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Assessment of Root-Associated *Paenibacillus polymyxa* Groups on Growth Promotion and Induced Systemic Resistance in Pepper

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Twenty-nine *P. polymyxa* strains isolated from rhizospheres of various crops were clustered into five genotypic groups on the basis of BOX-PCR analysis. The characteristics of several plant growth-promoting factors among the isolates revealed the distinct attributes in each allocated group. Under gnotobiotic conditions, inoculation of pepper roots with *P. polymyxa* isolates significantly increased the biomass in 17 of total 29 treated plants with untreated plants. Experiments on induced systemic resistance (ISR) against bacterial spot pathogen *Xanthomonas axonopodis* pv. *vesicatoria* in pepper by *P. polymyxa* strains were conducted and only one isolate (KNUC265) was selected. Further studies into ISR mediation by the KNUC265 strain against the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora* in tobacco demonstrated that the tobacco seedlings exposed to either bacterial volatiles or diffusible metabolites exhibited a reduction in disease severity. In conclusion, ISR and plant growth promotion triggered by *P. polymyxa* isolates were systemically investigated on pepper for the first time. The *P. polymyxa* KNUC265 strain, which elicited both ISR and plant growth promotion, could be potentially used in improving the yield of pepper and possibly of other crops.

Keywords: *Paenibacillus polymyxa*, ISR, PGPR, *Xanthomonas axonopodis*, *Erwinia carotovora*

Paenibacillus polymyxa is a plant growth-promoting rhizobacterium (PGPR) commonly found in the rhizospheres of various plant species [1, 3, 24]. Results from studies described in the literature have demonstrated the beneficial effects of *P. polymyxa* treatments upon plant growth promotion under *in vitro* and *in situ* conditions [7, 10, 14, 23, 24]. Such improvements in crop yield were adopted to involve many characteristics of *P. polymyxa*, including nitrogen fixation, plant-disease suppression, soil phosphorous solubilization, increase in soil porosity, and the production of antibiotics, hydrolytic enzymes, and phytohormones. Besides the secretion of plant growth promoting factors like major PGPR, *P. polymyxa* exhibits the ability to control phytopathogens in various plants under both greenhouse and field conditions. Treatments with *P. polymyxa* on seeds, leaves, or roots of plants significantly reduced the disease symptoms caused by complex soil-borne pathogens in sesame, wheat, strawberry, and potato [2, 4, 6, 22]. However, the latter experimental trials were concerned only with the direct antagonism between biocontrol agents and pathogens due to antibiosis or nutrient competition; they did not refer to the induction of defense responses in plants.

One of the major benefits of *P. polymyxa* application on plants is the associated enhancement of resistance to plant diseases, caused by fungi, bacteria, and parasites, *via* simultaneous elicitation of a number of mechanisms. The activity of PGPR as well as *P. polymyxa* capable of controlling plant diseases is sometimes known as "biocontrol". Mechanisms responsible for the biocontrol activity of PGPR include either previously well-studied antibiosis actions consisting of competition for nutrients, niche exclusion, and production of antifungal metabolites, or induced systemic resistance (ISR). The ISR phenomenon has been observed since 1991 when the antagonistic activity of *Pseudomonas fluorescens* strain WCS417 was being tested against *Fusarium oxysporum* f. sp. *dianthi* on carnation [26]. The experiment found that although the rhizobacteria and the pathogenic fungus were not in direct contact each other, the occurrence of a "protective" effect was still observed [26]. To date, ISR is mainly known to be

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elicited by PGPR or nonpathogenic bacteria that stimulate a plant's existing set of defensive responses. In the case of *P. polymyxa* research on ISR, pretreatment of *Arabidopsis* plants with the bacterium induced the jasmonic acid (JA)-responsive *Atvsp* (a vegetative storage protein acid phosphatase), the ethylene (ET)-responsive *Hel* (hevein), and the salicylic acid (SA)-responsive pathogenecity-related protein 1 (*PR-1*) genes, thereby suggesting that ISR was probably being induced in the plants by *P. polymyxa* [24]. Moreover, pretreatment of *Arabidopsis thaliana* roots with *P. polymyxa* triggered ISR in plants against the pathogens *Pseudomonas syringae* pv. *maculicola* and *Erwinia carotovora* [20, 24]. So far, there have been only these two above-mentioned citations in the literature to date in which ISR was shown to be mediated by *P. polymyxa* in *A. thaliana* under *in vitro* conditions.

To defend themselves against phytopathogens by means of any type of ISR mechanism, plants are usually thought to invest energy and allocate the limited resources to a defensive system, which leads to the possibility that plants cannot in turn use these resources for growth or other fitness-related processes. These theoretical concepts, called "fitness cost", can be explained by the continuous assumption of many elements in plants, which provide a helpful basis to interpret findings of induced plant responses against pathogens. To compensate the loss of energy requirements/fitness cost during the responses to pathogens of plants, the utilization of PGPR that can mediate ISR is probably an appropriate solution. Rhizobacteria possessing both plant growth promoting and ISR activities, hence, are able to suppress plant diseases greater and constantly enhance the fitness of plants.

In South Korea, peppers are a staple for a majority of the population and therefore are important agricultural products. However, each year the national pepper productivity has been hampered because of the bacterial pathogen *Xanthomonas axonopodis*. Thus, the current study was mostly carried out to screen and investigate for the first time *P. polymyxa* isolates as elicitors of plant growth promotion and ISR against *X. axonopodis* pv. *vesicatoria* in peppers, under gnotobiotic conditions. In addition, a promising selected ISR-inducing and plant growth promoting strain (KNUC265) was further assessed to better define the nature of bacterial ISR determinants that suppressed the outgrowth of the pathogen *E. carotovora* subsp. *carotovora* on tobacco. The data obtained could help assess the potential application of *P. polymyxa* in the growth of peppers as well as other crops.

Materials and Methods

Bacterial Strains and Growth Conditions

Several *P. polymyxa* isolates used in the current study were isolated from the roots of peppers cultivated in Daegu Province, South Korea, using a formerly described method [8]. Other *P. polymyxa* strains were collected from the *Paenibacillus* sp. collection produced by related work associated with the latter report [3]. For the amplification and sequencing of bacterial 16S rDNA genes, a pair of universal primers, GF1 (5'-TAACACATGCAAGTCGAACG-3') and GR1 (5'-GGTGTGACGGGCGGTGTGTACAAG-3'), was used. *P. polymyxa* isolates and *Bacillus subtilis* GB03 were grown at 28°C, in tryptic soy agar (TSA; Difco, Detroit, MI, USA) medium. The type-strain *P. polymyxa* ATCC842 was used as a reference bacterium. Bacterial pathogens *X. axonopodis* pv. *vesicatoria* and *E. carotovora* subsp. *carotovora* strain SCC1 were grown at 28°C in Luria–Bertani (LB) agar medium supplemented with the antibiotics rifampicin 50 μg/ml and ampicillin 100 μg/ml, respectively. All bacteria were stored in 15% glycerol stocks held at −70°C for long-term preservation.

For the preparation of PGPR inocula, *P. polymyxa* strains and *B. subtilis* GB03 were cultured in tryptic soy broth (TSB; Difco, Detroit, MI, USA) medium for 24 h, centrifuged, and resuspended in sterile distilled water (SDW) to the necessary concentrations. For the preparation of pathogenic infection, *X. axonopodis* pv. *vesicatoria* and *E. carotovora* subsp. *carotovora* strain SCC1 were cultured in LB medium for 20 h, centrifuged, washed with SDW, and resuspended in saline solution to the required concentrations of 10^5 and 10^8 CFU/ml, respectively. Bacterial concentrations corresponding to a measurement of a certain optical density value at 600 nm were confirmed by counting colonies on agar plates.

BOX-PCR Genomic Fingerprinting and *nifH* Gene Detection

Fingerprinting patterns from bacterial genomic DNA were generated with the BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3'). PCR was performed using a reaction mixture containing 30 ng of template DNA; 25 pmol of BOXA1R primer; a PCR premix kit, including 2.5 U *Taq* DNA polymerase; 250 μM dNTPs; 10 mM Tris-HCl, pH 9.0; 40 mM KCl; 1.5 mM $MgCl_2$ stabilizer; and tracking dye to a final volume of 50 μl (Bioneer, South Korea). The following temperature profile was used for PCR: 1× (5 min, 94°C); 30× (1 min, 94°C; 1 min, 53°C; 8 min, 72°C); and 1× (15 min, 72°C). Samples of the BOX-PCR products were separated by electrophoresis on horizontal 1.5% (w/v) agarose gels, followed by ethidium bromide gel staining. The BOX-PCR bands on the agarose gels were scored, rearranged, and computationally analyzed by the unweighted pair group method with arithmetic means (UPGMA).

The *nifH* genes of the *P. polymyxa* strains were detected using Southern hybridization [3] and by nested PCR using IGK/NDR-1 primers for the first PCR and PolF/PolR for the second PCR [19].

Detection of Siderophore Production

The ability of *P. polymyxa* strains to produce siderophores was determined by the O-CAS universal method, as previously described [17]. Bacteria were first streaked on the iron-free defined agar medium DM [18] and grown at 28°C for 48 h. The O-CAS blue agar was prepared as follows: chrome azurol S (CAS), 60.5 mg; hexadecyltrimethyl ammonium bromide (HDTMA), 72.9 mg; Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 30.24 g; 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl, 10 ml; and agar, 0.9% (w/v). Siderophore production was observed after laying 10 ml of O-CAS blue agar over the agar plates containing cultivated *P. polymyxa* strains. Changes in color of the overlaid medium surrounding bacterial colonies after 15 min of reaction were recorded. The color changes from blue to purple or

orange indicated siderophore production of catechol or hydroxamate types, respectively.

Quantification of IAA Production

IAA produced by *P. polymyxa* isolates was assayed by measuring the IAA concentration in the bacterial supernatant. Bacteria were cultivated for 20 h in DM medium without tryptophan (Trp); a 20-µl aliquot was then transferred into 5 ml of fresh DM medium supplemented with 0.01% Trp, obtaining a 10^5 colony-forming units (CFU)/ml final cell density. After 28 h of cultivation, the cell density of cultures was measured spectrophotometrically at 600 nm, and the bacterial cells were removed from the culture medium by centrifugation (5,000 ×*g*, 10 min), after which 80-µl aliquots of the supernatant were mixed with 160 µl of Salkowski's reagent ($FeCl_3$ 0.5 M:$HClO_4$ 35%; 2:100) in a 96-well plate [5]. After incubating at room temperature (RT) for 25 min, the absorbance of the mixture at 530 nm was measured in a Sensident Scan (Labsystems, Helsinki, Finland). The IAA concentration in the culture was determined by comparison with a standard curve for IAA purchased from Sigma (St. Louis, MO, USA).

Elicition of ISR and Plant Growth Promotion by *P. polymyxa* Strains in Peppers

Hot pepper (*Capsicum annuum* L. cv. Bukang) seeds were surface-sterilized by soaking in 1.2% (v/v) sodium hypochlorite (NaOCl) for 20 min, followed by rinsing seven times in SDW; they were then held for 24 h at RT in darkness. For germination, seeds were sown in a 1% agar plate at a ~5-mm depth and held at 25°C for 10 days in darkness. Well-germinated and similarly sized seedlings were transplanted at a 2-cm depth in polystyrene foam pots filled with sterilized commercial soil containing vermiculite and perlite. Pepper seedlings were grown in a growth chamber (16 h of light and 8 h of darkness at 25°C) and watered daily with sterile tap water as needed.

Two weeks after transplantation, 10 ml of the *P. polymyxa* suspensions (5×10^8 CFU/ml) was used to drench the pepper roots. To analyze the plant growth-promoting effects of bacterial treatments, pepper fresh weight and height were measured three weeks later. For ISR bioassays, assessments were made seven days after applying PGPR to the pepper roots, at which time the suspension of pathogen *X. axonopodis* pv. *vesicatoria* of 10^5 CFU/ml was pressure-infiltrated into leaf tissues using a needleless syringe [8]. The severity of the bacterial spot disease on leaves was evaluated after seven days of pathogenic infection on a five-point scale: 0= symptomless leaf; 1=few lesions to 10% of leaf area affected; 2= 10%–25% of leaf area affected; 3=25–50% of leaf area affected; 4 =50%–75% of leaf area affected; 5=>75% of leaf area affected by lesions, or dead leaf [9]. In trial tests to detect the presence of *P. polymyxa* on pathogen-infected pepper leaves, the aerial parts of non-infected leaves and stems were homogenized, diluted in saline solution, and plated on TSB agar plates.

Asessment of *P. polymyxa*-Mediated ISR on Tobacco

Tobacco (*Nicotiana tabacum* L. cv. Bukang) seeds were surface-sterilized by soaking in 1.0% (v/v) NaOCl for 30 min, rinsed four times in SDW, and placed on 0.8% agar plates containing half-strength Murashige and Skoog (MS) basal medium [16] supplemented with 1.5% sucrose. After vernalization for two days at 20°C in darkness, seedlings were transferred to MS agar I-plates, the plastic Petri dishes (100×15 mm) that contain a center partition (I-plates; Fisher Scientific, USA).

To assay ISR mediated by volatiles produced by *P. polymyxa* KNUC265, nine tobacco seedlings were placed onto one side of the I-plates, and one 5-mm-diameter filter paper disc was placed on the other, after which 20 µl of *P. polymyxa* suspension (10^8 CFU/ml) was applied dropwise to each paper disc. Agar plates tested for volatile-inducing ISR were sealed with a polyvinyl membrane and maintained in a growth chamber at 25°C under 12/12 h light/dark conditions. To bioassay diffusible ISR determinants from the KNUC265 strain, a filter paper disc containing 20 µl of *P. polymyxa* suspension (10^8 CFU/ml) was placed onto the edge of I-plates, and nine tobacco seedlings were placed at 2-cm intervals from the paper disc, with three seedlings per line. Fourteen days after applying *P. polymyxa*, 5 µl of a 10^8 CFU/ml suspension of *E. carotovora* subsp. *carotovora* strain SCC1 was dropped onto each leaf of the tobacco seedlings. Additionally, SDW was applied to the paper disc of the control I-plate as a negative control; the synthetically systemic-resistance inducer benzothiadiazole (BTH) and *Bacillus subtilis* GB03, a proven bacterium producing volatile ISR-determinant types, were used as positive controls [21]. Soft-rot symptoms on the tobacco leaves on I-plates were assessed by visual inspection at 24 h of pathogenic post-challenge. The numbers of symptomatic leaves per seedling were counted as a measurement of disease severity [21].

Statistical Analysis

All experiments with pepper plants were repeated three times with five replicates, and each replicate contained one plantlet. Experiments with tobacco were carried out three times with three replicates, and each replicate containing one I-plate. Data were statistically analyzed by analysis of variance (ANOVA), and the means compared using the Duncan's multiple range test ($P\leq0.05$) with the software package SPSS v.14.1 for Windows (SPSS Inc., Chicago, IL, USA).

Nucleotide Sequence Accession Number

The 16S rDNA gene sequence of the strain *P. polymyxa* KNUC265, as reported in this paper, has been deposited in the GenBank database under Accession No. EU239165.

RESULTS

BOX-PCR Fingerprinting of *P. polymyxa* Strains

All 29 isolates used in the current study were partially classified as *P. polymyxa* strains by analyzing the sequences of 16S rRNA genes (data not shown). The results of bacterial genomic DNA fingerprints that relied on BOX-PCR showed genetic differences among the *P. polymyxa* isolates (Fig. 1). By analyzing the phylogenetic tree of the polymorphic PCR patterns based on UPGMA, the isolates were allocated into five groups when employing a cut-off value of 60% similarity. Twenty-two (76%) and 15 (52%) of the total strains showed common PCR bands of 0.7 kb and 1.3 kb, respectively. Bacterial strains, therefore, were dominantly categorized into group A (52%); the other groups (B, C, and E) respectively contained 2, 6, 5 strains,

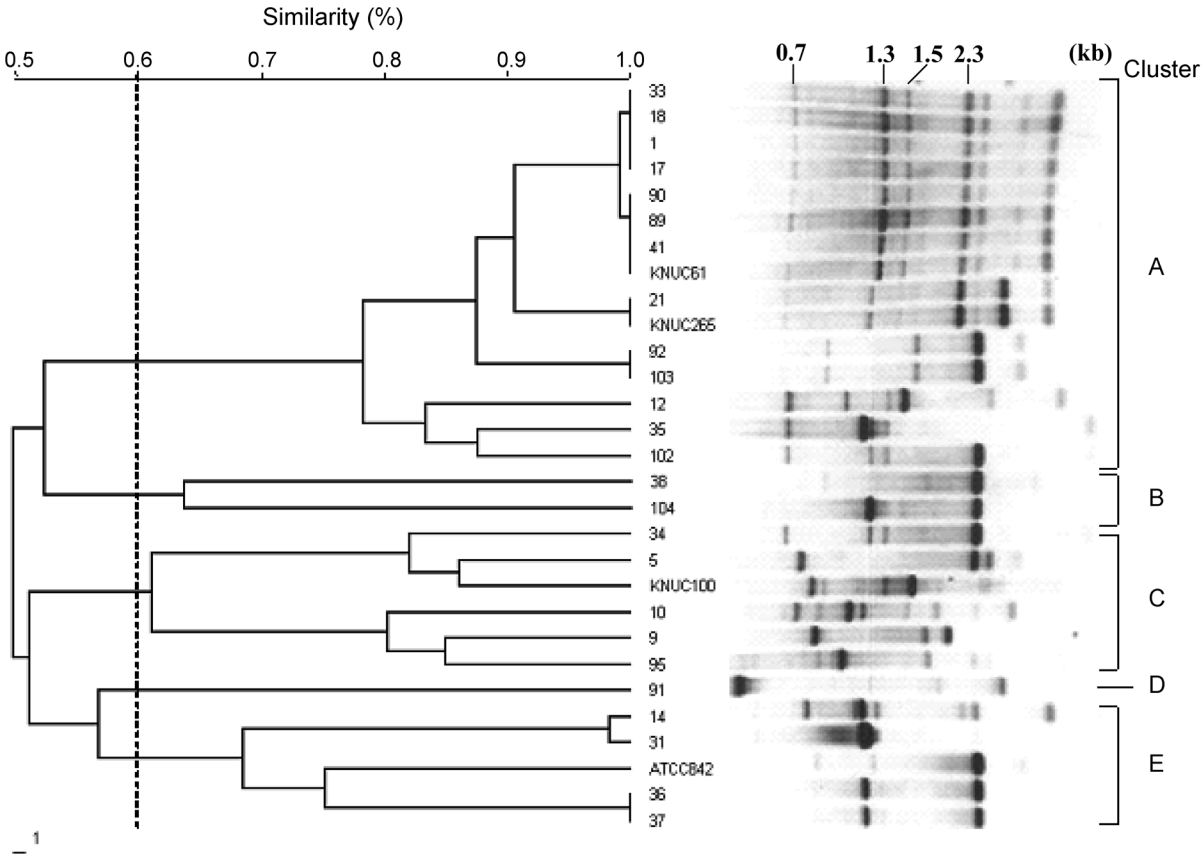

Fig. 1. Dendrogram of genetic relationship among *P. polymyxa* isolates, based on data from BOX-PCR using the single primer BOX1AR.

The BOX-PCR bands on the agarose gels were scored, rearranged, and computationally analyzed by the unweighted pair group method with arithmetic means (UPGMA). Strains were clustered into five groups, with the similarity level >60%.

whereas the isolate 91 delineated a separate position on the dendrogram (Fig. 1).

Siderophore Production

Among isolates tested for their siderophore production ability by detecting O-CAS reactivity in the iron-depleted defined medium, two isolates (33%) in group C were found to have siderophore-producing capabilities (Table 1a and 1b); strains in other bacterial groups were not found to synthesize siderophore (Table 1b). Both mentioned isolates in the group C were able to generate a color change from blue to orange on the examined O-CAS plates, indicating siderophore production of the hydroxamate type (data not shown).

Production of IAA and Detection of *nifH* Gene

Since the Trp-dependent IAA-producing ability of *P. polymyxa* strains has been cited elsewhere [10], all isolates were analyzed for the productivity of accumulating IAA in defined DM medium supplemented with Trp (Table 1a). All bacterial strains showed the ability to produce IAA ranging from 7.5 to 25.9 μg/ml. When comparing IAA productivity among the isolates in allocated groups, the isolates in group C had the lowest IAA production activity, with an average of 10.0 μg/ml (Table 1b). The isolates in other groups (A, B, D, and E) synthesized IAA at average levels of 17.5, 15.3, 18.8, and 17.9 μg/ml, respectively.

Detection of *nifH*, a nitrogen-fixing gene that encodes nitrogen on the chromosome of *P. polymyxa* showed that 53% and 60% of the isolates in groups A and E, respectively, were found to possess the *nifH* gene (Table 1b), whereas the isolates in groups B, C, and D did not appear to contain the gene (Table 1a and 1b).

Assessment of Antagonistic Activity Against Plant Pathogens

In vitro antagonism of *P. polymyxa* strains toward the common phytopathogens *E. carotovora* subsp. *carotovora* and *X. axonopodis* pv. *vesicatoria* was determined by using the paper disc method of Cheong *et al.* [3]. Strains in groups A, B, E, and D showed highly antagonistic activity towards the plant soft-rot pathogen *E. carotovora* subsp. *carotovora* strain SCC1, whereas isolates in group C exhibited low activity (Table 1b). For *in vitro* antagonism

Table 1a. Characterization of *P. polymyxa* strains isolated from winter crops, from the southern part of South Korea.

Isolates	Siderophore production	Antagonistic activity[a]		*nifH* gene[b]	IAA production[c] (μg/ml)	BOX-PCR group	Source
		E. carotovora	*X. axonopodis*				
33	–	++	–	+	23.6‡	A	[3]
18	–	++	–	+	16.2*†	A	[3]
1	–	–	++	+	12.6§	A	[3]
17	–	++	–	–	20.4†	A	[3]
90	–	++	++	+	25.9‡*	A	[3]
89	–	+	++	+	12.5§	A	[3]
41	–	+	++	+	18.0*	A	[3]
KNUC61	–	+	–	+	16.3*†	A	Current study
21	–	++	–	–	17.8*	A	[3]
KNUC265	–	++	+	–	19.9†	A	Current study
92	–	++	–	–	18.5*	A	[3]
103	–	+	+++	–	14.4*‡	A	[3]
12	–	–	–	+	20.8†	A	[3]
35	–	+	–	–	14.8*‡	A	[3]
102	–	–	–	–	16.5*†	A	[3]
38	–	+++	–	–	12.2§	B	[3]
104	–	++	–	–	18.3*	B	[3]
34	–	++	–	–	12.2§	C	[3]
KNUC5	–	–	–	–	12.4§	C	Current study
KNUC100	+	–	–	–	9.0†‡	C	Current study
10	–	–	–	–	8.9†‡	C	[3]
9	–	–	–	-	10.1†‡	C	[3]
95	+	–	–	–	7.5*†‡	C	[3]
91	–	++	–	–	18.8*	D	[3]
14	–	+	–	+	21.5†	E	[3]
31	–	++	–	–	14.1*‡	E	[3]
ATCC 842	–	+	–	–	15.1*‡	E	American Type Culture Collection
36	–	+	–	+	16.5*†	E	[3]
37	–	++	–	+	22.7‡	E	[3]

[a] *In vitro* antagonisms of the *P. polymyxa* strains against bacterial phytopathogens were tested, based on the diameter of the inhibition zone on the scale: –: no inhibition; +: 6–10 mm; ++: 11–15 mm; +++: >16 mm.

[b] *nifH* genes from *P. polymyxa* strains were detected by Southern hybridization [3]. The *nifH* genes in strains KNUC265, KNUC100, KNUC61, KNUC5, and ATCC842 were detected by nested PCR, using two pairs of primers; IGK (5'-TACGGYAARGCBGGYATCGG-3') and NDR-1 (5'-TTGGAGCCGGCRTANGCRCA-3') for the first PCR, and PolF (5'-TGCGAYCCSAARGCBGACTC-3') and PolR (5'-ATSGCCATCATYTCRCCGGA-3') for the second PCR [19]. +: detected; –: not detected.

[c] Values of IAA assays showing the same symbol(s) were not significantly different ($P<0.05$).

Table 1b. Statistical characteristics of each *P. polymyxa* group, summarized from Table 1a.

Statistical data	Group A	Group B	Group C	Group D	Group E
IAA production (μg/ml)	17.5	15.3	10	18.8	17.9
Anti-*Erwinia* sp. (%)	80	100	17	100	100
Anti-*Xanthomonas* sp. (%)	40	0	0	0	0
nifH gene presence (%)	53	0	0	0	60
Siderophore production (%)	0	0	33	0	0

Underlined values indicate distinct characteristics of certain bacterial group(s) that are different from those of any of the other bacterial groups used in the current study.

toward phytopathogen *X. axonopodis* pv. *vesicatoria*, 40% of isolates in group A exhibited antibacterial ability in bioassays, whereas the strains in groups B, C, D, and E did not exhibit this ability (Table 1b).

Plant Growth-Promoting Effects on Pepper

Individual inoculation of peppers with the majority of *P. polymyxa* isolates promoted the growth of plantlets, as reflected in increases in plant height, root fresh weight, and total fresh weight, compared with the untreated controls (Table 2a). The overall result of treatments showed that 17 of the total of 29 P. *polymyxa* isolates tested (59%) were significantly effective in promoting plant growth versus their control counterparts (Table 2a). Among the five bacterial groups, those in group C showed low enhancement in development of plant root and height, compared with untreated controls (Table 2b).

Assessment of ISR Against Pathogen *X. axonopodis* pv. *vesicatoria* in Peppers

Bioassays for the elicitation of ISR in peppers by 29 *P. polymyxa* strains showed that only the isolate KNUC265 significantly reduced the severity of bacterial spot disease, compared with untreated controls (data not shown). When examining the presence of *P. polymyxa* KNUC265 on aerial parts of pathogen-infected pepper plants in ISR experiments, no correlative colony of the KNUC265 strain was found on selective agar plates (data not shown).

Assessment of ISR Against *E. carotovora* subsp. *carotovora* on Tobacco

In order to further understand the mechanisms underlying the phenotypic ISR triggered by strain KNU265 in pepper, experiments on the degree of ISR response produced against infections of the soft-rot pathogen *E. carotovora* subsp. *carotovora* in tobacco were established. Data demonstrating the ability to trigger ISR *via* the application of diffusible and/or volatile metabolites from the isolate KNUC265 against *E. carotovora* subsp. *carotovora* in tobacco are shown in Fig. 2. A reduction of disease severity was triggered in tobacco seedlings that were directly in contact with *B. subtilis* GB03, *P. polymyxa* KNUC265, or BTH. Furthermore, putative volatile organic compounds (VOCs), including 2,3-butanediol and acetoin, released by *B. subtilis* GB03 and non-identified VOCs of *P. polymyxa* KNUC265 significantly reduced the severity of soft-rot disease [21]. By visual observation on I-plates, the diffusible metabolites and VOCs from GB03 and KNUC265 were found to significantly enhance plant growth, as indicated by the leaf area of the plants, in either pathogenic or nonpathogenic infected treatments (data not shown). BTH treatment, meanwhile, caused an inhibition in plant growth, compared with the SDW control treatment.

Table 2a. Effects of *P. polymyxa* treatments on growth promotion in pepper seedlings.

Treatments	Height (cm)	Root fresh weight (g)	Total fresh weight (g)
33	14.13	0.38	4.09*
18	13.57	0.35	3.25
1	14.41*	0.36	3.43
17	13.83	0.33	3.14
90	14.22	0.41*	3.33
89	14.42*	0.34	3.63*
41	12.23†	0.33	2.71†
KNUC61	13.29†	0.35	3.22
21	15.22*	0.41*	4.41*
KNUC265	15.74*	0.42*	4.63*
92	16.03*	0.44*	4.97*
103	14.11	0.34*	3.12
12	15.36*	0.41*	4.14*
35	15.62*	0.46*	5.11*
102	15.17*	0.43*	4.39*
38	12.56†	0.35	2.65†
104	15.24*	0.43*	4.21*
34	13.53	0.39	3.34
KNUC5	14.55*	0.39	3.69*
KNUC100	12.67†	0.35	2.52*
10	13.76	0.36	2.96
9	13.53	0.34	2.73†
95	15.08*	0.39	4.13*
91	15.46*	0.38	3.69*
14	16.23*	0.41*	4.84*
31	14.15	0.37	3.71*
ATCC 842	13.76	0.32†	2.98
36	14.87*	0.41*	3.94*
37	17.13*	0.43*	5.43*
Control	13.85	0.36	3.21

Data are the mean of three replicates; each replicate represents average data from five plantlets.

*† Data in a column were significantly higher and lower than the corresponding untreated control, respectively, by using Duncan's multiple range test ($P \leq 0.05$).

Table 2b. Statistical characteristics of each *P. polymyxa* group on promoting pepper growth, summarized from Table 2a.

Statistical data	Group A	Group B	Group C	Group D	Group E
Increase height (%)	53	50	<u>33</u>	100	60
Increase root weight (%)	53	50	<u>0</u>	<u>0</u>	60
Increase total weight (%)	53	50	50	100	80

Underlined data indicate particular pepper growth-promoting characteristics of bacterial group(s) that are different from those of other bacterial groups compared in the current study.

DISCUSSION

In the current study, results of BOX-PCR and constructed dendrogram analysis revealed genetic variations among the isolates, leading to a classification division into five bacterial groups (Fig. 1). When comparing the plant growth-promoting characteristics of the isolates in each allocated BOX-PCR group, *P. polymyxa* strains showed

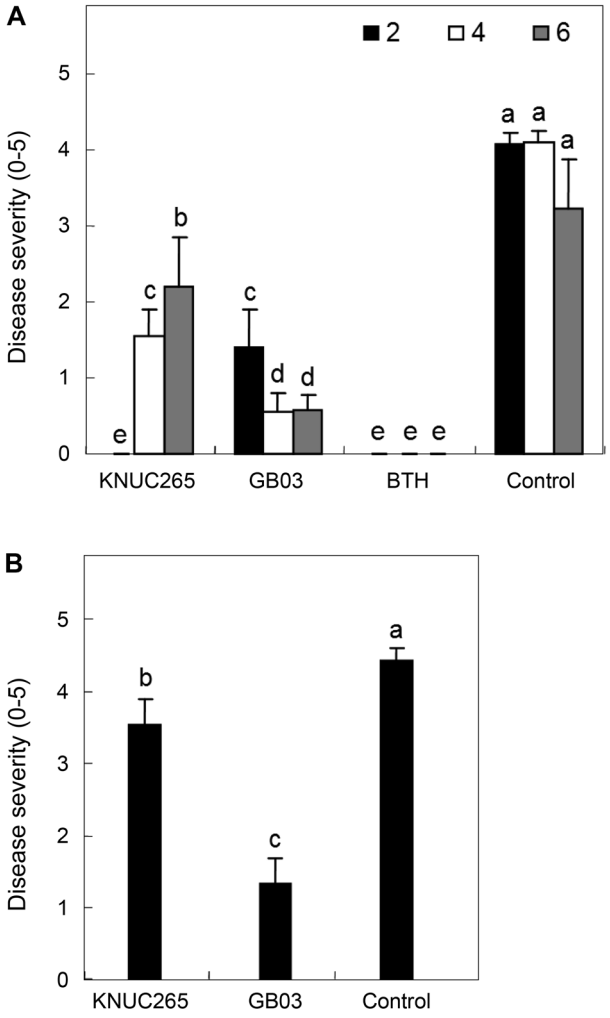

Fig. 2. Induction of systemic resistance mediated by *P. polymyxa* KNUC265 and *B. subtilis* GB03 against soft-rot pathogen *E. carotovora* subsp. *carotovora* strain SCC1 in tobacco seedlings, on MS agar plates.

A. Plants exposed to diffusible ISR determinant(s) released by bacteria on MS agar plates. Different shading of bars indicate the tobacco seedling placed at 2-cm intervals from the bacterial-treated paper discs: 2 cm (black boxes), 4 cm (white boxes), and 6 cm (gray boxes). **B**. Plants exposed to volatile compounds produced by bacteria on MS agar I-plates. Commercial strain *B. subtilis* GB03 was used as the positive control and a SDW treatment was used as the negative control. Mean data showing the same letter were not significantly different, according to Duncan's multiple range test ($P<0.05$).

some common attributes (Table 1a and 1b). Only bacteria in group A exhibited antagonistic activities against both phytopathogens *E. carotovora* subsp. *carotovora* and *X. axonopodis* pv. *vesicatoria*. The isolates in groups B, D, and E exhibited only antibacterial activity against *X. axonopodis* pv. *vesicatoria*, whereas isolates in group C did not display antagonistic activity against the pathogens (Table 1b). All isolates in group C brought about lower IAA productivity compared with the isolates in the groups A, B, D, or E (Table 1b). On the other hand, only two strains in group C exhibited siderophore production (Table 1b); group C, therefore, would appear to be rather different taxonomically and phenotypically from the other four groups identified in this study. Moreover, 53% and 60% of the isolates in groups A and E, respectively, contained the *nifH* gene on their chromosomal genomes, but none of the isolates in the other groups had this gene (Table 1a and 1b). Based on the evidence obtained, there were remarkable differences in the plant growth-promoting characteristics among *P. polymyxa* isolates in different bacterial groups. Compared with the untreated control, 17 of the total 29 *P. polymyxa* treatments promoted growth in peppers (Table 2a). An assessment of the percentage of isolates able to promote pepper growth in each group revealed that groups A (53%) and E (80%) showed better promotion of plant growth than groups B (50%) and C (50%). These positive observed growth effects are likely to be associated with the combination of high IAA production abilities and the presence of *nifH* genes among isolates in groups A and E (Tables 1a and 2a). Furthermore, these results are consistent with previously observed improvements in crop yield when applying the IAA-producing and nitrogen-fixing rhizobacteria to plant roots [7]. Meanwhile, isolates in each of groups A, B, and E enhanced the development of pepper roots, but none of the treatments with bacteria in group C resulted in improvements to root growth in inoculated plants, compared with their untreated control counterparts (Table 2b). These results could be explained by the low IAA productivity demonstrated by isolates in group C; however, an enhancement in total fresh weight in plants treated with bacteria in group C was still evident (Table 2b), suggesting that other growth-promoting factors besides IAA of *P. polymyxa* isolates likely affected plant growth promotion. These results demonstrate the likelihood of a certain level of diversity of plant growth-promoting capabilities among genetic-clustered groups of *P. polymyxa*. In the current study, 59% of all *P. polymyxa* treatments were found to promote growth in pepper plants, indicating that the majority of *P. polymyxa* strains could have the potential to improve pepper crop yield.

To date, *P. polymyxa* has been found to mediate ISR against pathogens *E. carotovora* and *P. syringae* pv. *maculicola* in *A. thaliana* [20, 24]. The data of the current study show an ability by *P. polymyxa* isolates to elicit ISR against *X. axonopodis* pv. *vesicatoria* in peppers. Of all the *P. polymyxa* treatments, protective effects against bacterial spot disease in peppers were recorded in the case of plants inoculated with the KNUC265 strain (data not shown). By definition, ISR is mediated by PGPR or nonpathogenic bacteria that trigger reactions throughout the plant and systemically enhance the defensive capacity of distant tissues against subsequent infection by the pathogens [25]. In the current study, an examination of pathogen-infected pepper leaves in ISR bioassays did not detect any *P.*

polymyxa KNUC265 colonies on the selective agar plate, indicating that a typical ISR had probably occurred.

Several ISR-eliciting rhizobacteria were found to be effective in several plant species, whereas some other strains exhibited a narrow specificity in plant hosts [11, 15]. Although the main objective of the current study was to evaluate *P. polymyxa* isolates as elicitors of plant growth promotion and ISR in peppers, further bioassays for the selected ISR-inducing KNUC265 strain *vis-à-vis* triggering ISR against soft-rot pathogen *E. carotovora* subsp. *carotovora* in tobacco on I-plates were carried out. This was done to determine the nature of the bacterial diffusible and/or volatile ISR determinants. The significant suppression of disease severity in tobacco – observed when plants were either directly or indirectly contacted with metabolites from the KNUC265 strain – demonstrated the possible involvement of both diffusible and volatile ISR determinants from the bacterium (Fig. 2). In a previous study by Ryu *et al.* [21], the spore-forming bacteria *B. amyloliquefaciens* IN937 and *B. subtilis* GB03 were found to produce 2,3-butanediol, which is effective in suppressing bacterial spot disease caused by *E. carotovora* subsp. *carotovora* on *Arabidopsis*. Moreover, volatile metabolite 2,3-butanediol produced by *P. polymyxa* in culture is known to be able to diffuse into aqueous medium, as described by Marwoto *et al.* [13]. In the current study, the *P. polymyxa* KNUC265 elicitation of both diffusible and volatile ISR determinants suggests that ISR-determinant type(s) produced by the isolate KNUC265 probably shared the same characteristics with those from the aforementioned *Bacillus* spp.

Assessments of disease severity on tobacco seedlings that had been in direct contact with KNUC265 on agar plates demonstrated a significant reduction of disease incidence when plants were placed at a smaller distance from bacterium-treated paper discs (Fig. 2). Therefore, the various concentrations of diffusible ISR determinants produced by *P. polymyxa* KNUC265 probably mediated the different ISR levels in tobacco. Tobacco seedlings in direct contact with metabolites released by GB03 strains, however, showed an inverse impact on reduction of disease severity, with higher antipathogen ISR levels being found at larger distances from the paper discs (Fig. 2). These results thus indicate the difference between properties of the ISR-determinant types produced by two genera of spore-forming rhizobacteria. Formerly, some PGPR were proven to elicit ISR in different plants through more than a single determinant, such as lipopolysaccharides, flagella, and siderophores [11, 12, 15]. As the ISR-mediating bacterium *P. polymyxa* KNUC265 has been shown not to produce siderophores (Table 1a), the bacterial ISR determinant(s) was presumably not related to siderophores.

Although no *P. polymyxa* isolate besides the KNUC265 strain has yet been assayed for ISR activity in tobacco, both the selected KNUC265 isolate, as well as *P. polymyxa* strains, may have potential applications in pathogenic suppression and plant growth promotion in peppers and other crops. Future research should therefore focus on characterizing the ISR determinant(s) from bacteria and studying the application of the ISR-mediating rhizobacterium *P. polymyxa* to other plants in greenhouse and field experiments.

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