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Colonization and Population Changes of a Biocontrol Agent, *Paenibacillus polymyxa* E681, in Seeds and Roots

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***Paenibacillus polymyxa* E681, with its plant growth promotion and root colonization ability, has been proven to be a promising biocontrol agent of cucumber and barley. This study investigated the attributes related to the movement of bacteria from the seed to the radicle and to the whole root system. It also illustrated the existing form and population changes of the bacteria on seed and root using the scanning electron microscope and confocal laser scanning microscopy. The bacteria invaded and colonized the inside of the seed coat while the seeds were soaked in bacterial suspension. Almost the same number of bacteria on seed surface invaded the inside of the seed coat right after seed soaking. The population densities of E681 increased greatly inside as well as on the surface of the seed before the radicle emerged. The bacteria attached on the emerging radicle directly affected the initial population of newly emerging root. The colonized cells on the root were arranged linearly toward the elongation of the root axis. In addition to colonizing the root surface, strain E681 was found inside the roots, where cells colonized the intercellular space between certain epidermal and cortical cells. When the cucumber seeds were soaked in bacterial suspension and sown in pot, the bacterial populations attached on both the surface and inside of the root were sustained up to harvesting time. This means that E681 successfully colonized the root of cucumber and sustained its population density up to harvesting time through seed treatment.**

***Keywords* :** root colonization, *Paenibacillus polymyxa* E681, seed treatment, population change

Although there have been numerous reports of successful biological control agents, the disease suppression and/or growth promotion afforded by these treatments are frequently not consistent in the field (Weller, 1988). The commercial application of biological control agents has not been extensively pursued partly because of the lack of a delivery system with which the agents can be carried conveniently to the target site (Chao et al., 1986), and because there are currently few commercially available products for the control of the plant disease (Xi et al., 1996). In many cases, variable performance of introduced rhizobacteria has been attributed to insufficient root colonization, a process whereby the bacteria inoculated onto seed attach to the root surface, and colonize the developing root system (Weller, 1988). As the root elongates, bacterial multiplication at the tip ideally would permit transport of bacteria as long as the roots grow but without multiplication, transport would occur only until the initial inoculum at the root tip (Kang and Park, 1997). Then, the bacteria spread locally and proliferate to the limits of the niche in competition with indigenous organisms, and survive (Howie et al., 1987). While the relationship between the bacterial population developing on the seed and root in seed treatment is important for effective biocontrol, little information is available regarding movement of microorganisms from the seed to the rhizosphere. Especially, bacteria, to be effective biocontrol agents, may be required to rapidly and extensively colonize seed and quickly become active to protect seeds from pathogen colonization and infection. This short-time period before infection may be a critical period for successful seed protection (Hood et al., 1998; Nelson et al., 1986). Thus, events in seed attachment, movement from seed to emerging root, and colonization on root by the bacterium seem to be relevant in understanding the biocontrol mechanism.

The purpose of this study was to investigate the attributes related to the movement of bacteria from the seed to the radicle and to the whole root system, as well as to develop enhanced seed inoculation techniques for consistent and effective biocontrol. It also aimed to illustrate the existing form and population changes of bacteria on seed and root using selective markers labeled on the bacteria and the fluorescent antibodies, and to observe such changes under the scanning electron microscope and confocal laser scanning microscopy.

Materials and Methods

Bacterial strains. The biocontrol bacterium used in this study

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was *Paenibacillus polymyxa* E681 (Ryu and Park, 1997) from the collection of the Laboratory of Soil Borne Diseases and Biocontrol of the Department of Agricultural Biology, Gyeongsang National University. *P. polymyxa* E681 is a promising plant growth promoting rhizobacterium (PGPR) and a spontaneous rifampicin mutant. *P. polymyxa* E681 was cultured on trypticase soy agar (TSA, BBL) at 30ºC for 30 hours.

Seed treatment. Seeds of cucumber (*Cucumis sativus* L. cv 'Shinhugjinju') were used for bacterial seed treatment. The cell suspension of E681 used for seed treatment was fixed to 10^8 cells per ml. The seeds of cucumber were soaked in the bacterial suspension for 1 hour for seed treatment.

Population analysis of bacteria on the seed. To analyze the population densities of the bacteria attached to the seed, two different methods were employed. One was surface disinfection with 1% sodium hypochlorite right after seed treatment. Soaked seeds were surface disinfected for 5 minutes with 1% sodium hypochlorite, then washed with sterile water. Non-sterilized seeds were used as population density in the outside. The other method was removal of the seed coat after seed treatment with bacteria. After the seeds have been divided into seed coat removed and unremoved, the population densities of bacteria on the surface and inside the seed were determined by dilution plate counting.

Population analysis of bacteria on root. Bacterial densities colonized on the roots from the seeds with treatment of surface sterilized, non-sterilized, coat excised, and unexcised were enumerated by DLF method (Bae et al., 1990). To examine population change of bacteria from seed to root, both intact and coat excised seeds were transferred onto plate containing wet filter paper and were incubated for 48 hours at 27ºC. The population density of bacteria on the root tip was determined by dilution plate counting.

The cucumber seeds were treated with E681 and sowed in plastic pot (30-cm in diameter). The roots of cucumber treated with E681 were collected from the pot at 15-day intervals. Half of the root preparation was transferred at 100 ml 0.1 M $MgSO_4$ solution and shaken at 250 rpm for 1 hour. At the same time, the other half of the root preparation was surface disinfected for 10 minutes with 1% sodium hypochlorite. Both root samples were macerated in sterile mortar with pestle. The population density was determined as described above.

Microscopical observation. E681 colonized on cucumber seeds and roots were obtained by the DLF method and using the soil medium described by Ahmad and Baker (1987). Both intact and coat-removed seeds and root segments were fixed in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) and the specimens were post-fixed in 2% OsO4 (Sigma, U.S.A) in 0.05 M phosphate buffer solution for 2 hours. The specimens were dehydrated through a series of ethanol gradient (20-100%) and coated by Ion coater with gold. Prepared specimens were observed with the use of Scanning Electron Microscopy (JSM-6400, Jeol Co. Japan) at 10, 15, and 20 kV.

The polyclonal antibody (PAb) was obtained from a 12-month-old male New Zealand white rabbit which had been immunized seven times with 10^9 living cells/ml of 0.9% phosphate-buffered saline. The homology of antisera was tested by agar gel diffusion method and the titer was determined through cell binding with antibody and fluorescein isothiocyanate (FITC), labeled goat anti-rabbit immunoglobulins under immunofluorescence microscopy (D-07740 Jena, Carl Zeiss, Germany).

For the observation of the rhizoplane under immunofluorescence microscopy, the cross-section of the roots was made by hand with a razor blade. The root samples were treated with 1:1000 dilutions of primary antisera of E681. The samples were treated with the secondary antiserum consisting of FITC, labeled goat anti-rabbit immunoglobulins (Sigma, U.S.A). All slides were viewed on an LSM 410 inverted confocal laser scanning microscopy (Carl Zeiss, Germany) equipped with an Argon ion laser (488 nm/10 mW). All combining and processing of the images were performed with the standard software provided by Zeiss.

Results

Population density of the bacteria on the surface and inside of seeds and emerging roots. Through the series of experiments detecting bacterial population on the seed surface after sterilization, it was confirmed that some of the bacteria inoculated on seeds of cucumber, pepper, rice, and sesame invaded and colonized the inside of seed coat during the seed soaking. On the surface of non-sterile cucumber seed, the initial population of E681 was 3.3×10^4 cfu/seed, while on the surface of sterile seed, no population was detected. When the population density of E681 on the root was measured, almost the same population density was detected from the surface sterile and non-sterile seeds. Some of sterile seeds showed even more population density. The same results were obtained from rice, pepper, and sesame (Table 1).

The results were similar with that of seed-coat excised cucumber seed after soaking in bacterial suspension. The initial population of E681 on both intact and coat-excised seeds was 10^4 cfu/seed and the root colonizing population

Table 1. Root colonizing population of *P. polymyxa* E681 from surface sterilized and non-sterilized seeds after various seeds were soaked in bacterial suspension for 1 hour

Crop	Seed surface sterilization	Population density ($\times 10^4$ cfu/root tip)	
		Seed surface	Root
Cucumber (*Cucumis sativus*)	none	3.3	28
	sterile	–	240
Hot pepper (*Capsicum annum*)	none	0.8	100
	sterile	–	370
Rice (*Oryza sativa*)	none	5.0	5.4
	sterile	–	2.2
Sesame (*Sesamum indicum*)	none	0.3	17
	sterile	–	52

Table 2. Population densities of *P. polymyxa* E681 on the seeds and roots of cucumber from seed coat removed and intact seeds after soaking in bacterial suspension

Treatment	Population density ($\times 10^4$ cfu/root tip)	
	Seed	Root
Intact seed	3.3	28
Coat removed seed	3.5	30

of both seeds was 10^5 cfu/root (Table 2).

Population change of bacteria from seed to root. Initial population of E681 on intact seed and coat-excised seed was around 10^4 cfu/seed and was not significantly different. The bacterial population of E681 on intact and coat-excised seed was slowly increased. The cucumber root started to emerge after 16 hours of bacterial treatment. The initial population of E681 on emerging root from intact and coat-excised seed was similar. Also, the population density colonized on the root from both intact and coat-excised seeds was similar and remained constant after 32 hours (Fig. 1).

Existing form of the bacteria on the surface and inside of cucumber seed and emerging root. Immediately after inoculation, the cells of E681 were randomly scattered on the surface of the cucumber seed. Also, the cells of E681 were penetrated up to the inside of the seed coat during the seed soaking period. After 6 hours of treatment, seed coat was excised and the cells of E681 were observed on endothelium of seed and radicle. The cells of E681 abundantly

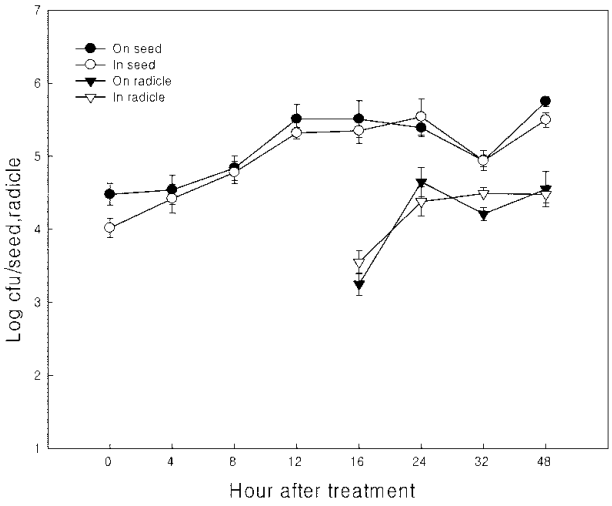

Fig. 1. Sequential population change of *P. polymyxa* E681 on and inside of the seeds and emerging roots of cucumber after the seeds were soaked in the bacterial suspension.

proliferated and aggregated on the groove of endothelium (Fig. 2A). Also, a lot of the cells already moved to the radicle after 10 hours when the seed coat and endothelium were excised from the seed. By this time, the radicle and the cotyledons were formed completely. Numerous cells of E681 aggregated on the upper parts of the radicle and cells were arranged linearly along the junction in between radicle cells (Fig. 2B). By 20 hours, most bacteria were organized in micro-colonies on the radicle epidermis, and most micro-colonies and individual cells were located in the junctions

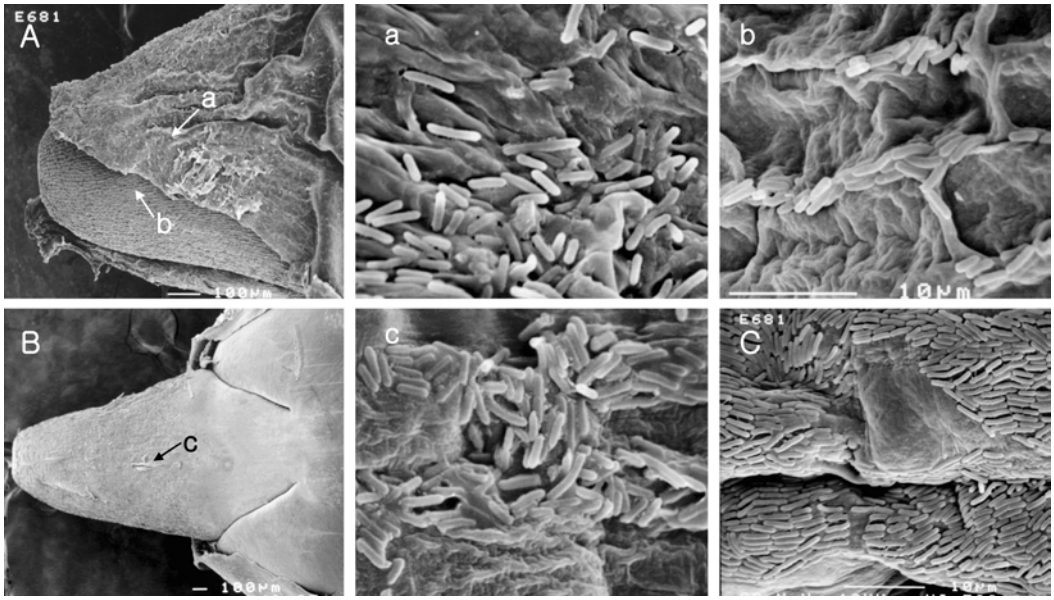

Fig. 2. Scanning electron microscopic observation of *P. polymyxa* E681 on cucumber seed and emerging roots. (**A**) Overall view of radicle and endothelium of cucumber seed after 6 hours seed bacterization. (**B**) Newly emerging root of cucumber when the seed coats were excised after 10 hours seed bacterization. (**C**) Cells of E681 on the root after 20 hours of seed bacterization: i) cells of E681 on a radicle; ii) cells of E681 on the endothelium of seed; iii) cells of E681 on an emerging root.

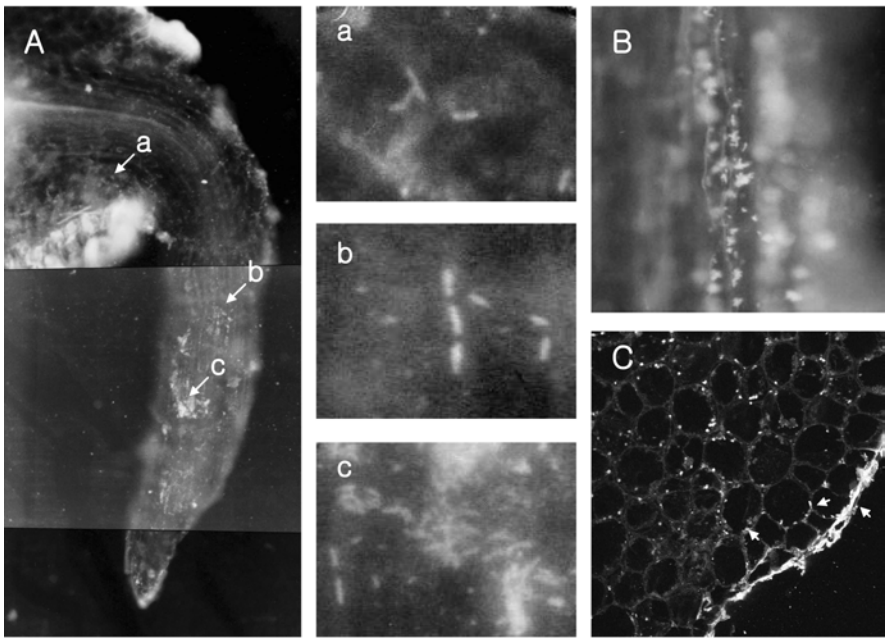

Fig. 3. *P. polymyxa* E681 on the emerging root of cucumber observed through immunofluorescence microscopy and confocal laser scanning microscopy (CLSM). (**A**) Overall view of lateral root emerging and cells of *P. polymyxa* E681 on the inside of root and surface of lateral root. (**B**) Cells and microcolonies of *P. polymyxa* E681 at the surface of cucumber root. (**C**) Image of E681 distributed in intercellular space of root captured by CLSM: i) cells of *P. polymyxa* E681 adjacent to emerging lateral root; ii and iii) cells of *P. polymyxa* E681 on the surface of lateral root. Arrows indicate cells of E681.

in between the epidermis cell or in deeper parts of the root epidermis (Fig. 2C).

Using immunofluorescence microscopy (IF) and confocal laser scanning microscopy (CLSM), attachment of E681 on the seeds and roots was directly examined. The primary antiserum raised against E681 was effective to detect the cell of E681 on the roots and seeds. After 72 hours of seed treatment, the cells of E681 were colonized on the surface of cucumber root (Fig. 3B). The lateral root surface was covered by E681 cells, while the epidermal cells were covered with bacteria. Also, some of the cells were located adjacent to the lateral root that emerged (Fig. 3A). Cells of E681 were also present both on the surface and inside the root, where cells colonized the intercellular space between certain epidermal and cortical cells (Fig. 3C).

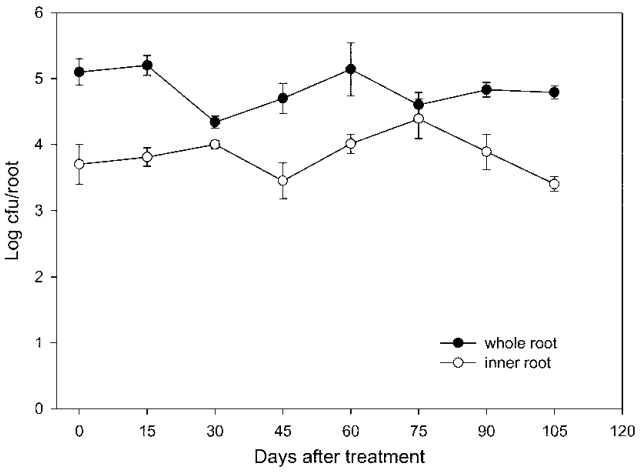

Fig. 4. Sequential change of *P. polymyxa* E681 on the whole and inner root of cucumber grown in pot. The population density of the inner root was estimated after surface sterilization with 1% NaOCl for 10 min.

Population analysis of introduced bacteria in the field. The colonized population density of E681 on the whole root and inside root of cucumber were maintained at 10^3-10^5 cfu per root up to fruit bearing (Fig. 4).

Discussion

Treatment of seed with good root colonizing bacteria effectively protected the plant root from plant deleterious soil-borne microorganisms. Root colonization by introduced bacteria is an important step in the interaction of bacteria with the host plant (Weller, 1988). Results of this study indicate population changes of the bacteria from seed to root system. It was confirmed that the bacteria invaded and colonized the inside of the seed coat while the seeds were soaked in bacterial suspension, through seed surface sterilization and/or coat excised seed (Tables 1, 2). Almost the same number of bacteria on seed surface invaded the inside of the seed coat right after seed soaking. These bacteria are assumed to have invaded through basal pore with water

imbibitions of seed. Bacteria that invaded the seed coat colonized the inner plane of seed and proliferated until seed germination. The bacteria that proliferated inside of the seed coat may significantly affect root colonization.

Studies related to root colonization mostly by Gram-negative bacteria (Bull et al., 1991; Chin-A-Weong et al., 1997) have been reported. This current study, however, investigated the root colonization of Gram-positive bacterium belonging to *Paenibacillus polymyxa*. The population densities of E681 increased greatly inside, as well as on the surface of the seed before the radicle emerged, and the bacteria attached to the emerging radicle affected directly the initial population of newly emerging root (Fig. 1).

Previous studies exploring seed treatment of rhizobacteria have been generally focused on attachment and survivability of rhizobacteia on the surface of the seed (Caesar and Burr, 1991; Hood et al., 1998; Shah-smith and Burns, 1996). However, in this experiment, colonizing bacteria inside the seed is more important to root colonization rather than the surface of the seed. Therefore, the method of soaking in bacterial suspension, especially at the initial root colonization, is likely to be a critical factor for effective rhizobacteria treatment.

Based on the observation with the use of the SEM, it was confirmed that bacterial cells that invade the seed coat move directly to emerging root. After seed treatment, cells of bacteria moved from the endothelium of the seed to the emerging radicle, and increased abundantly on emerging radicle. The cells were arranged linearly toward elongation root axis (Fig. 2).

Immunofluorescence microscopy and CLSM proved to be a valuable tool to monitor in detail the colonization of cucumber roots by *P. polymyxa* E681, and showed that the particular bacterium is an endophyte. The cells of E681 were found preferentially at the junction of epidermal cell, a microhabitat with enhanced exudation, humidity, and mucigel (Fig. 3A). In addition to colonizing the root surface, strain E681 was also found inside the roots, where cells colonized the intercellular space between certain epidermal and cortical cell (Fig. 3C). Hallmann et al. (1997) reported that bacterial preferences for colonization of specific plant areas seem to be strain and species specific.

When the cucumber seeds were soaked in bacterial suspension of E681 and sown in experimental pot, the colonized bacterial populations on the whole and inside of the root were sustained from the beginning up to harvesting time (Fig. 4). This means that E681 successfully colonized the root of cucumber and sustained population density up to harvesting time with only seed treatment. The root population of *Pseudomonas* sp. strain B10, which is not effective to plant, was shown by Loper et al. (1985) to be markedly decreased with distance from the initial seed piece. In contrast, plant growth promoting strain *Pseudomonas* sp. strain B4 was distributed over the whole root at relatively uniform population. These results support the finding that the ability of biocontrol agent in colonizing the root system is closely related with suppression of soil-borne pathogens and enhancement of plant growth.

The results obtained in this study provide some basic information about commercial application of biological control agents by seed treatment. The SEM data and continuous population changes of the bacteria from the inside of the seed to the root system can be used to investigate effective seed treatment of biocontrol agent and root colonization of rhizobacteria. Immunofluorescence microscopy and CLSM monitored in detail the colonization of E681 in the root system, showing that this bacterium plays a role as an endophyte. Further studies are required to determine the role of E681 endophyte.

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