root colonizing abilities of bacterial isolates were examined by DLF method (2). The cell densities of the suspension was adjusted to 60% transmittance at 660 nm with spectro- photometer (spectronic 20, Milton Roy Co.).

Population densities on main root and lateral root of cucumber. The population densities of selected root colonizing isolates on main root and lateral root of cucumber were examined by DLF method. After 72 hours of incubation, the top 1 cm-segment of the root was taken with sterile scalpel. Lateral root was detached from top 1-cm segment of root. Each of the root segments were transferred into a test tube containing 9 m*l* 0.1 M $MgSO_4$. The test tubes containing root segment were vigorously stirred on a vortex mixer. The colony forming units were determined by plating a series of 10-fold dilution on KB agar. Cell colony number was counted after 3 days incubation.

Scanning electron microscopy. Cucumber roots colonized by bacteria were obtained by DLF. The root segments were fixed overnight in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) at 4℃. The specimens were carefully rinsed three times with sterile distilled water, post-fixed in 2% OsO_4 (Sigma Co., U.S.A) in 0.05 M phosphate buffer (pH 7.0) solution for 2 h at room temperature and rinsed carefully three times with sterile distilled water. The specimens were dehydrated through a series of ethanol gradient (20, 40, 60, 80, 90, 100%) and then dehydrated twice through 100% amylacetate. Dehydrated specimens were dried in Critical-Point Drier (E3300, BioRad, USA) with CO_2 gas, and coated by an Ion Coater (Jec-1100, Jeol Co., Japan) with gold. Prepared specimens were observed in a Scanning Electron Microscope (JSM-6400, Jeol Co., Japan)in 10 Kv.

RESULTS

Growth rate of the selected isolate *in vitro*. The results obtained from the preliminary experiments, 4 fluorescent *Pseudomonas* isolates such as B16, V13, MC07 and X01 showed almost the same characteristics in many aspects except root colonizing ability. Generally, the selected isolates were fast growing bacteria. They reached maximum stationary phase within 17 hrs. However, The cell growth of B16 and V13 started earlier than those of MC07 and X01. The growth of X01 did not enter the logarithmic growth phase upto 11 hrs after inoculation (Fig. 1).

Root colonizing abilities of the selected isolates. The bacterial population dentisities of the isolates tested rapidly increased on the surface of cucumber seed-coat 24 h after seed inoculation. The population densities of the good colonizing isolates B16 and V13 on seed coat were much higher than that of poorly colonizing isolates, MC07 and X01 By the time most of the seeds were fully germinated, bacterial cells of the isolates tested moved to newly emerging root tips. The transfer rate of isolates B16, V13, MC07 and X01 from the seed-coat to the radicle were 3.80%, 18.89%,

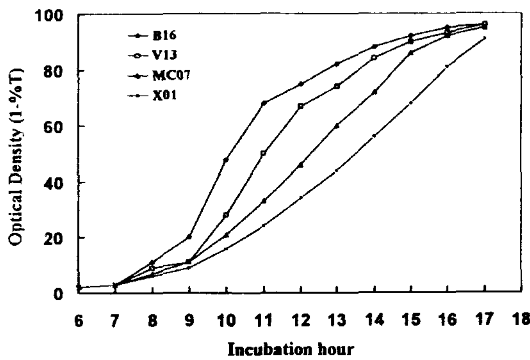

Fig. 1. Growth curves of isolates tested in the King's B broth at 27℃.

0.89% and 0.33%, respectively (Table 1).

The population densities of B16 and V13 colonized on cucumber roots were much higher than those of MC07 and X01 at 72 hr after seed treatment. Generally, colonizing bacterial densities were sharply diminished with the increase of root depth. The colonizing population of B16 and V13 on first 1-cm of root segment were 3.66×10^6 and 30.64×10^6 cfu, respectively. While the population densities of MC07 and X 01 on first 1-cm of root segment were 1.70×10^6, 0.43×10^6 cfu, respectively. The population densities of B16 and V13 on all part of cucumber root were higher than that of MC07 and X01. Especially the bacterial population of B16 and V13 on the last 1-cm segment part of cucumber roots were much higher than those of MC07 and X01. The colonizing density of V13 was higher than that of any other colonizing strains on all part of the root surface (Table 2).

At 72 hrs after seed treatment, bacterial populations

Table 1. Population densities of the isolates tested on the seedcoats and radicles of cucumber plant 24 hr after seed inoculation

Isolate	Population densities			Translocation rate (%)
	Seedcoat ($\times 10^5$ cfu/unit)		Radicle ($\times 10^2$ cfu/unit)	
	0 h[a]	24 h	24 h	
B16	1.67[a]	9.75[a]	37.05[b]	3.80[b]
V13	6.00[a]	5.77[b]	109.00[a]	18.89[a]
MC07	1.67[a]	3.02[c]	2.68[c]	0.89[c]
X01	2.00[a]	1.00[c]	0.33[c]	0.33[c]

[a] In each column, values followed by the same letter do not differ significantly (P=0.05) according to Duncan's mul- tiple range test.

Table 2. Spatial differences of population densities of the isolates tested that colonized on each part of the cucumber root 72 h after seed inoculation

Isolate	Population density on root ($\times 10^4$ cfu/cm)		
	First 1-cm[a]	4-5 cm (middle part)	Last 1-cm
B16	366.0[b]	3.88[b]	0.54[b]
V13	3064.0[a]	6.16[a]	1.48[a]
MC07	170.0[bc]	0.92[c]	0.15[c]
X01	43.0[bc]	0.47[c]	-

[a] In each column, values followed by the same letter do not differ significantly(P=0.05) according to Duncan's multiple range test.

were detectable on lateral roots. Less than 1/100 of the population colonized on the main root moved to lateral roots (Table 3). The isolates V13 and B16 showed relatively higher populations on lateral roots in proportion to their population density on the main roots. About half of the total populations colonizing the first 1-cm root segments were missing when the bacterial population of main and lateral roots were analysed separately.

Existing patterns of B16 on the cucumber seedcoat and emerging roots. Soon after seed inoculation of B16, the cells of bacteria were randomly scattered on cucumber seed coat. At 24 h after seed treatment, the cells of B16 were greatly increased, and new cells were gathered on the groove of cucumber seed coat. Then bacterial cells moved to the emerging radicle from the seed. Some bacterial cells already colonized and proliferated on root cap of emerging radicles. With the progress in time, the bacterial cells of B16 were arranged linearly toward the elongated root axis (Fig. 2).

Existing pattern of root colonizing isolates on the

Table 3. Population densities of tested isolates colonized on the top 1-cm segments main and lateral root

Isolate	Population density on root ($\times 10^5$ cfu/1-cm root)		
	Main root	Lateral root	Total
B16	32.02[b]	2.45[b]	68.90[ab]
V13	533.33[a]	3.55[a]	576.00[a]
MC07	11.87[bc]	1.39[bc]	32.10[bc]
X01	4.20[bc]	0.09[c]	8.00[bc]

[a] In each column, values followed by the same letter do not differ significantly (P=0.05) according to Duncan's mul- tiple range test.

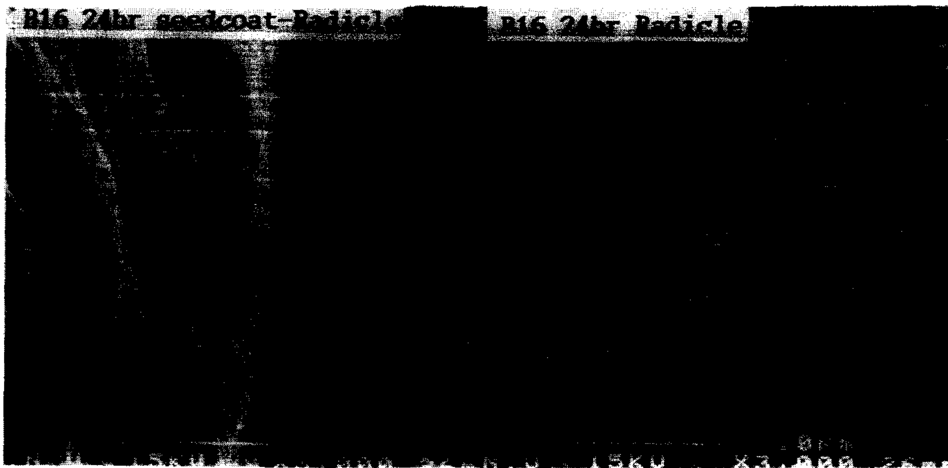

Fig. 2. Scanning electron microscopy of the cells of *Pseudomonas fluorescens* B16 on cucumber seed-coat and radicle ($\times$3000).

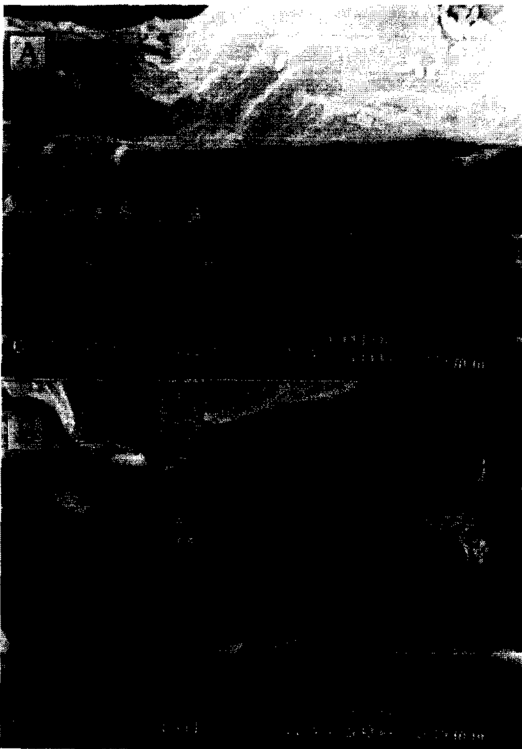

Fig. 3. Scanning electron microscopic observations of the cells of root-colonizing isolates on middle part of cucumber rhizoplane 96 hr after seed inoculation($\times$3500). A) the cells of the good colonizing isolate B16. B) the cells of the poorly colonizing isolate X01.

cucumber rhizoplane. Bacteria inoculated to the seed began to move along the emerging root and colonize the root surface. Fig. 3. illustrated the different patterns of rhizosphere colonization by the good colonizing isolate B16 and the poorly colonizing isolate X01 in the middle part of cucumber roots. The isolate B16 had most of its cells inside the grooved lines of the rhizoplane and much higher numbers of bacterial cells were arranged linearly and parallel along the growing root axis (Fig. 3A). In contrast, the cells of isolate X01 were randomly associated with smooth surface of roots and the cell numbers were much less than that of B16 (Fig. 3B).

When primary root axis began to give rise to lateral root after 48 hrs, the bacterial cells moved along to the newly emerging lateral root axis. The good colonizing isolate B16 not only grew on the top 1-cm segment of the cucumber rhizoplane, but also readily moved to the lateral roots. In top 1-cm segment of root, the cells of B16 vigorously multiplied on the whole root surface and many cells moved to lateral roots and also arranged linearly along the axis of root growth (Fig. 4A, 4C). But, quite a few cells of isolates X01 were in lateral roots. They were not arranged continuously along the axis of root growth (Fig. 4B, 4D).

DISCUSSION

Biocontrol agent should colonize the surface of the plant which it protects, and colonization is widely believed to be essential for biocontrol (9). Generally, the term rhizosphere competence or root colonization ability of bacteria and fungi are practically used for quan-

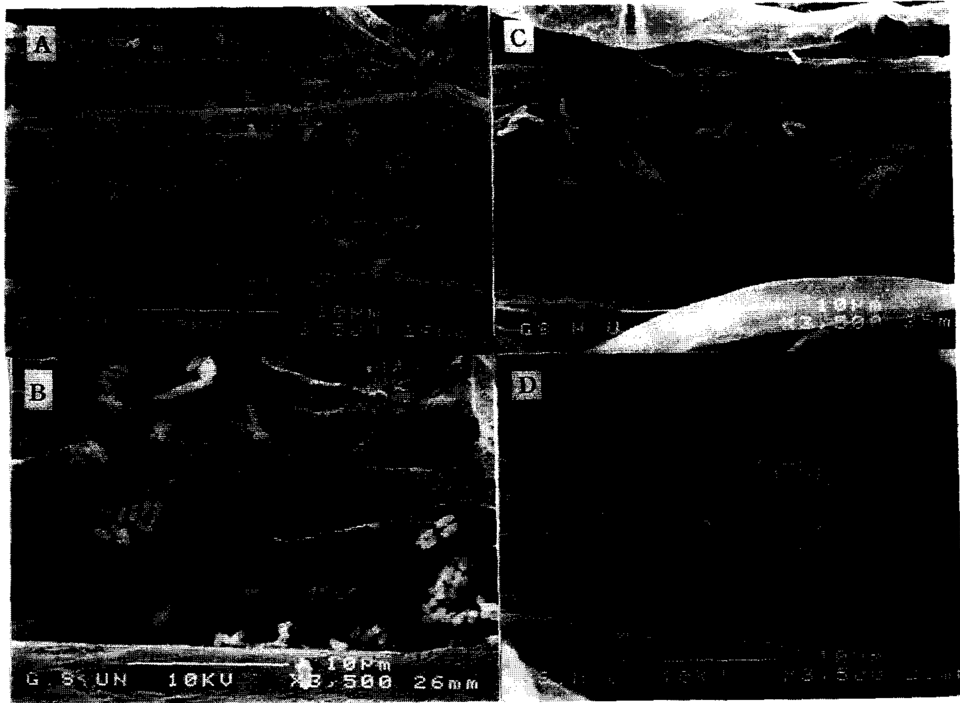

Fig. 4. Scanning electron microscopy of the root-colonizing bacterial isolates on the main(A, B) and lateral(C, D) roots of cucumber 96 hr after seed inoculation. A) and C) cells of the good colonizing isolate B16. B) and D) cells of the poorly colonizing isolate X01 (×3500).

tification of the numbers of colony forming units establishing on the root system per unit of root such as length, weight, number of roots, and so on (1, 2, 14). In this study, we employed the DLF method permitting convenient assessment of population densities of tested isolates colonized on the specific site of the root system, especially in the early stage of growth (2). It assumed that rapid multiplication and adherence on the seed surface was probably the first step of the root colonization. Harman *et al.* (7) also reported that root colonizing bacteria were determined during the first 24 hr after plating. The seed treated with the good root colonizing bacteria effectively protected the plant from plant deleterious soilborne microorganisms.

The rhizosphere competent isolates B16 and V13 showed rapid growth on the surface of seed coat and moved to the newly emerged roots. The population densities of good colonizing isolates on seed coat near emerging root already exceeded the carrying capacity to the root.

The rhizosphere competent isolates B16 and V13 showed higher population densities and rapid movement along with the root tips and the lateral roots. It could be assumed that the root colonizing bacteria proliferate at the juncture or lateral roots because rich organic materials were available.

Biocontrol agents are needed to protect the juncture or lateral roots because this sites are very important for the root pathogens to invade host plants (8, 10, 12, 13). Loper *et al.* (11) suggested that the ability of rhizosphere bacterial strain to establish a significant population size along an elongation root system is a key determinant in its effect on plant growth. In our previous studies, rhizosphere competent isolates also showed consistently better plant growth and more efficient disease suppression than rhizosphere incompetent isolates (9, 14).

Scanning electron microscopy of bacterial colonization on rhizoplane are generally conducted in a static state. Foster *et al.* (6) observed that only 7~15% of the actual root surface is occupied by microbial cells which were on the grooves of epidermal cells of the host roots, despite huge numbers of bacteria were existed in the rhizosphere.

The cells of isolate B16 were linearly arranged and parallel with growing root axis, while the cells of poor-

ly colonizing isolate X01 were randomly scattered on the root surface, and the number of bacterial cells was also limited. The parallel arrangement of bacterial cells with the root growing axis partially illustrated that rhizosphere competent bacteria were not aggregated at certain spots, but continuously move to the newly growing root system when they multiply in the rhizoplane.

Our results suggest that the root colonizing bacteria might have a distinct effect of expelling other microorganisms from the colonization on plants. Therefore, the bacteria should be applied to the plants in the germinating stage. Because the seed application procedure makes the bacteria reach firstly to the roots, so that the root growth promoting action by the bacteria can be significant.

요 약

전국에서 채집한 균주들 중 근권정착능력이 있는 균주를 선발하였다. 선발된 대부분의 균주들은 형광성 *Pseudomonas*속에 속하는 균주들이었고 이들 중 근권정착능력이 아주 우수한 B16과 V13을, 정착능력이 낮은 MC07과 X01을 시험에 공시하였다. 근권정착능력이 우수한 균주 B16과 V13은 종자 발아 초기단계에서의 증식 능력이 우수할 뿐만 아니라, 종자가 발아를 시작하여 유근이 나오는 시기에 이들의 정착밀도가 높았으며, 96시간이 지난 후에도 뿌리의 근단 부위에서 V13과 B16 균주들의 정착밀도가 높았다. 주사전자현미경(SEM)을 통하여 이들의 근권에서의 정착양상을 관찰한 결과, 정착능력이 우수한 균주들은 뿌리의 전반적인 부분에서 증식하고 근단 부위로 이동하는 것을 관찰할 수 있었다. 그러나 정착능력이 낮은 균주들은 유근의 표면에 산발적으로 흩어져 증식한 것을 볼 수 있었다.

감사의 글

본 연구의 수행은 1995년도부터 한국학술진흥재단 (Korea Research Foundation)의 중점과제로 경상대학교 부설 유전공학연구소(Genetic Engineering Institute)에 지원된 연구비로 연구되었음.

REFERENCES

1. Ahmad, J. S. and Baker, R. 1988. Implications of rhizosphere competence of *Trichodema harzianum. Can. J. Microbiol.* 34 : 229-234.
2. Bae, Y. S., Kim, H. K. and Park C. S. 1990. An improved methods for rapid screening and analysis of root colonizing biocontrol agents. *Korean J. Plant Pathol.* 6 : 325-332.
3. Brown, M. E. 1974. Seed and root bacterization *Annu. Rev. Phytopathol.* 12 : 181-197.
4. Bull, C. T., Weller, D. W. and Thomashow, L. S. 1991. Relationship between root colonization and suppression of *Gaemannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. *Phytopathology* 81 : 954-959.
5. Chao, W. L., Nelson, E. B., Harman G. E. and Hoch, H. C. 1986. Colonization of rhizosphere by biological control agents applied to seeds. *Phytopathology* 76 : 60-65.
6. Foster, R. C., Rovira, A. D. and Cock, T. W. 1983. Ultra structure of the root soil interace. APS press, St Paul, Minn. 157pp.
7. Harman, G. E., Taylor, A. G. and Stasz, T. 1989. Combining effective strains of *Trichoderma harzianum* and solid matrix priming to improve biological seed treatment. *Plant Disease.* 73 : 631-637.
8. Harman, G. E. 1989. Deployment tactics for biocontrol agent in Plant Pathology. In new directions in biological control: Alternatives for suppressing Agricultural Pests and disease. UCLA symp. Mol. Cell Biol., ed. R. Backer, P. Dunn, 112 : 779-92. New York: Liss)
9. Jang, S. S., Jeong, M. J., Park, C. S. and Kim, H. K. 1993. Significant attribute biocontrol agents for colonizing ability at corresponding - infection site of soil-borne plant pathogens. *Korean J. Plant Pathol.* 9 : 7-11.
10. Kloepper, J. W., Scher, F. M., Lalibertes, M. and Zaleska, I. 1985. Measuring the spermosphere colonizing capacity(spermosphere competence) of bacterial inoculates. *Can. J. Microbiol.* 31 : 926-929.
11. Loper, J. E., Hankock, C. and Schroth, M. N. 1985. Population dynamic of soil pseudomonads in rhizosphere of potato (*Solanum tuberosum* L.) *Appl. Environ. Microbiol.* 49 : 416-422.
12. Park, C. S. 1994. Impact of rhizosphere competence of biocontrol agent upon disease suppression and plant growth promotion Proc. Int. Symp. on Biological Control of Plant Disease pp. 27-49 held in Suweon Korea.
13. Park, C. S., Paulitz, T. and Baker, R. 1988. Attributes associated with increased biocontrol activity of fluorescent pseudomonads. *Korean J. Plant Pathol.* 4 : 218-225.
14. Park, C. S. and Yeom, J. R. 1994. Biological control of cucumber damping off and enhancement of see-

dling growth by low temperature-tolerant *Pseudomonas fluorescens* M45 and MC07. pp. 54-56 in pro. PGPR Workshop held in Adelaide, Australia 15.
15. Parke, J. L. 1990. Population dynamics of *Pseudomonas cepacia* in the pea spermosphere in the relation to biocontrol of *Pythium*. *Phytopathology* 80: 1307-1311.
16. Parke, J. L. 1991. Root colonization by indigenous and introduced microorganisms. In The Rhizosphere and Plant growth, Keister, D. L. and Cregan, P. B. eds. (Boston, MA: Kluwer Academic Publishers) pp 33-42.
17. Schroth, M. N. and Khancock, J. G. 1992. Disease-suppressive soil and root colonizing bacteria. *Science* 206: 1376-1381.
18. Weller, D. M. and Cook, R. J. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* 26: 376-407.

(Received March 20, 1997)