



Plant Growth-Promoting Rhizobacteria Stimulate Vegetative Growth and Asexual Reproduction of *Kalanchoe daigremontiana*

Yong-Soon Park^{1,2}, Kyungseok Park², Joseph W. Kloepper³ and Choong-Min Ryu^{1,4*}

¹Molecular Phytobacteriology Laboratory, Superbacteria Research Center, KRIBB, Daejeon 305-806, Korea

²Agricultural Microbiology Division, NAAS, RDA, Wanju 565-851, Korea

³Department of Entomology and Plant Pathology, Auburn University, Alabama 36849, USA

⁴Biosystems and Bioengineering Program, University of Science and Technology (UST), Daejeon 305-350, Korea

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Certain bacterial species associate with plant roots in soil. The plant growth-promoting rhizobacteria (PGPR) stimulate plant growth and yield in greenhouse and field. Here, we examined whether application of known bacilli PGPR strains stimulated growth and asexual reproduction in the succulent plant *Kalanchoe daigremontiana*. Four PGPR strains *B. amyloliquefaciens* IN937a, *B. cereus* BS107, *B. pumilus* INR7, and *B. subtilis* GB03 were applied to young plantlets by soil-drenching, and plant growth and development was monitored for three months. Aerial growth was significantly stimulated in PGPR-inoculated plants, which was observed as increases in plant height, shoot weight, and stem width. The stimulated growth influenced plant development by increasing the total number of leaves per plant. Treatment with bacilli also increased the total root biomass compared with that of control plants, and led to a 2-fold increase in asexual reproduction and plantlet formation on the leaf. Collectively, our results firstly demonstrate that *Bacillus* spp. promote vegetative development of *K. daigremontiana*, and the enhanced growth stimulates asexual reproduction and plantlet formation.

Keywords : asexual plant reproduction, *Bacillus* spp., *Kalanchoe daigremontiana*, plant growth, plant growth-promoting rhizobacteria (PGPR), rhizosphere

The rhizosphere is a zone surrounding plant roots, which supports a large and diverse microbial community (Kloepper et al., 2004; Yang et al., 2009). Some rhizosphere bacteria have been studied because of their beneficial inter-

actions with plants. The plant growth-promoting rhizobacteria (PGPR), especially *Pseudomonas* spp. and *Bacillus* spp., colonize roots of monocots and dicots, directly or indirectly promote plant growth, and elicit induced systemic resistance (Kloepper et al., 2004). For example, treatment of cucumber plants with *B. pumilus* INR7 significantly promoted the growth and numbers of the main runners and leaves of plants grown under field conditions, and reduced the disease severity caused by angular leaf spot and anthracnose after inoculation of plants with *P. syringae* pv. *lachrymans* and *Colletotrichum orbiculare*, respectively (Wei et al., 1996). In addition, plant growth was stimulated and disease severity was reduced in cucumber plants treated with *B. amyloliquefaciens* IN937a and *B. subtilis* GB03 (Raupach and Kloepper, 2000). The growth promotion and disease reduction effects elicited by treatments with *B. amyloliquefaciens* IN937a and *B. subtilis* GB03 also were observed for *Arabidopsis thaliana* seedlings grown in culture medium (Ryu et al., 2003). Treatment with *B. cereus* BS107 increased shoot height but not shoot biomass, and elicited induced systemic resistance to *Xanthomonas axonopodis* pv. *vesicatoria* in pepper (*Capsicum annuum*) (Yang et al., 2009) and *Pectobacterium carotovorum* subsp. *carotovorum* in tobacco (Yang et al., 2011). Although extensive research indicates that PGPR influence plant growth, development, and disease resistance in a variety of plant species, any reports have never explored the effects of PGPR on asexual reproduction.

Kalanchoe is a succulent plant that reproduces asexually by producing plantlets along the edge of mature leaves. *Kalanchoe* is a useful and important model plant for research in three fields. First, it is an important model for investigations of crassulacean acid metabolism (CAM), which plays an important role in photosynthetic carbon assimilation (Garcês and Sinha, 2009). Second, natural compounds extracted from *Kalanchoe* have important applications due to

*Corresponding author.

(Phone) +82-42-879-8229, FAX) +82-42-860-4488

E-mail) cmryu@kribb.re.kr

their anti-tumor, anti-inflammatory, and insecticidal properties (Supratman et al., 2000, 2001). Third, *Kalanchoe* species are used for studies on asexual reproduction (Batygina et al., 1996; Garcês et al., 2007, 2014). Asexual reproduction generates new individuals by reprogramming resident cells in different organs (Guo et al., 2015). These plantlets possess roots, stems, and leaves, and develop asexually at the margin of plant leaves (Guo et al., 2015). The plantlets are only formed under stress conditions in some species, or are spontaneously produced in others. *Kalanchoe daigremontiana* is a constitutive plantlet-forming species (Garcês et al., 2007).

In this study, we investigated whether previously identified PGPR affected the vegetative growth and asexual reproduction of *K. daigremontiana*. We tested four strains of bacilli (*B. amyloliquefaciens* IN937a, *B. cereus* BS107, *B. pumilus* INR7, and *B. subtilis* GB03) for positive effects on *K. daigremontiana* growth and reproduction. This could be a useful strategy to stimulate propagation of the mother plants. The results showed that shoot biomass production was strongly promoted by treatments with these bacilli strains. The most significant effect on shoot biomass production was observed in plants inoculated with *B. pumilus* INR7. Root biomass also increased after treatment with three of the bacilli, but not in plants treated with *B. cereus* BS107. The highest root weight was measured in plants treated with *B. pumilus* INR7. The asexual production of plantlets on the leaf margins was stimulated by all four bacilli strains, especially *B. pumilus* INR7 and *B. subtilis* GB03. Taken together, our data clearly demonstrate that PGPR bacilli positively affect the growth, development, and asexual reproduction of *K. daigremontiana*.

To investigate whether previously reported PGPR modulated the plant growth and resistance to pathogens, we collected satellite plantlets from the leaf margins of *K. daigremontiana*. Using the satellite plants for experiments rather than seeds saved time and money. Before performing the experiments, the plantlets were surface sterilized with 6% sodium hypochlorite (Yuhan Corp., Seoul, South Korea) for 5 min, and subsequently washed with sterile distilled water (SDW) at least five times. The sterilized seedlings were sown in soilless potting medium (Punong Co. Ltd., Gyeongju, South Korea). Plants were grown under natural light conditions, and three different temperatures (21, 24, and 28°C) were tested to optimize growth. Plants grew best at 28°C. To evaluate differences between experimental treatments, analysis of variance was performed for all data using JMP software v 5.0 (SAS Institute, Cary, NC, USA). Significant effects were determined by the magnitude of the *F* value ($P=0.05$). When a significant *F* test was ob-

tained, separation of means was performed by Fisher's protected least significant difference (LSD) at $P=0.05$.

The *B. amyloliquefaciens* IN937a, *B. cereus* BS107, *B. pumilus* INR7, and *B. subtilis* GB03 were grown in tryptic soy agar (TSA) containing rifampicin (100 µg/ml final concentration) overnight and then the plates were scrapped out. The bacterial suspensions of these bacilli were adjusted (1×10^8 cfu/ml). The *K. daigremontiana* plantlets were soil-drenched once a week for first 3 weeks with suspensions (10 ml of 1×10^8 cfu/ml). As a control, some plantlets were treated with 0.33 mM benzothiadiazole (BTH) at the same time as application of bacilli. BTH, salicylic acid (SA), methyl jasmonate, and β -aminobutyric acid (BABA) in-

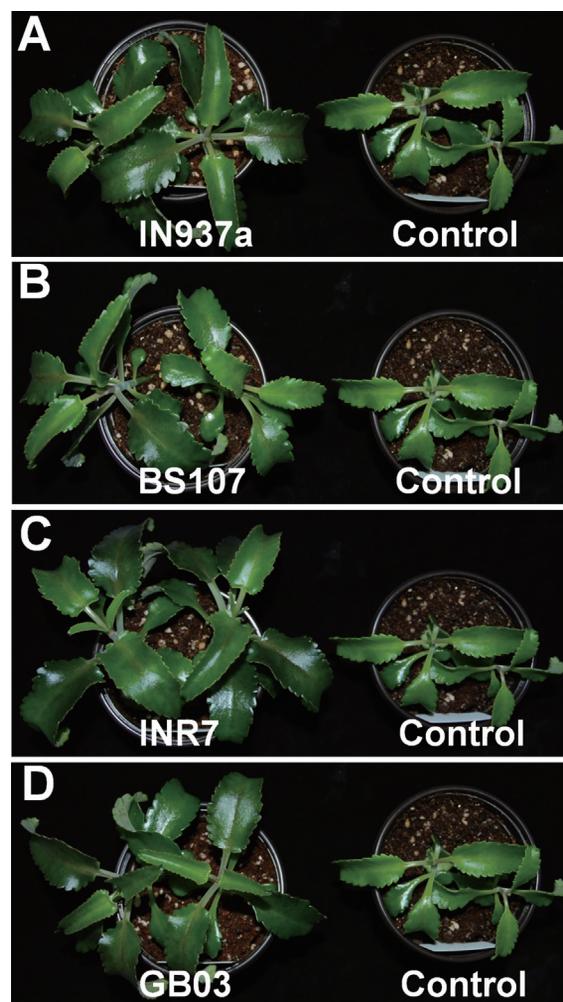


Fig. 1. Representative images of *Kalanchoe daigremontiana* after inoculation of *Bacillus* spp. *K. daigremontiana* seedlings were drenched once a week for the first 3 weeks with suspensions (1×10^8 cfu/ml) of (A) *B. amyloliquefaciens* IN937a, (B) *B. cereus* BS107, (C) *B. pumilus* INR7, and (D) *B. subtilis* GB03, and grown at 28°C for 90 d. Six biological replicates were used for each application of bacilli ($n = 6$).

duce disease resistance in plants (Chen et al., 1993; Davis and Ausubel, 1989; Epple et al., 1997; Vallad and Robert, 2004). More specifically, BTH activated plant defense machinery but had a negative effect on plant growth, such as the significant reduction in yield of BTH-treated wheat under field conditions (Heil et al., 2000). Our previous studies showed that aerial and root biomass of pepper plants were significantly reduced by BTH treatment (Park and Ryu, 2014; Yang et al., 2011). These results suggest that BTH treatment may affect the balance between physiological growth and activation of defense machinery (Heil et al., 2000; Heil and Baldwin, 2002). Consistent with previous results, we observed a significant reduction of *K. daigremontiana* plantlet growth after BTH treatment (data not shown), and the plantlets were killed after 5 weeks of BTH application (data not shown). By contrast, the initial growth of plantlets inoculated with bacilli was not significantly different from that of control plantlets (data not shown).

We assessed phenotypic changes of *K. daigremontiana* plantlets treated with the four bacilli. At 90 d after inoculation, plants treated with *B. amyloliquefaciens* IN937a, *B. cereus* BS107, *B. pumilus* INR7, and *B. subtilis* GB03 were taller and healthier than control plants (Fig. 1). *B. pumilus* INR7 had the most significant effects on promoting plant growth (Fig. 1C). We investigated the effects of bacilli treatment on plant height, shoot weight, stem width, and

total leaf number. Plant height was increased by at least 20% in bacilli-inoculated plants compared with control plants, and was statistically equivalent for all bacilli (Fig. 2A). Shoot weight increased by approximately 2.3-, 4.8-, and 3.1-fold in plants treated with *B. amyloliquefaciens* IN937a, *B. pumilus* INR7, and *B. subtilis* GB03, respectively; *B. cereus* BS107 treatment did not promote shoot weight relative to control plants (Fig. 2B). Stem width increased by at least 1.3-fold in bacilli-inoculated plants compared with that of control plants (Fig. 2C). The shoot weight and stem width trends were similar, suggesting that stem width was positively correlated with shoot weight. The leaf number increased by at least 20% in bacilli-inoculated plants compared with controls, but did not increase in *B. amyloliquefaciens* IN937a-inoculated plants (Fig. 2D). Taken together, these results indicated that PGPR bacilli strains promote *K. daigremontiana* growth and development. The most significant effects were observed in plants treated with *B. pumilus* INR7 (Fig. 1C and Fig. 2).

We also monitored root architecture at 90 d after the bacilli inoculation. Plants treated with bacilli had longer primary roots and more secondary and lateral roots than control plants (Fig. 3A). Root weight increased by approximately 3.0-, 2.1-, 5.0-, and 3.1-fold after treatment with *B. amyloliquefaciens* IN937a, *B. cereus* BS107, *B. pumilus* INR7, and *B. subtilis* GB03, respectively (Fig. 3B). These

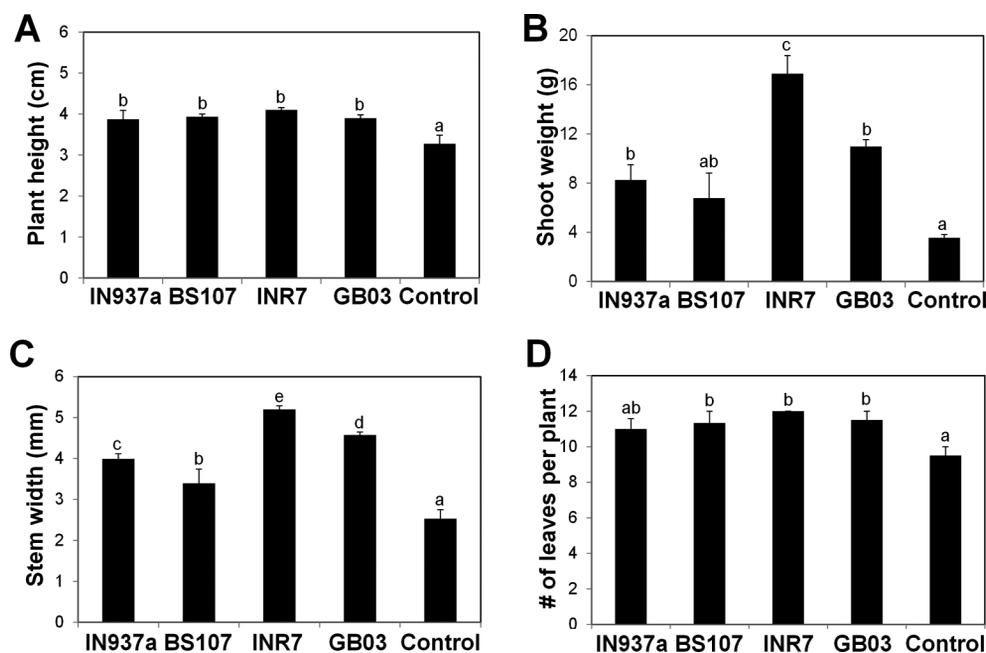


Fig. 2. *Kalanchoe daigremontiana* growth and development is stimulated by inoculation of *Bacillus* spp. *K. daigremontiana* seedlings were inoculated with four *Bacillus* spp. and grown as shown in Fig. 1. (A) Plant height, (B) shoot weight, (C) stem width, and (D) total leaf number per plant were evaluated at 90 d after the bacilli inoculation. Six biological replicates were used for each treatment ($n = 6$). Error bars indicate standard error of the mean and different letters indicate significant differences between treatments ($P = 0.05$).

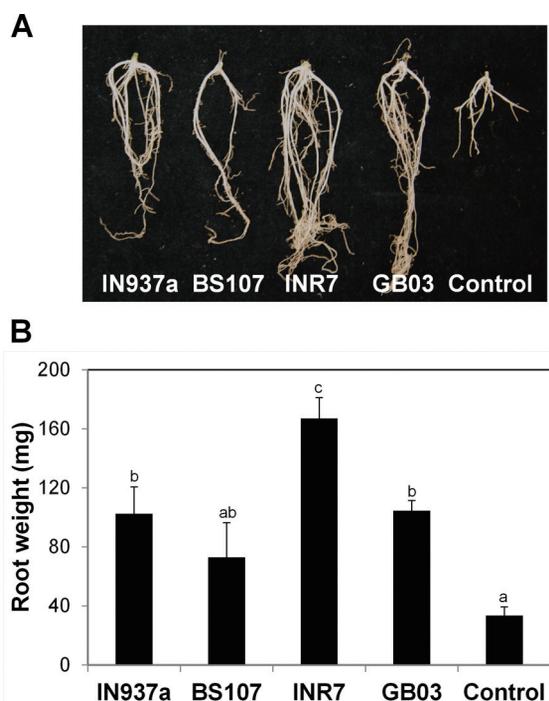


Fig. 3. Root biomass production is stimulated by treatment with *Bacillus* spp. *Kalanchoe daigremontiana* seedlings were inoculated with four *Bacillus* spp. and grown as shown in Fig. 1. (A) Representative images of root growth in bacilli-inoculated and control plants. (B) Total root weight was greater in most of bacilli-inoculated plants than in control plants. Six biological replicates were used for each test ($n = 6$). Error bars indicate standard error of the mean and different letters indicate significant differences between treatments ($P = 0.05$).

data indicate that the bacilli positively stimulate root biomass production. We conclude that the four bacilli strains promote aerial and root growth of *K. daigremontiana* plantlets. The most significant effects on growth were observed in plants inoculated with *B. pumilus* INR7.

The effects on plant growth and development may be mediated by volatile organic compounds (VOCs) emitted by bacilli. Previous research discovered that the VOCs 2,3-butanediol and acetoin emitted by *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a promoted *Arabidopsis* plant growth (Ryu et al., 2003). Additional analyses using diverse signaling mutants of *Arabidopsis* revealed that the cytokinin pathway is involved in responding to VOCs emitted by *B. subtilis* GB03 (Ryu et al., 2003). Although there was the difference of the population density of *Methylobacterium* spp. in bacilli-treated and control plants, *Methylobacterium* spp. were isolated from inside the leaves of *K. daigremontiana* plants (data not shown). *Methylobacterium* spp. are reported to stimulate seed germination,

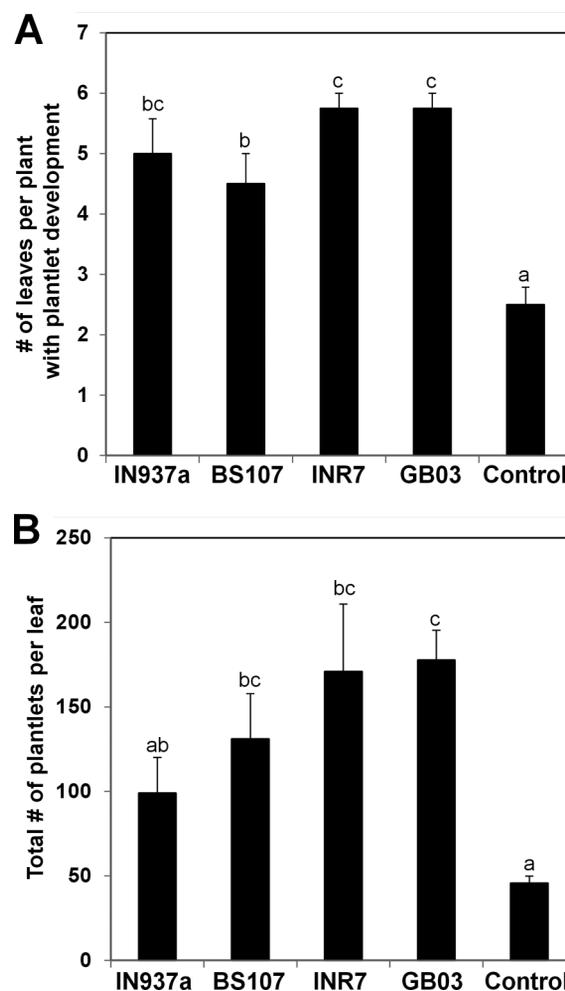


Fig. 4. Asexual reproduction in *Kalanchoe* plants treated with *Bacillus* spp. *K. daigremontiana* seedlings were inoculated with four *Bacillus* spp. as shown in Fig. 1 and grown for 120 d. (A) Total number of leaves with plantlet development per plant. (B) Total number of plantlets per leaf. Six biological replicates were used for each treatment ($n = 6$). Error bars indicate standard error of the mean and different letters indicate significant differences between treatments ($P = 0.05$).

plant development, and phytohormone production (Lidstrom and Christoserdova, 2002). Our results suggest that *Methylobacterium* spp. population density, or any compounds released from *Methylobacterium* spp. in response to bacilli VOCs, may positively affect *K. daigremontiana* growth.

Asexual reproduction occurs in many *Kalanchoe* species (Batygina et al., 1996; Garcês et al., 2007, 2014). To investigate whether bacilli-mediated promotion of *K. daigremontiana* growth affects asexual reproduction, we examined asexual generation of plantlets on leaf margins at 120 d after bacilli inoculation. The total number of leaves

in bacilli-inoculated plants was at least 1.5 times higher than that in control plants (Fig. 4A). The total number of plantlets produced on each leaf of bacilli-treated plants was 2-fold higher than that in control plants (Fig. 4B). The strongest positive effects on asexual reproduction were observed after treatment with *B. pumilus* INR7 and *B. subtilis* GB03. Plantlet formation is regulated by shoot meristemless (*STM*) and leafy cotyledon1 (*LECI*), which are central regulators of organogenesis and embryogenesis (Garcês and Sinha, 2009). Recent work shows that a truncated *LECI* gene (*KdLECI*) is required for asexual plantlet production of *K. daigremontiana* (Garcês et al., 2014). Our results suggest that *Bacillus* spp. may positively regulate asexual reproduction via *KdLECI*.

To investigate whether the soil-drenched bacilli colonized roots of Kalanchoe, we measured total population of each of bacilli in rhizosphere of Kalanchoe plants. Since bacterial suspension was applied onto plants once a week for first 3 weeks, the roots of Kalanchoe were harvested and macerated for counting bacterial population. The bacterial colonies were calculated on selection media (TSA + rifampicin). Unexpectedly, we observed the difference of initial population density at day 0 between the bacilli strains even though we adjusted the solution of each bacilli as $OD_{600} = 1$ (1×10^8 cfu/ml). At this moment, we could not precisely answer for this phenomenon. The fact that the highest and the lowest densities were shown by INR7 and BS107, respectively (Table 1) led us to speculate that this initial colonization ability of the each *Bacillus* spp. used in this study may be a key factor for plant growth and asexual reproduction. As showing in Table 1, the bacilli population was observed at three different time points (0, 5, and 10

Table 1. Population density of the introduced bacilli on the *Kalanchoe* roots

Treatments	Population density (Log cfu/g fresh root) at days after PGPR inoculation		
	Day 0	Day 5	Day 10
IN937a	6.26 c	6.40 c	6.00 c
BS107	5.41 b	5.50 b	5.63 b
INR7	7.22 d	7.14 d	7.21 e
GB03	6.50 c	6.43 c	6.47 d
Control	0.00 a	0.00 a	0.00 a

Numbers represent the mean of five replications per treatment. Bacterial numbers were counted 0, 5 and 10 days after inoculation of *B. amyloliquefaciens* IN937a, *B. cereus* BS107, *B. pumilus* INR7, and *B. subtilis* GB03 on the roots. To select targeted bacterial population, an antibiotic 100 µg/ml rifampicin was amended in the tryptic soy broth agar. Different letters indicate significant differences using Fisher's protected LSD test at $P=0.05$.

days after application of the bacilli at third times in a row). These results may support that promoting plant growths and developments are affected by biological stimulation of the bacilli than just fertilization effect.

In conclusion, this study provides new insights into the effects of bacilli PGPR on *Kalanchoe* growth, development, and asexual reproduction. (1) We optimized conditions for using four *Bacillus* spp. to promote *K. daigremontiana* growth. (2) We observed a positive association between aerial and root biomass production stimulated by *Bacillus* spp. PGPR. (3) The PGPR-induced enhancement of *K. daigremontiana* growth ultimately promoted asexual reproduction and plantlet formation. (4) Among the tested *Bacillus* spp., *B. pumilus* INR7 had the greatest effect on both vegetative growth and asexual reproduction in *K. daigremontiana*.

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