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Improvement of biological control capacity of *Paenibacillus polymyxa* E681 by seed pelleting on sesame

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Abstract

Sesame is an important vegetable crop for the production of oil in Korea. The main obstacle of sesame cultivation is the occurrence of damping-off diseases and wilt caused by a complex of soil-borne pathogens in fields cultivated for two or more successive years. To protect sesame seedlings against these diseases, *Paenibacillus polymyxa* E681, a plant growth-promoting rhizobacterium (PGPR) previously shown to suppress disease incidence and promote growth on cucumber and pepper in the greenhouse and field experiments, was evaluated for its capacity for biological control and growth promotion *in vitro* and *in situ*. Seed treatment with strain E681 alone did not show consistent protection. Therefore, seed pelleting with strain E681 was attempted to increase the seed size and improve the stability and effectiveness of biocontrol capacity by strain E681. Through screening of pelleting materials, a combination of clay and vermiculite was selected for further experiments to enhance seed germination and root colonization of strain E681 on sesame. In greenhouse trials, formulations of strain E681 reduced disease incidence in disease-conducive soil. In the field, pelleting of sesame seeds with strain E681 significantly reduced pre- and post-emergence damping-off compared to the non-treated or pelleting alone controls; pelleting also promoted the plant growth and the grain yield. Furthermore, the efficacy of strain E681 for biological control and plant growth promotion was improved by sesame seed pelleting compared to the treatment with strain E681 alone. Hence, the application of strain E681 via seed pelleting offers potential to overcome some of the problems associated with successive years of sesame cultivation.

Keywords: Plant growth-promoting rhizobacteria; *Paenibacillus polymyxa*; Biological control

1. Introduction

Sesame (*Sesamum indicum* L.) is one of the longest cultivated plants having been grown for edible oil and food since 2350 B.C. (Ashri, 1989). In Korea, sesame seeds and sesame oil containing about 47% oleic and 39% linoleic acid are used in cooking and baking (Ashri, 1989; Weiss, 1983). Farmers in Korea have cultivated sesame because it is a well-adapted crop for Korea that develops an extensive seedling root system during drought or rainy conditions and can be harvested 100 days after seeding. However, continuous cultivation of sesame results in several problems, including decreased healthy stands, resulting in reduced yields (Ashri, 1989; Kang and Kim, 1989; Weiss, 1983). In particular, the planting of sesame in a field for successive years leads to heavy losses in yield due to complex foliar and soil-borne diseases. The most common diseases are leaf spot, leaf and stem blights, Fusarium wilt, charcoal rot, and root rot (Ojiambo et al., 1999; Weiss, 1983). Recently, mosaic disease caused by a potyvirus, *Sesame mosaic potyvirus*, was also reported in the United States (Pappu et al., 1997). By 2003 in Korea, a total of 25 diseases, including three viral, two phytoplasmas, two bacterial, and 18 fungal pathogens, have been reported in sesame (Choi et al., 2004). The main issue is a complex of soil-borne or leaf pathogens such as *Fusarium* spp., *Rhizoctonia*

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solani, *Ralstonia solanacearum*, *Alternaria sesame*, *Cercospora sesame*, and *Odium sesami*, which cause symptoms such as damping-off, severe wilt, and leaf damage (Cho and Shin, 2004; Jeong et al., 2003). To solve this problem, various disease management methods such as biological control have been used (Kang and Kim, 1989).

Plant growth-promoting rhizobacteria (PGPR) are a group of root-colonizing bacteria in the rhizosphere of many plant species that exert beneficial effects on plants (James and Olivares, 1998; Kim et al., 1997a,b, 1998; Kloepper, 1992; Park et al., 1988). During the past several decades, many researchers have studied various PGPR strains for their capacity to increase the plant growth and yield, and to control the plant pathogens under greenhouse and field conditions (Kloepper et al., 2004; Leeman et al., 1995; McSpadden-Gardener, 2004; Weller, 1988). To date, many mechanisms have been proposed for growth promotion by PGPR strains. First, enhancement of plant growth by PGPR was elucidated by the production of compounds that mimic plant hormones or other plant stimulants (James and Olivares, 1998; Lucy et al., 2004; Ryu et al., 2003). Second, mechanisms for growth promotion that decrease microbial populations of pathogenic or deleterious microorganisms through antibiosis have been proposed (Handelsman and Stabb, 1996; Jetiyanon and Kloepper, 2002; Kloepper et al., 2004). These mechanisms have been referred to as both direct and indirect mechanisms (Glick, 1999).

In addition to these mechanisms, PGPR strains must effectively colonize roots in order to provide consistent results under greenhouse or field conditions (Cho and Shin, 2004; Emmert and Handelsman, 1999; Kim et al., 1997a; Maplestone and Campbell, 1989; Yan et al., 2003). Some evidences have been presented for a positive correlation between high populations of introduced bacteria and increased disease control. Bull et al. (1991) obtained an inverse correlation of wheat grown in a growth chamber for a period of three weeks to root colonization and suppression of take-all. *Bacillus* sp. strain L324-92, which colonized wheat roots, exhibited antagonism against *Gaeumannomyces graminis* var. *tritici*, *R. solani*, *R. oryzea*, and *Pythium* spp., resulting in increasing yield in the field (Kim et al., 1997a,b). *Bacillus cereus* UW85 grew and spread on soybean roots, and it persisted in the rhizosphere until seed harvesting (Emmert and Handelsman, 1999; Halverson et al., 1993). However, optimal root colonization for disease suppression may be different, depending on the strategy of pathogenesis of the pathogen to be controlled. Many procedures have been used to improve the capacity of introduced bacteria on the seed; these include processes such as seed formulation and pelleting with talc and soil (Jacobsen et al., 2004). Pelleting makes a small seed like sesame larger and therefore easier to handle and plant accurately (Connick et al., 1990; Schisler et al., 2004; Taylor and Harman, 1990).

The objective of this study was to assess the disease management potential of a biocontrol agent under greenhouse and field conditions. We used seed pelleting to enhance the capacity of the biocontrol agent, *Paenibacillus polymyxa* strain E681. To date, there are no reports of PGPR strains that act as biological control agents against the complex of soil-borne sesame diseases.

2. Materials and methods

2.1. Bacterial and soil preparation

PGPR strain *Paenibacillus polymyxa* E681 was applied to sesame seeds (*S. indicum* L.), variety Suwongae, to elucidate the suppression of disease incidence in vitro and in vivo. Strain E681 has previously been shown to elicit plant growth promotion and biological control on several crop plants such as barley, hot pepper, Arabidopsis, and cucumber (Ryu and Park, 1997; Ryu et al., 2005a,b). For experimental use, the bacterium were streaked onto Tryptic soy broth agar (TSA; Difco Laboratories, Detroit, MI, USA) plates and incubated at 28 °C in the absence of light for 24 h. Bacterial cells were harvested from TSA plates in sterile distilled water (SDN) to yield 10^9 cfu/ml, determined by the optical density. For long-term storage, bacterial cultures were maintained at −80 °C in Tryptic soy broth containing 20% glycerol. For greenhouse experiments, soil was collected from Gyeongsang National University (GSNU) experimental field at Daegok, Jinju, GyeongNam, Republic of Korea, where sesame was grown the previous year.

2.2. In vitro antibiosis assay

To test the antagonism of strain E681 against plant pathogenic fungi, we used nine fungal isolates: *Pythium debaryanum*, *R. solani*, *Fusarium oxysporum*, *Botrytis cinerea*, *B. allii*, *Cladosporium fulvum*, *P. ulimum*, *Phytophthora capsici*, and *Aspergillus* sp. These fungi were originally isolated from various symptomatic crops, including sesame cultivated in the field, and were previously classified by our laboratory to be members of the above species. Strain E681 was spot-inoculated on the edge of a Petri dish with equal spacing around the perimeter of PDA (Potato dextrose medium, Difco, Detroit, MI, USA) one day before placing one fully grown mycelial disk ($d = 1$ cm) on the center of each PDA plate. All fungi except *F. oxysporum* f. sp. *cucumerirum* were grown on PDA for 3 days prior to inoculation. *Fusarium oxysporum* f. sp. *cucumerirum* was incubated on the PDA medium at 27 °C for 6 days. Five days after the incubation of bacteria, suppression of fungal growth was measured as the distance of the clear zone between the bacterial colony and each fungus.

2.3. Screening pelleting materials

To select suitable pelleting materials, we screened soil-based materials (clay, vermiculite, and talc) or the combination treatments on sesame seeds, as listed in Table 2. For coating the materials on the surface of sesame seeds, 1% polyvinylalcohol as a binder was sprayed on sesame seeds

in the coating machine (Seed Pelleting Manufacture Machine, Samheung Engineering Co., Republic of Korea). During formulation, a suspension of strain E681 was applied at 10^8 cfu/ml with 1% polyvinylalcohol as a positive control. The pelleting seeds were dried at room temperature for at least 2 days prior to use. To assess the germination rate of each pelleting material or its combination treatments in vitro, 100 pelleted seeds were placed on filter paper in a Petri dish with 4.5 ml sterile water, and the treated Petri dish was then incubated at 27 °C. After six days, the number of germinated seeds per 100 seeds was counted. This experiment was designed as a complete randomized design with three replications.

2.4. Root colonization assay

To assess the root colonizing capacity in vitro, we used the Double Layer Filter Paper (DLF) method (Bharathi et al., 2004; Ryu et al., 2005b). For this experiment, strain E681 was used as a spontaneous rifampicin-resistant mutant, as described previously (Ryu et al., 2005b) Five sesame seeds pelleted with strain E681 or water were placed on the bottom layer of filter paper (Whatman No. 2) in a Petri dish ($d = 11$ cm) and covered with a same size filter paper. Sterile water (4.5 ml) was added to the two layers of filter papers, and dishes were sealed with polypropylene wrap to maintain constant moisture. The sealed Petri dishes were incubated vertically at 27 °C in the dark to allow straight growth of the emerging roots. For comparing the root colonizing capacity of strain E681 applied in different pelleting materials, each 1 cm root-tip segment was placed in a test tube containing 10 ml of 0.1 M $MgSO_4$, which was vigorously stirred with a vortex mixer for 20 s. The bacterial numbers per root segment or pelleting seed were determined as root colonization capacity by plating on 1/10 TSA containing 100 μg/ml rifampicin for the selection of only strain E681 by the dilution plating method and by incubating plates for 3 days at 27 °C. Water treatment was used as negative control. We also assessed root colonization in vivo. The pelleted seeds were sown in field soil in a plug nursery pot (3 × 10 holes, 10 cm in diameter). The emergence rate was assessed at 3, 6, 9, 12, 15, and 30 days after sowing. This experiment was designed as a completely randomized design with three replications, one plug nursery pot per replication, including 30 holes, with one seed per hole. This experiment was conducted two times. Results of repeated trials were similar. Hence, one representative trial of each experiment is reported here.

2.5. Biological control of damping-off and plant growth promotion by pelleting of strain E681 in the field

To evaluate the effect of strain E681 pelleting on the sesame seeds under field conditions in terms of the suppression of pre- or post-emerging damping-off disease, we conducted experiments at GSNU Research Farm, Daegok, Jinju, where sesame had been cultivated for two successive years, resulting in serious yield loss in the previous year. Through preliminary screening, a combination of clay and vermiculite was used as pelleting material for strain E681 formulation. A range of two to five sesame seeds were directly seeded on the mulching row (120 × 5000 cm), which was covered with black polyvinyl with holes at every 50 cm on May 27, 1997. This experiment was designed as a randomized complete block (RCB) with five replications, one row per replication including 100 plants. The emergence rate was recorded by counting the germinated seedlings per 100 holes in each treatment 15 days after seeding. After assessing the germination rate, all of the seedlings except one plant per hole were removed. For assessing healthy stands after flowering (early July), 4-week-old sesame seedlings without severe stem symptoms, such as wilt, early leaf senescence, and collapsed leaves, were counted in each treatment.

Growth promotion elicited by strain E681 was evaluated as indicated by the number of capsules per plant, weight of 1000 seeds, weight of 1-L seeds, and grain yield per 10 are (0.10 ha) after harvesting.

2.6. Data analysis

Data were subjected to analysis of variance using JMP software version 4.0.4 (SAS Institute, Inc., Cary, NC). The effect of strain E681 treatment was considered significant according to the magnitude of the F value ($P = 0.05$). When a significant F test was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD).

3. Results

3.1. In vitro antibiosis of strain E681 against fungal pathogens

Strain E681 inhibited the growth of nine of the tested fungal strains (Table 1). An inhibition zone (clear zone between strain E681 and fungal hyphae) of greater than 6 mm was exhibited against *P. debaryaum*, *R. solani*,

Table 1
Antibiotic capacity of *Paenibacillus polymyxa* strain E681 against plant pathogenic fungi under in vitro conditions

Fungal isolates	Degree of inhibition
Pythium ultimum	++
Pythium debaryanum	+++
Rhizoctonia solani	+++
Fusarium oxysporum	+++
Phytophthora capsici	++
Botrytis cinerea	+++
Botrytis allii	+++
Cladosporium fulvum	+++
Aspergillus sp.	++

Suppression of fungal growth by strain E681 was measured according to the diameter of the clear zone caused by inhibition of fungal growth; ++ = 4–5 and +++ = 6 mm or above.

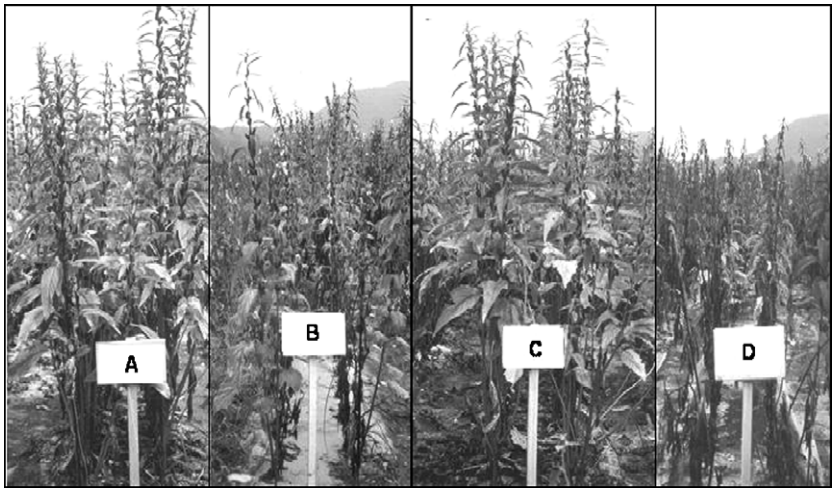

Fig. 1. Enhancement of biological control of *Paenibacillus polymyxa* strain E681 in the field. The pictures were taken 2 months after seeding. A = E681 + pelleting; B = pelleting; C = E681 + non-pelleting; D = non-pelleting.

F. oxysporium, *B. cinerea*, *B. alli*, and *C. fulvum*. The growth of *P. ulimum*, *P. capsici*, and *Aspergillus* sp. was suppressed by strain E681 with an inhibition zone of 4–5 mm.

3.2. Screening pelleting materials

Previous to the application of *P. polymyxa* strain E681 on sesame seeds, we screened for an adequate pelleting material. Seven single materials or combinations of materials were assessed on sesame seeds with 1% polyvinyl alcohol as a binder to coat pelleting material onto sesame seeds. Observations were made on seed shape, uniformity, and hardness. Of the tested materials, talc, clay + talc, and vermiculite + talc resulted in better shapes of pelleted seeds than other materials. Uniformity was increased when talc, vermiculite + talc, and clay + vermiculite + talc were pelleted on the sesame seeds. For hardness, vermiculite, talc, clay + vermiculite, vermiculite + talc, and clay + vermiculite + talc were selected for further study (Table 2). In addition to these combinations, clay + vermiculite treatment was selected based on the germination test. Collectively, talc, clay + vermiculite, clay + talc, or clay + vermiculite + talc were selected as the best pelleting materials for strain E681.

To test whether selected pelleting materials were compatible to sesame seeds (not harmful to sesame seed germination), the germination rate after seed pelleting with five different selected materials (talc, clay + vermiculite, clay + talc, clay + talc, and clay + vermiculite + talc) on the sesame seeds was assessed on the wet filter paper in the Petri dish (Fig. 2). Treatment with talc alone resulted in the best germination rate (93%) among the treatments. In contrast, the combination of clay + vermiculite + talc showed the lowest germination rate (76%) compared to the untreated control (87%) (Fig. 2). None of the treatments other than clay + vermiculite + talc differed statistically in germination rate of formulated sesame seeds with different pelleting materials.

Table 2
The shape, uniformity, and hardness of the pelleting either with clay, vermiculite, talcum, or peatmoss alone or in combinations

Pelleting materials[a]	Shape[b]	Uniformity[b]	Hardness[b]	Germination rate (%)
Cl	+	+++	++	62.3b
Ve	+	+++	+++	58.0bc
Ta	+++	+++	+++	82.0a
Cl + Ve	++	++	+++	74.0ab
Ve + Ta	+++	+++	+++	32.0c
Cl + Ta	++	++	++	82.0a
Cl + Ve + Ta	++	+++	+++	43.0c
Non-pelleting	—	—	—	91.0a

Numbers represent mean of three replications per treatment, 100 seeds per replication. Seed germination was counted 2 weeks after the sowing of each pelleting material treatment on sesame seeds. Different letters within columns indicate significant differences using Fisher's protected LSD test at $P = 0.05$.

[a] Cl = clay; Ve = vermiculite; Ta = talc, and non-pelleting = polyvinyl alcohol alone.

[b] Shape, uniformity and hardness were presented as +++ = excellent; ++ = good; + = bad.

3.3. Root colonization of strain E681 pelleted on sesame seed

To test the effect of pelleting materials on root colonization of strain E681, we assessed bacterial population densities on the seed coat and root system two weeks after sowing pelleted seeds in Petri dishes using the DLF method. We attempted to assess bacterial populations in the pelleting material and 1 cm of root tip segments after seed germination. Treatment with talc, clay + vermiculite, clay + talc, or clay + vermiculite + talc did not affect bacterial population on the seed coat; however, the non-pelleted treatment had the lowest populations compared to other treatments (Fig. 3). Root colonization of strain E681 was greatest in the clay + vermiculite treatment (0.5×10^4 cfu/root segment). The bacterial population on the clay + vermiculite + talc-treated plants was 58% higher than on plants receiving other pelleting material

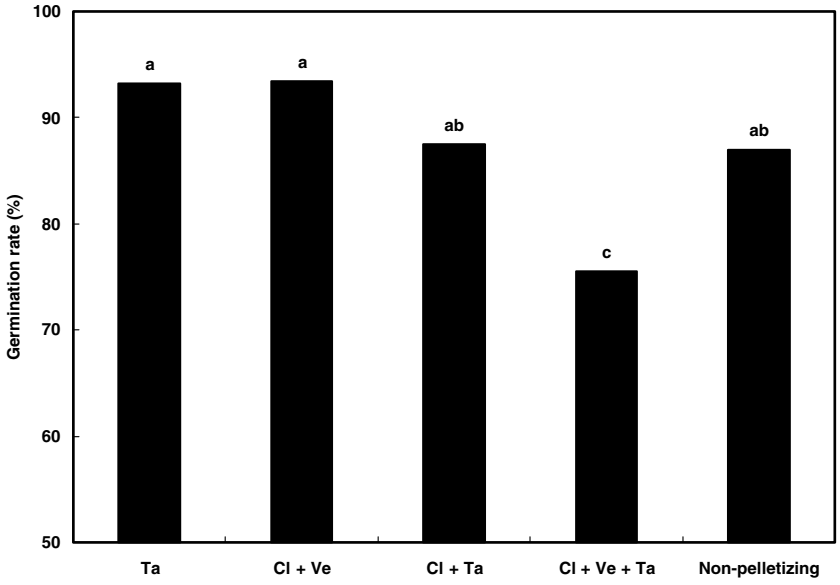

Fig. 2. Effect on the germination rate of sesame seeds by various pelleting materials. Germination rates were measured by calculating the number of germinated seeds per 100 for each type of seed treated with the different indicated materials. Non-pelleting = polyvinyl alcohol alone. Different letters indicate significant differences using Fisher's protected LSD test at $P = 0.05$.

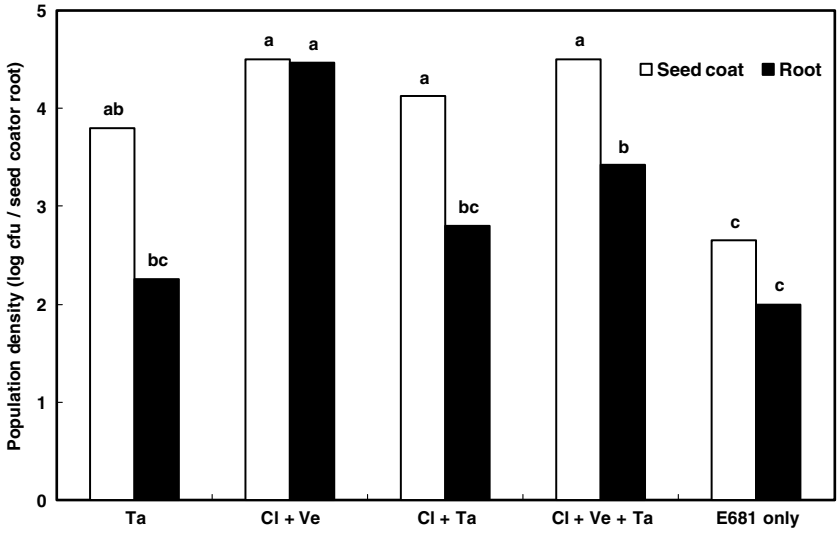

Fig. 3. Root colonization of *Paenibacillus polymyxa* strain E681 after seed pelleting with various pelleting materials in vitro. Different letters indicate significant differences using Fisher's protected LSD test at $P = 0.05$.

treatments (Fig. 3). The root colonization capacity by strain E681 on the talc alone or clay + talc treatment was intermediate between the non-pelleted control and the clay + vermiculite treatment (Fig. 3). In addition to in vitro assay, we evaluated the root colonization of sesame by strain E681 in disease-carrying soil and recorded 8.0×10^4 cfu/g root at 30 days after sowing (data not shown). In contrast, when strain E681 was applied to seeds without pelleting treatment, no bacteria were detected. The results indicate that pelleting with clay + vermiculite showed greater root colonization and competency of *P. polymyxa* strain E681 compared to other pelleting treatments, particularly in vivo.

3.4. Biological control of seedling diseases by seed pelleting in the field

We assessed two types of soil-borne diseases: pre-emergence damping-off, indicated as emergence rate 15 days after seeding, and healthy stands after the flowering stage (40 days after seeding) in the field. Upon seed pelleting with strain E681, a 92% greater emergence rate was obtained. Sesame seeds without any treatment in the re-planted soil germinated very poorly (<30%) (Figs. 1 and 4A) and eventually showed severe symptoms of damping-off in the field (data not shown). Treatment of strain E681 alone (without pelleting) enhanced the early emergence rate of sesame to a

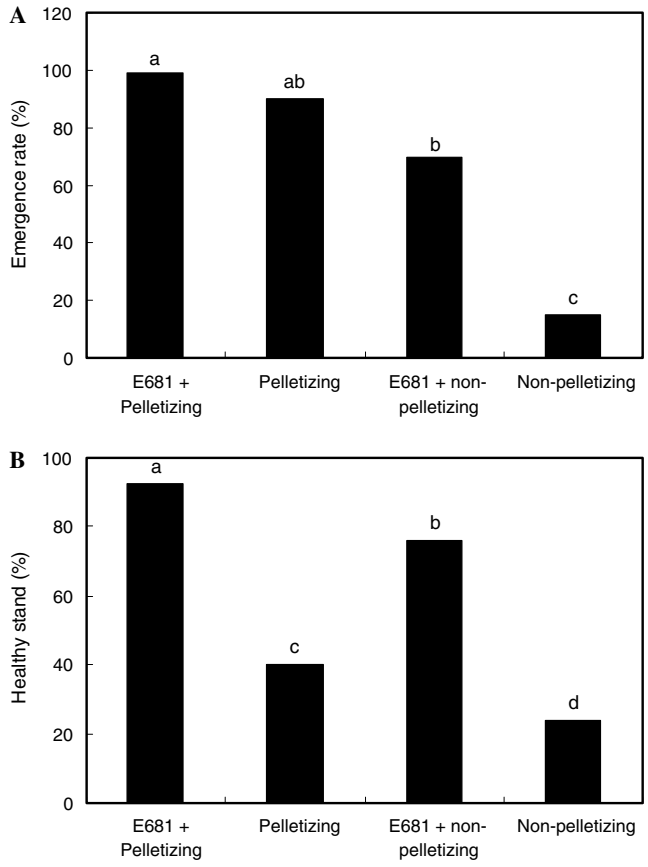

Fig. 4. Emergence rate and proportion of healthy stands after seed pelleting of *Paenibacillus polymyxa* strain E681 in the field. E681 + pelleting = sesame seed pelleting with strain E681; non-pelleting = polyvinyl alcohol alone. Different letters indicate significant differences using Fisher's protected LSD test at $P = 0.05$.

higher degree than that of pelleted seeds. However, non-pelleted seeds could not protect themselves against post-emergence damping-off in the future. We counted the number of healthy plants two months after seeding. E681 + pelleting resulted in a greater percentage of healthy stand (92%) than pelleting alone or non-pelleted seed treatment with E681, 40 and 24%, respectively (Figs. 1 and 4B). Seed treatment of strain E681 with pelleting significantly increased the percentage of healthy stand compared to pelleting alone or non-pelleting treatment (Figs. 1 and 4B). Collectively, seed pelleting improved the capacity of strain E681 to increase the seed emergence rate and the percentage of healthy stand.

3.5. Plant growth promotion by pelleting seed with strain E681

As indicators of growth promotion by seed pelleting of strain E681, we measured three parameters; weight per 1000 seeds, weight of 1 l of seeds, and grain yield per 10 are (Table 3). Pelleting + E681 treatment significantly increased the weight per 1000 seeds, weight of 1 l, and grain yield by 12.4, 10.2, and 10.2%, respectively, compared to non-pelleting treatment. The weight per 1000 seeds of pelleting + E681 treatment was significantly heavier than that of treatment with E681 alone.

Table 3
Plant growth promotion by seed pelleting with *Paenibacillus polymyxa* strain E681 in the field

Treatments[a]	Yield parameters		
	Weight of 1000 seeds (g)	Weight of 1 L (g)	Yield (kg/10 are[b])
E681 + pelleting	2.35b	634.3d	77.4b
Pelleting	2.32b	602.8b	66.8b
E681 + non-pelleting	2.29b	620.4c	69.9b
Non-pelleting	2.09a	575.4a	24.5a

Numbers represent the mean of four replications per treatment, 20 plants per replication. Different letters within columns indicate significant differences using Fisher's protected LSD test at $P = 0.05$.

[a] E681 + pelleting = sesame seed pelleting with strain E681 and non-pelleting = polyvinyl alcohol alone.

[b] 10 are = 1/10 ha.

4. Discussion

The results reported here indicate that *P. polymyxa* strain E681 effectively controlled pre-emergence and post-emergence damping-off diseases on sesame plants. This is the first report to find that PGPR can control soil-borne diseases on sesame under greenhouse and field conditions.

Korean farmers follow the adage that sesame cannot be planted in the same field in two consecutive years. Such successive planting inevitably leads to very poor germination and abundant post-emergence damping-off. Surviving plants are stunted, wilted in the field. In Korea, the main disease affecting these plants is believed to be wilt caused by *F. oxysporum* or *R. solani* (Cho and Shin, 2004).

In this study, we evaluated whether a PGPR strain, E681, which previously showed growth promotion and biological control activity on different crop systems (Ryu and Park, 1997; Ryu et al., 2005b), suppressed early seedling damping-off in sesame cultivation and increased healthy stands in the field where sesame was grown during the previous years. From in vitro testing to field trials, strain E681 has been found to be a promising biocontrol agent, as well as a promoter of plant growth and increased grain yield (Fig. 4; Table 3).

In the application of biological control agents into seeds or seedlings in order to protect plants against soil-borne pathogens or to increase plant growth, an inconsistent effect of introduced biocontrol agents has often been cited as the main obstacle to large-scale implementation in agriculture (Schisler et al., 2004; Weiss, 1983). Our results also demonstrate that using a formulation system based on soil matrix-based pelleting material may improve the efficacy of strain E681. Emergence rates and healthy stands were greater when sesame seeds were treated with strain E681 with pelleting than when it was applied without pelleting (Fig. 4). These results suggest that seed pelleting can maximize the potential of PGPR

colonization capacity. We expected that seed pelleting would help the PGPR to more efficiently establish bacterial populations in competition with indigenous microorganisms during germination, thereby resulting in enhanced populations on roots. In sesame cultivation, the early establishment of the introduced biocontrol agent plays a critical role in the growth of healthy stands because it allows them to escape disease under field conditions. Moreover, seed pelleting with strain E681 significantly improved seed yield (Table 3). These findings are important because they suggest that pelleting of PGPR may be a useful strategy to help overcome the inconsistency of field application of biocontrol agents.

We could not define the exact mechanism of strain E681 in terms of its protection of sesame against soilborne pathogens. *Paenibacillus polymyxa* strain E681 showed a broad spectrum of antagonistic capacity against pathogenic fungi of plants (Table 1). Previous reports have shown that *P. polymyxa* produces many antagonistic substances and controls many soil and foliar pathogens in the greenhouse and the field (Dijksterhuis et al., 1999; Helbig, 2001; Lebuhn et al., 1997; Mavingui and Heulin, 1994). Furthermore, recent genomic and biochemical analysis of strain E681 revealed that it produces several types of antibiotics, including polymyxins, fusaric acid, and polyketides (unpublished data). The production of these antibiotics could provide an advantage for establishing the population during the germination of seeds. Plant growth promotion may be an indirect effect of this antibiotic production through the suppression of plant diseases in disease-carrying soil. We cannot exclude the possibility that strain E681 directly promoted plant growth by producing plant mimic hormone. One of the possible explanations for growth promotion by strain E681 is the increase in plant growth by strain E681 is directly related to *P. polymyxa* species, which have also been reported to produce many plant growth stimulators, including auxin, cytokinin, and 2,3-butanediol (Lebuhn et al., 1997; Nakashimada et al., 2000; Ryu et al., 2003; Timmusk et al., 1999).

Collectively, our results suggest that pelleting with PGPR can result in broad-spectrum antagonism against various plant pathogens and may be a good means by which to control damping-off caused by complex organisms in the field. Application of strain E681 in seed pelleting can be used to manage pre- and post-emergence damping-off in sesame plants. Given the severe losses that result from successive years of cultivation of sesame in Korea, this result is of great importance.

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