

Biological Seed Treatment : Viable Population Changes of *Paenibacillus polymyxa* on Seed and Root and its Antagonistic Activity after Seed Formulation

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ABSTRACT

We analyzed the viable population changes of *Paenibacillus polymyxa* E681, a plant growth-promoting rhizobacterium, on seeds and roots after bioformulation at varying time intervals during the storage. The viable population of E681 on tested crop seeds sustained log 4-5 cfu/seed after 300 days of seed treatment. The ability of root colonization and inhibition of fungal mycellial growth was not influenced even after 300 days of seed treatment. The seed-soaking treatment returned better results than powder formulation, in increasing the initial population of E681 on plant roots. Collectively, it was found that E681 is a durable and stable biological control agent for application to crop seeds.

Key words - Seed formulation, Endospore, Root colonization, *Paenibacillus polymyxa* E681

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I. INTRODUCTION

Seeds are the most compact forms of plants, and hence less inoculum or space is needed to apply biological control agents (BCAs) at this early stage (Chao *et al.*, 1986). Seed treatment is an attractive method for introducing BCAs into the soil-plant environments. After seed treatment, BCAs can multiply on or within seeds, and proliferate to root system, eventually colonizing whole root system (Brown, 1974; Suslow & Schroth, 1982; Choi *et al.*, 2004).

Many research groups have carried out studies on fluorescent Pseudomonads as a promising BCA, though the vegetative cells of Pseudomonads are sensitive to adverse environmental conditions, including dry, radiation, and temperature, and hence retain a relatively short shelf-life on seeds and rhizospheres (Weller, 1988; Pierson & Weller, 1994; Shah-smith & Burns, 1996). Recent reports have provided evidence that *Bacillus* or *Paenibacillus* also elicited plant growth promotion or suppression of plant diseases (Ryu *et al.*, 2003, 2005, 2006). The major benefit of using bacilli in this respect is their endospore formations, that are more stable and durable under unfavorable environmental conditions. Endospore formation can make bacilli easy to formulate and commercialize, because of their a long-term shelf-life. This characteristic has been consistently attracted the attention of major research groups attempting to develop BCAs for practical applications. For example, several commercial products such as Quantum, Kodiak, and BioYield originated from bacilli, and are available in the USA and other countries (Cook, 1993; Brannen & Kenney, 1997; McSpadden-Gardener, 2004; Lucy *et al.*, 2004). However, optimal biological control strategies for disease suppression may be different depending on the specific nature of the target

pathogen. To improve the biological activity of introduced bacteria, seed application through various soil components was applied (Kloepper & Schroth, 1980; Connick *et al.*, 1990; Taylor & Harman, 1990; Jacobsen *et al.*, 2004; Schisler *et al.*, 2004).

It was previously found that *Paenibacillus polymyxa* E681, with its plant-growth promotion and root-colonizing ability, has been proven to be a promising BCA to control major soil-borne pathogens (Choi *et al.*, 2004; Ryu *et al.*, 2005). In order to apply E681 onto the seed, a powder formulation was also developed (Ryu *et al.*, 2005). This study was conducted to evaluate the potential of E681 as a BCA by characterizing its antifungal activity and root colonizing ability after 300 days of seed formulation.

II. MATERIALS AND METHODS

2.1 Bacterial isolates

The bacterial isolates, *Pseudomonas fluorescens* B16 (Kim *et al.*, 2003) and *Paenibacillus polymyxa* E681 (Choi *et al.*, 2004), were used in this experiment. *Ps. fluorescens* B16 was cultured on King's B agar (KB; 10 g proteose peptone No. 3, 1.5 g K₂HPO₄, 1.5 g MgSO₄·7H₂O, 10 ml glycerol, 20 g agar, 1 l distilled water) at 28°C. *P. polymyxa* E681 was cultured on 1/10 strength of Trypticase Soy Agar (TSA; 3g BBL Co. trypticase soy broth, 20g agar, 1 l distilled water) at 37°C. Rifampicin resistant strains of these bacteria were used for population changes. When required, 100 µg/ml rifampicin was added to the medium. Both bacterial strains were maintained at -80°C with glycerol (25%) for long-term storage.

2.2 Tested plant seeds

Seeds of cucumber (*Cucumis sativus* L. cv Shinheukjinju; Seminis), hot pepper (*Capsicum anuum*

L. cv Nokwang; Seminis), tomato (*Lycopersicon esculentum* Mill, cv Gwangsu; Seminis), sesame (*Sesum indicum* L., cv Suwon 6), barley (*Hordeum vulgare* L., cv Oweol), wheat (*Triticum aestivum* L. cv Woori), and rice (*Oryza sativa* L. cv Ilpum) were used.

2.3 Bioformulation with powder materials

Powdered bentonite, perlite, and talc (Sigma Chemical Company, St. Louis, Mo) were used for formulation and application of the BCA to seeds. Bacterial suspensions prepared as 10^9 cells per ml in 10 mM MgSO₄ solution were mixed with an equal volume of autoclaved 20% xanthan gum. Independently, bentonite, perlite, and talc powder were added to bacterium-xanthan gum mixtures. After drying, the bacterial powder mixtures were grounded in a Waring Blender, and screened to remove lumps. Seeds were sprayed with a 1.5% (w/v) solution of polyvinyl alcohol, and were then either hand-shaken or mechanically rolled in the powder prepared above. The pelletized-seeds were then air-dried at room temperature and stored in plastic containers with lids. The seed-soaking treatment in the bacterial suspension was used as a control.

2.4 Assessment of longevity of bacterial strains on seeds

Seeds formulated with powder materials were tested at 10-day intervals for strain B16 and 50-day intervals for E681 to assess bacterial survival. The colony forming units (CFU) of each treatment were measured with a dilution plate method. Briefly, a volume (0.1 ml) of a diluted suspension was spread over the surface of KB plate for strain B16 and TSA for E681 using a sterile glass spreader. The plate was then incubated until colonies appear, and the number of colonies was counted. Initial populations of a bacterial

strain on seed were measured immediately after seed formulation.

2.5 Root colonization assay

Bacterial cells colonized on roots were examined by DLF method (Bae *et al.*, 1990). Seeds treated with soaking treatment in bacterial suspension or powder-formulating materials were randomly taken at varying time intervals. After 40 h of incubation at 28°C, the root tip was cut into the 1 cm segment with a sterile scalpel. Then root segments were collected and were re-suspended in 30 ml of 10 mM MgSO₄ solution. The test tubes were vigorously stirred with a vortex mixer. The colony forming units were determined by plating a series of 10-fold dilution on KB for B16 or 1/10 strength of TSA for E681 supplemented with 50 µg/ml rifampicin (Sigma Chemical Company, St. Louis, MO). Bacterial colony number was counted after 3 days of incubation at 28°C for strain B16 and 37°C for E681.

2.6 *In vitro* inhibitory activity against fungal pathogens

The colonies of *P. polymyxa* E681 were transferred on eight spots of PDK (12 g Difco potato dextrose broth, 5 g proteose peptone No. 3, 15 g agar, 1 l distilled water) media plate with equal spacing around the perimeter, and incubated at 28°C. One day after incubation, 0.5 cm diameter of mycellial disks of *Fusarium oxysporum*, *Pythium ultimum*, *Phytophthora capsici*, and *Rhizoctonia solani* which were grown on Potato Dextrose agar (PDA; 24 g Difco potato dextrose broth, 20 g agar, 1 l distilled water) was placed in the center and equal distant from the 8 colonies of bacteria. After incubation for 5 days, the inhibitory activity was examined, and determined as the width of the clear zone between the bacterial colony and fungal pathogens.

2.7 Scanning electron microscopy (SEM)

Seed and root samples were fixed in 5% glutaraldehyde in 50 mM phosphate buffer (pH 7.0) for overnight at 4°C. The specimens were then carefully rinsed three times with 50 mM phosphate buffer solution, after which they were post-fixed in 2% OsO₄ (Sigma, USA) in 50 mM phosphate buffer (pH 7.0) solution for 2 h at room temperature and rinsed carefully three times with 50 mM phosphate buffer solution. The specimens were dehydrated through a series of ethanol gradient 20, 40, 60, 80, 90, and 100% and additional dehydration were made with 100% amylacetate twice, and were then coated with gold using an Ion coater. Prepared specimens were observed using Scanning Electron Microscopy (SEM) (JSM-6400, JEOL Co. Japan) at 10, 15, and 20 kV.

2.8 Data analysis

All determinations were conducted in triplicate and standard deviation values were estimated. Data were subjected to analysis of variance using SIGMA PLOT (Systat Software Inc., Richmond, CA, USA), and significant differences between treatments were determined using the Duncan's multiple range test.

III. RESULTS AND DISCUSSION

3.1 Viable population changes of *Ps. fluorescens* B16 and *P. polymyxa* E681 on seeds after bioformulation

The viable population of B16 on cucumber seeds after the seed-soaking treatment was sharply decreased within a week. Survival of B16 in bentonite formulation was extended, for a total period of up to 45 days (Fig. 1A). This result coincides with the experimental observations. Fluorescent *Pseudomonads* as biocontrol agents will be

similar to those that have been faced in rhizobia, which are sensitive to drying and heat (Mary *et al.*, 1985; Osa-Afiana & Alexander, 1982).

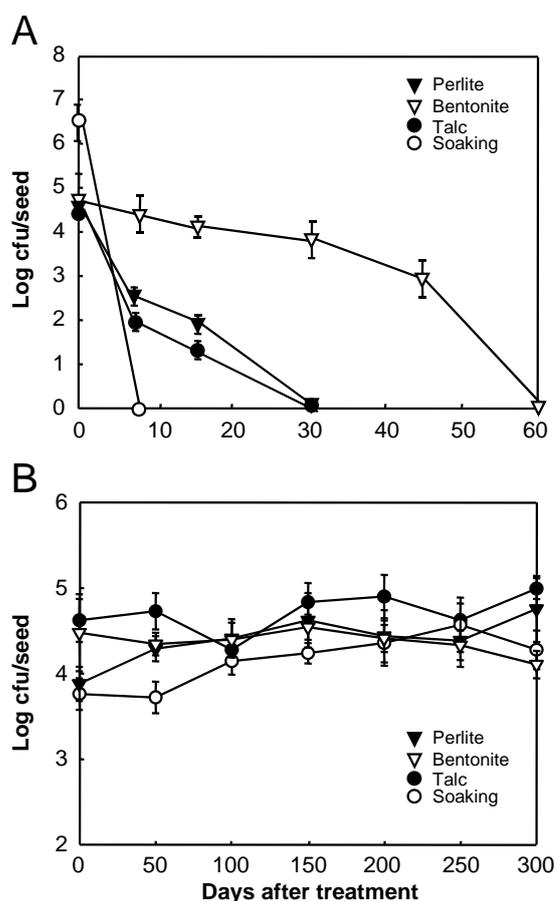


Fig. 1. Viable population of *Pseudomonas fluorescens* B16 (A) and *Paenibacillus polymyxa* E681 (B) on cucumber seeds right after bioformulation. The bacterial cells were formulated with powder materials, bentonite, perlite, and talc. Soaking denotes seed-soaking treatment in the bacterial suspension. Error bars represent the standard deviation.

Survival of E681 in all treatments was maintained more than 300 days. The viable population of E681 on the surface of cucumber seeds was $3\text{-}5 \times 10^4$ cfu/seed up to 300 days (Fig. 1B). The results

indicated that cells of E681 were able to survive on cucumber seeds after 300 days of bioformulation.

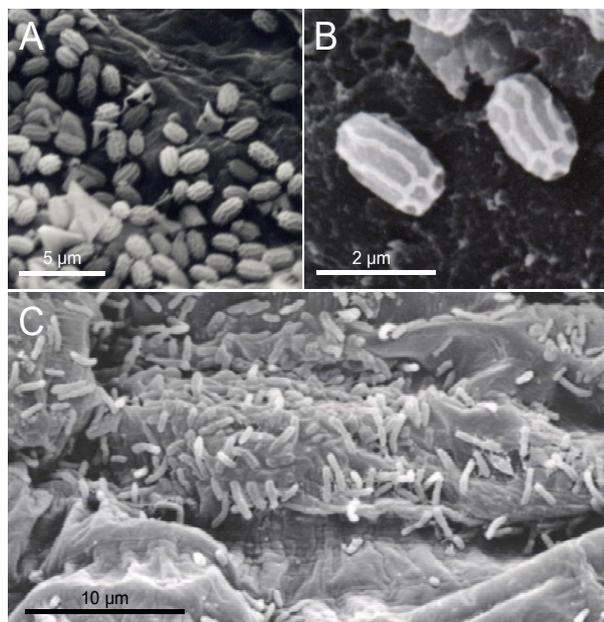


Fig. 2. Endospores (A) of *Paenibacillus polymyxa* E681 on crop seeds 300 days after bioformulation. Enlarged endospores (B). Vegetative cells (C) of E681 germinated from endospores on an emerging radicle.

Endospore-forming bacilli are able to survive for a long time because endospores are stable and durable structures formed under starvation or unfavorable environmental conditions (Madigan *et al.*, 2008). Accordingly, this led to the observation of the endospore formation of E681. Based on the observation with the use of the SEM, it was confirmed that the endospores of E681 were randomly scattered on the surface of the seeds (Fig. 2A). The shape of endospores was oval and longitudinal. Several contours of the spore coat were seen to be orderly arranged on the surface of the endospores (Fig. 2B). Hence, it was suggested that the endospore of E681 derives its a long-term shelf-life.

In our experiment, *Ps. fluorescens* B16 did not survive more than 7 days on cucumber seeds when the bacteria were inoculated on the seed by soaking method. Seven days survival on seeds was not enough for commercial applications of bacteria and was needed to develop an enhanced method to increase survivability of B16. In contrary, *P. polymyxa* E681, a endospore forming bacterium, had a long shelf-life up to 300 days. For this point of view, E681 was a desirable candidate for practical applications.

3.2 Population changes of B16 and E681 on roots

Population densities of B16 and E681 on cucumber roots were assessed after their application with various powder formulation. The population densities of B16 and E681 colonized on cucumber roots through treatment with powder formulation were lower than that of the seed-soaking treatment (Fig. 3). Root-colonized populations of both bacterial strains were significantly different between powder formulation and seed-soaking treatment (Fig. 3). Higher populations in seed-soaking treatments appears to be due to the higher populations within seeds. This data is consistent with previously reported results; when crop seeds were soaked in bacterial suspension, the bacteria entered seeds through pores at the base, and colonized within the seeds (Choi *et al.*, 2004). The seed-soaking treatment for E681 is confirmed to be a more effective treatment than powder formulation in increasing the initial populations on plant roots.

To further confirm whether the root-colonizing ability of E681 was significantly influenced by longer storage times, we assessed the root-colonization ability of E681 re-isolated from seeds that were stored for 300 days at room temperature. The root-colonization ability of E681 was found to be sustained after 300 days of storage (Fig. 4). To investigate whether endospores of E681 were able to

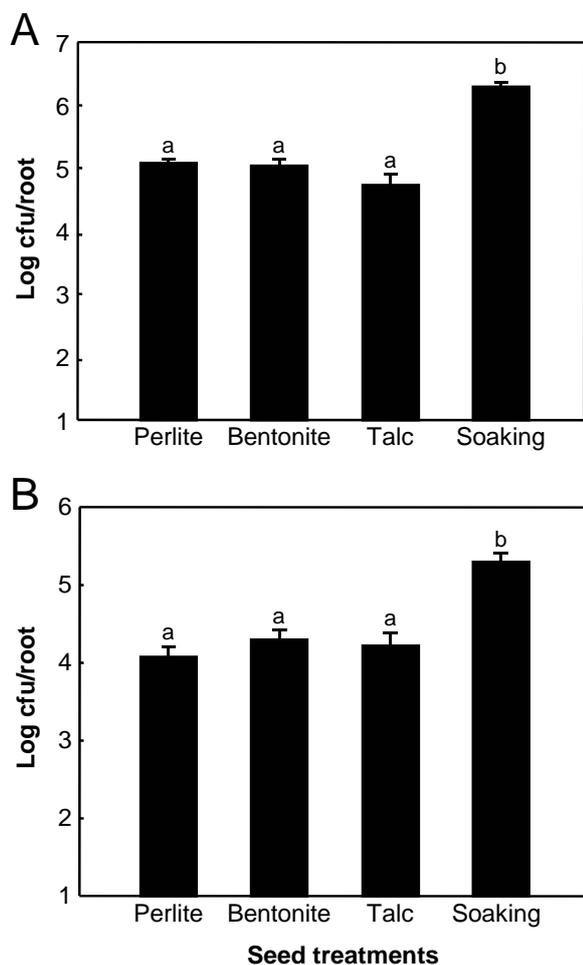


Fig. 3. Population densities of *Pseudomonas fluorescens* B16 (A) and *Paenibacillus polymyxa* E681 (B) on cucumber roots right after bioformulation. The bacterial cells were formulated with seed-pelleting materials, bentonite, perlite, and talc. Soaking denotes seed-soaking treatment in the bacterial suspension. Error bars represent the standard deviation. Means with different letters are significantly different ($P \leq 0.01$) according to the Duncan's multiple range tests.

germinate along with the seeds, root samples were observed under SEM after the emergence of the first shoots. The vegetative cells of E681 produced from endospores were abundantly proliferated and aggregated

on an emerging radicle (Fig. 2C). Our results are similar with those of studies of root colonization of *Ps. fluorescens* (Kang & Park, 1997; Bloemberg *et al.*, 2000). The bacterial cells on the grooves of epidermal cells of the host roots were linearly arranged and parallel with growing root axis.

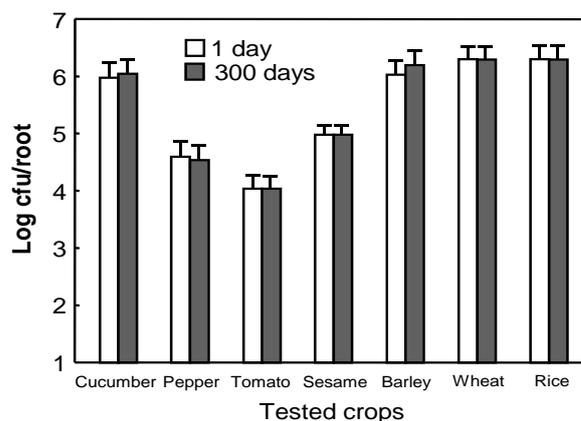


Fig. 4. Root-colonized population of *Paenibacillus polymyxa* E681 on tested crop seeds after 1 day and 300 days of seed-soaking treatment. Tested seeds were soaked in bacterial suspension buffered with 0.1 M $MgSO_4$ and air-dried at room temperature. Dried-seeds were kept at lab table for the test. Error bars represent the standard deviation.

3.3 *In vitro* inhibitory activity of E681 against fungal pathogens

To investigate whether the inhibitory activity of E681 against soil-borne fungal pathogens was significantly influenced by long term storage, we assessed the antagonistic activity of E681 re-isolated from seeds that were stored for 300 days at room temperature. The width of clear zone was 9 mm against *F. oxysporum*, 9 mm against *Ph. capsici*, 4 mm against *Py. ultimum*, and 11 mm against *R. solani*. The inhibitory activity of strain E681 was not

significantly influenced over the 300-day-storage period (Table 1).

Table 1. Inhibitory activity of *Paenibacillus polymyxa* E681 against some fungal soil-borne pathogens

Fungal pathogens	Before seed formulation	300 days after seed formulation ^b	
		from barley roots	from wheat roots
<i>Fusarium oxysporum</i>	++++ ^a	++++	++++
<i>Pythium ultimum</i>	+++	+++	+++
<i>Phytophthora capsici</i>	++++	++++	++++
<i>Rhizoctonia solani</i>	++++	++++	++++

^a +, 0-1; ++, 2-3; +++, 4-5; +++++, 6 (mm)<.

^b The 50 colonies were re-isolated from the roots of barley and wheat, which were stored for 300 days after seed formulation. One day after incubation, 1 cm diameter of mycelial disks of tested pathogens was placed in the center and equal distant from the eight colonies of bacteria. After incubation for 5 days, the inhibitory activity was examined as width of the clear zone between the bacterial colony and fungal pathogens.

The two possible research questions that will advance our understanding of biological control activities of E681 can be discussed. What environmental conditions might induce endospore formation? Many Gram-positive bacteria including *Bacillus* and *Paenibacillus* produce endospores during a process called sporulation. Endospores are differentiated cells that are resistant to harsh environmental conditions including extremes of temperature, drying, UV radiation, nutrient deletion, and harmful chemicals. Endospore formation is usually induced by a lack of nutrients such as carbon or nitrogen (Madigan *et al.*, 2008). Crop seeds are also dormant structures of plants lacking key nutrients and

water for bacterial growth. In the present study, bioformulated-seeds were air-dried at room temperature. Vegetative cells of E681 in/on crop seeds ceased growth owing to the lacking of essential nutrients and water. Thus, cells of E681 ceased vegetative growth and began sporulation when water and a key nutrient such as carbon or nitrogen became limiting.

What are specific triggers for E681 endospore germination? Endospores can be thought of as the dormant stage of a bacterial life cycle and remain dormant for years, but they can convert back to vegetative cells rapidly. In a process called germination, endospores are conditioned to germinate when placed in the presence of specific triggers such as amino acids (Madigan *et al.*, 2008). In the present study, converting back from endospores to vegetative cells of E681 on an emerging radicle was observed under SEM. Plant root and seed exudates consist of polysaccharides, sugars, amino acids, organic acids, phenolic compounds, and enzymes (Bertin *et al.*, 2003; Rovira, 1969). These compounds serve as food sources or signals for BCAs. It was thought that endospores of E681 on a radicle were conditioned to germinate when exposed to root and seed exudates containing specific triggers. It has been shown that root and seed exudates stimulate the growth of bacteria and that the stimulating agents were certain biologically active amino acids (Vančura *et al.*, 1969). We do not know what triggers influences endospore germination of E681, but it is believed that root and seed exudates can function as triggers. Therefore, it seems that effect of seed and root exudates on the growth of E681 require for further study.

Therefore, it is conclusive that endospores of E681 survived on seeds for a long time, and mycellial growth inhibition against some fungal pathogens and root colonization ability were not reduced after 300 days after seed formulation. These data suggested that

E681 is a durable and stable BCA. In addition, the seed-soaking treatment for E681 is better than powder formulation for increasing initial populations on plant roots.

IV. Acknowledgement

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