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Diversity of Root-Associated *Paenibacillus* spp. in Winter Crops from the Southern Part of Korea

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Abstract The genus *Paenibacillus* is a new group of bacilli separated from the genus *Bacillus*, and most of species have been isolated from soil. In the present study, we collected 450 spore-forming bacilli from the roots of winter crops, such as barley, wheat, onion, green onion, and Chinese cabbage, which were cultivated in the southern part of Korea. Among these 450 isolates, 104 *Paenibacillus*-like isolates were selected, based on their colony shape, odor, color, and endospore morphology, and 41 isolates were then finally identified as *Paenibacillus* spp. by 16S rDNA sequencing. Among the 41 *Paenibacillus* isolates, 23 were classified as *P. polymyxa*, a type species of the genus *Paenibacillus*, based on comparison of the 16S rDNA sequences with those of 32 type strains of the genus *Paenibacillus* from the GenBank database. Thirty-five isolates among the 41 *Paenibacillus* isolates exhibited antagonistic activity towards plant fungal and bacterial pathogens, whereas 24 isolates had a significant growth-enhancing effect on cucumber seedlings, when applied to the seeds. An assessment of the root-colonization capacity under gnotobiotic conditions revealed that all 41 isolates were able to colonize cucumber roots without any significant difference. Twenty-one of the *Paenibacillus* isolates were shown to contain the *nifH* gene, which is an indicator of N_2 fixation. However, the other 20 isolates, including the reference strain E681, did not incorporate the *nifH* gene. To investigate the diversity of the isolates, a BOX-PCR was performed, and the resulting electrophoresis patterns allowed the 41 *Paenibacillus* isolates to be divided into three groups (Groups A, B, and C). One group included *Paenibacillus* strains isolated mainly from barley or wheat, whereas the other two groups contained strains isolated from diverse plant samples. Accordingly, the present results showed that the *Paenibacillus* isolates collected from the rhizosphere of winter crops were diverse in their biological and genetic characteristics, and they are good candidates for further application studies.

Key words: *Paenibacillus*, rhizosphere, 16S rDNA, PGPR, nitrogen-fixation, BOX-PCR

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The genus *Paenibacillus* is a group of Gram-positive, spore-forming, rod-shaped, and facultative aerobic bacteria separated from the genus *Bacillus* [1]. *Bacillus polymyxa* strains isolated from plants were first described as early as 1908 [39]. In 1963, *B. polymyxa* strains were reported to promote the growth of wheat, maize, and tomatoes [29]. Since 1993, Ash *et al.* [1] proposed that members of "group 3" within the genus *Bacillus* should be transferred to a new genus, *Paenibacillus*, within which *P. polymyxa* was proposed as a new kind of species. The genus *Paenibacillus* currently contains more than 70 species, including *P. polymyxa* and *P. azotofixans* [1].

Many research groups have already studied *Paenibacillus* spp. to exploit its useful functions in agriculture systems, in particular in plant growth promotion and biological control against plant pathogens [11, 12]. *P. polymyxa* strains, which are generally recognized as plant growth-promoting rhizobacteria (PGPR), produce a wide variety of secondary metabolites, including plant growth-regulating substances, such as auxin or cytokinin, which directly increase plant growth [19]. Previous data have shown that *P. polymyxa* strains exhibit an antagonistic activity towards several plant pathogens, including fungi and bacteria [7, 10, 30], and such activity against soilborne pathogens has been associated with the production of antimicrobial substances, such as polymyxin, fusaricidin, and chitinase [21, 24, 25, 27, 36]. A *P. polymyxa* strain isolated from wheat rhizosphere was shown to have a clear antagonistic

effect against the take-all disease caused by the fungus *Gaeumannomyces graminis* var. *tritici* in wheat [12], whereas the treatment of seeds with *P. polymyxa* was found to increase the seedling survival and shoot dry weight against soilborne pathogens, such as *Pythium* spp. [16]. In addition to controlling soilborne pathogens, the application of *P. polymyxa* to strawberry leaves has been reported to have antagonistic effects against the foliar pathogen *Botrytis cinerea*, the casual agent of gray mold. In field trials over three years, treatment with a culture suspension of *P. polymyxa* isolate 18191 reduced the conidiophore density of *B. cinerea* and reduced the incidence of gray mold caused by *B. cinerea* in mature strawberry fruit after harvest by 24% to 36% [11].

Genotypic characterization based on DNA fingerprinting has been used to study microbial communities from several different environments [22, 33, 38], and families of repetitive DNA sequences, such as REP, ERIC, and BOX, have been determined to be sufficient to show differences between bacterial species [38]. Ever since the BOX element, consisting of differentially conserved subsequences (BOX A, BOX B, and BOX C), was identified as the first interspersed repetitive element from the bacterium *Streptococcus pneumoniae* [38], the BOX A subunit has been used to study bacterial diversity, including Gram-positive bacteria [38]. According to previous research, comparisons of *P. azotofixans* strains isolated from rhizosphere or bulk soil [34] and *P. polymyxa* strains from maize rhizosphere have been conducted using the BOX-PCR technique [40]. A comparison of 104 *P. azotofixans* strains from maize in two different soil types from Varzea and Cerrado, Brazil, revealed 25 statistically different groups among the bacterial strains [34], whereas the diversity of 67 *P. polymyxa* strains from the rhizosphere of maize planted in Cerrado soil revealed 18 significantly different groups, based on the BOX elements [40]. BOX-PCRs have also been successfully applied to studying the population diversity among *Paenibacillus* spp. from different soils and environments.

Accordingly, the current study investigated the biological and genetic diversity of *Paenibacillus* isolates collected from the rhizosphere of winter crops from the southern part of Korea. To assess the genetic diversity among the *Paenibacillus* spp., a comparison of the 16S rDNA and BOX element sequences was performed, thereby providing a deeper insight into the nature of root-associated *Paenibacillus* spp.

MATERIALS AND METHODS

Isolation of Endospore-Forming Rhizobacteria from Plant Samples

Root samples were collected from winter crops, such as barley, Chinese cabbage, green onion, sesame, onion, wheat, and radish, grown in the southern part of Korea from 1995 to 2002. The root samples were cut using flame-sterilized scissors, and then washed with tap water to remove large soil particles and reduce the root-colonizing bacteria. Thereafter, the roots were shaken in 100 ml of sterilized water in a 250-ml flask at room temperature for 30 min, then macerated using a sterile-mortar and pestle, resuspended in 30 ml of 0.1 M $MgSO_4$ solution in a test tube (3×20 cm), and heat-treated at 80°C for 30 min in a water bath to kill any non-spore-forming bacteria and fungal spores. After vortex mixing, 100 µl of the heat-treated suspension was spread on a 1/10 tryptic soy agar (TSA, BD Co, MD21152, U.S.A.) and incubated at 28°C to isolate a single colony. The colonies grown on the 1/10 TSA were collected, and the isolates were stored in 15% glycerol at −70°C for long-term preservation.

Amplification, Sequencing, and Phylogenetic Analysis of 16S rDNA Gene

To screen and efficiently identify *Paenibacillus* spp. from the isolates, the PCR detection method was used as described by Petersen *et al.* [26] with a slight modification. A pair of specific PCR primers, PPG1 (5'-TAACACGT-AGGCAACCTGCC-3') and PPG2 (5'-TTACACAATTG-AAGCCGTGGTTCCCATAGC-3'), was designed to generate a *Paenibacillus*-specific 16S rDNA fragment of 754 bp. Through a preliminary PCR experiment, it was confirmed that the primers PPG1 and PPG2 generated only the expected DNA fragment size for *Paenibacillus* spp. among the bacterial strains used: When the PCR primers were tested with six known *Paenibacillus* spp. (*P. polymyxa* E681, *P. azotofixans* KCTC 3740, *P. macerans* KCTC 1822, *P. polymyxa* ATCC 15970, *P. polymyxa* ATCC 39564, and *P. polymyxa* ATCC 842), two *Bacillus* spp. (*B. thuringiensis* serovar kurstaki strain 4D11 and *B. subtilis* 168), and three Gram-negative bacteria (two *Burkholderia*

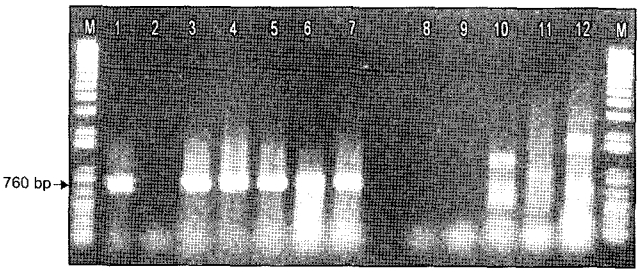

Fig. 1. Electrophoresis pattern, showing PCR products of 16S rDNA for several *Paenibacillus* strains and reference bacterial strains amplified using specific primers PPG1 and PPG2.
Lanes; M: Size marker; 1: *Paenibacillus polymyxa* E681; 2: H_2O; 3: *Paenibacillus azotofixans* KCTC 3740; 4: *Paenibacillus macerans* KCTC 1822; 5: *Paenibacillus polymyxa* ATCC 15970; 6: *Paenibacillus polymyxa* ATCC 39564; 7: *Paenibacillus polymyxa* ATCC 842; 8: *Bacillus thuringiensis* serovar kurstaki strain 4D11; 9: *Bacillus subtilis* 168; 10: *Burkholderia* sp.; 11: *Burkholderia* sp.; 12: *E. coli* DH10B.

spp. and *E. coli* DH10B), only the six *Paenibacillus* spp. showed DNA bands corresponding to a 754 bp fragment (Fig. 1). The PCR reaction mix consisted of 20 pmol each of primer (PPG1, PPG2), AccuPower® PCR PreMix [Taq DNA polymerase, dNTP, Tris-HCl, KCl, $MgCl_2$, a stabilizer, and tracking dye (Bioneer Co., Daejeon, Korea)], and

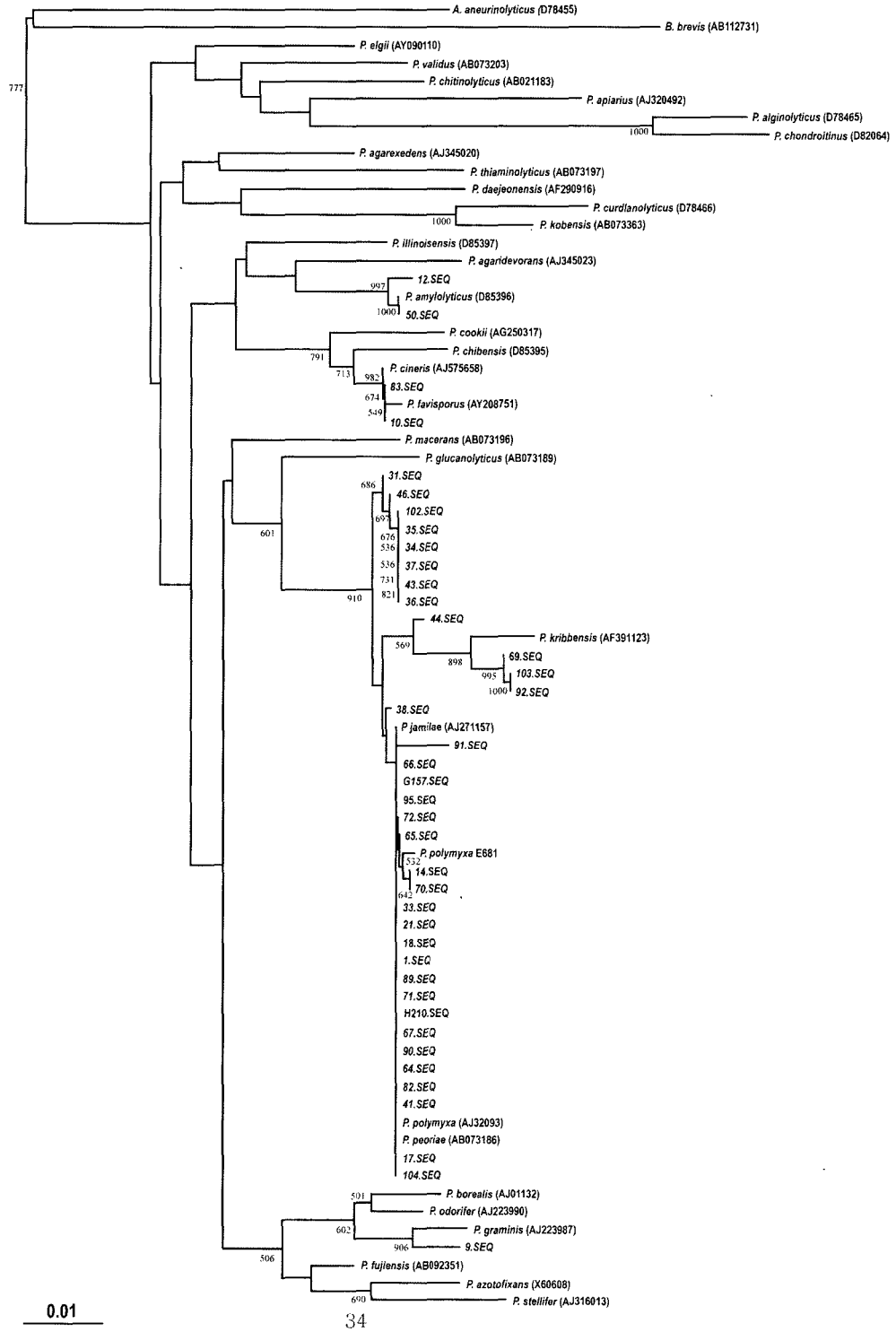

Fig. 2. Comparative sequence analysis of 16S rDNA for 41 *Paenibacillus* isolates collected from the rhizosphere of winter crops and representative strains of genus *Paenibacillus* in GenBank using the neighbor-joining method.
Significance of each branch is indicated by the bootstrap value calculated for 1,000 subsets.

20 μl of the colony suspension. The PCR process consisted of an initial preheating period (94°C for 5 min), 35 cycles (94°C for 1 min, 50°C for 1 min, 72°C for 1 min), and final extension (72°C for 5 min). The DNA amplification was carried out using a model GeneAmp PCR system 9700 (Perkin-Elmer Co., Foster City, CA, U.S.A.). The amplified 16S rDNA fragments were then eluted using a QIAquick® Gel extraction kit (Qiagen Sciences, MD, U.S.A.), and the DNA sequences were analyzed using a BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, U.S.A.), according to the manufacturer's instructions. The DNA sequence alignment, calculation of the nucleotide substitution rates, and construction of a neighbor-joining phylogenetic tree were all carried out using Clustal X program version 1.83. Alignment gaps and unidentified base positions were not taken into account in the calculations. The sequence identities shown in Fig. 2 were determined using njplotWin95 in the Clustal X program package.

Assessment of Antagonistic Activity Against Plant Pathogens

The assessment of the *Paenibacillus* strains isolated from the rhizosphere for antagonism against various microorganisms, including fungal and bacterial pathogens, was carried out in agar plate assays using petri dishes containing a PDK agar (20 g of potato dextrose broth, 10 g of Bacto peptone, 20 g of agar per 1 l sterile distilled water). To analyze the antagonistic activity against the fungal pathogens *Rhizopus oryzae*, *Botrytis cinerea*, *Rhizoctonia solani*, *Mucor ambigus*, *Sclerotium rolfsii*, *Sclerotinia* sp., *Alternaria* sp., *Phytophthora cactorum*, *Fusarium oxysporum*, *Fusarium solani*, and *Pythium aphanidermatum*, the *Paenibacillus* strains were first inoculated at a distance of 3.5 cm from the center of the agar plate using a toothpick, incubated at 30°C for one day, and then 1-cm diameter mycelial disks of fungal pathogens were placed in the center of the petri dish. Before loading, the fungal pathogens were grown on the PDK agar for 2 days. After 3 to 7 days of incubation at 30°C, the plates were examined for fungal growth inhibited zones around the *Paenibacillus* strains. To analyze the antagonistic activity against the fungi *Penicillium* sp. and *Aspergillus niger*, seven bacterial species (*E. coli*, *Agrobacterium tumefaciens*, *Burkholderia glumae*, *Pseudomonas syringae* pv. *tomato* DC3000, *Erwinia carotovora* subsp. *carotovora*, *Xanthomonas campestris* pv. *vesicatoria*, *Bacillus subtilis*), and *Saccharomyces cerevisiae*, the fungal spores, and vegetative cells of other microorganisms were suspended in a molten form of the PDK agar. After solidifying the agar, the *Paenibacillus* strains were then spot-inoculated using a toothpick and incubated at 30°C until appearance of a clear zone. The phytopathogenic fungi and bacteria used in these experiments were kindly obtained from the Korean Agricultural Culture Collection (KACC) at the National Institute of Agricultural Biotechnology, Suwon, Korea and Korean Collection for Type Cultures (KCTC), Daejeon, Korea.

Analysis of Plant Growth-Promoting Effect

Cucumber seeds were soaked in a suspension of each bacterial isolate at approximately 10^7 cfu/ml, and then planted in a rectangular pot (5 cm×5 cm×8 cm) containing a mixture of nursery soil and vermiculite (2:1 w/w). The pots were covered with wet paper to provide constant moisture until the seedlings emerged. No other nutrients were added during the experiment. The seeded pots were placed in an ordinary glasshouse. The foliar height of the cucumber seedlings was measured for 35 days after the seed germination. The experiments were conducted twice with three replications containing five plants.

The resulting data were subjected to an analysis of variance using JMP software (SAS Institute Inc., Cary, NC, U.S.A.). The significance of the effect of bacterial treatment was determined according to the magnitude of the F value at P=0.05. When a significant F value was obtained, a separation of the means was accomplished using Fisher's protected least significant difference (LSD) at P=0.05. The results for the repeated trials of each experiment outlined above were similar. Hence, only one representative trial of each experiment is reported in the results section.

Assessment of Root-Colonizing Ability

Cucumber seeds (*Cucumis sativus* L. cv 'Manchuncheongjang,' Seminis Korea Co., Seoul, Korea) were surface disinfected using 1% sodium hypochlorite (NaOCl) for 10 min and rinsed three times with distilled sterile water, followed by air-drying for 1 h in a clean hood. A loopful of a fresh bacterial colony grown on TSA was suspended in 0.1 M $MgSO_4$ solution for the seed treatment. The cucumber seeds were dipped in 1 ml of the cell suspension for 30 min, and then air-dried for 30 min on sterilized filter paper. Five cucumber seeds were placed on a bottom layer of filter paper (Whatman No. 2, Whatman International Ltd, Maidstone, England) in an 11 cm (diameter) petri dish, covered with another layer of filter paper, and 4.5 ml of sterile water were added. The dishes were then sealed with a polypropylene wrap to maintain constant moisture, placed in a cardboard box, and incubated at 28°C for 72 h in the dark. Thereafter, 1 cm of the root tips was cut using a sterile surgical blade, and each segment was transferred to an eppendorf tube containing 1 ml of 0.1 M $MgSO_4$ solution. The eppendorf tubes containing the root tips were then vigorously stirred using a vortex mixer, and the colonies on each root segment were determined by plating a 10-fold dilution on TSA, followed by incubation at 30°C for 36 h.

Determination of Extracellular Enzyme Activity

The extracellular enzyme activities of the *Paenibacillus* isolates were determined by inoculating on agar media

containing an appropriate substrate and observing the clear zone around a colony with or without staining after incubation for 3 days. Amylase activity was determined on 2% soluble starch medium with iodine staining, whereas protease activity was determined on skim milk-TSA (1% skim milk, 1.5% TSB, and 2% agar) by direct observation of the clear zone, and cellulase activity was determined on 0.5% carboxylmethyl cellulose-TSA, where the clear zone was observed after staining with 0.5% Congo red solution.

Detection of the *nifH* Gene by PCR Amplification and Southern Blotting

To detect the presence of the *nifH* gene in the *Paenibacillus* isolates, the total DNA of the isolates was extracted using the method described by Sambrook *et al.* [32], precipitated with ethanol, air-dried, and finally dissolved in 50 μl of TE buffer (10 mM Tris, 25 mM EDTA, pH 8.0) with RNase (20 μg/ml). The sequences of the primers used for the PCR amplification of the *nifH* gene were GCIWTYTAYGGIA-ARGGIGG for 19F and AAICCRCCRCAIACIACRTC for 407R [37]. The letter I represents inosine, R represents A or G, W represents A or T, and Y represents C or T. The amplification of the *nifH* gene was conducted using 100 ng of genomic DNA from each *Paenibacillus* isolate. The reaction process was 5 min at 94°C, 1 min at 50°C, and 0.5 min at 72°C for 40 cycles. Gel electrophoresis of the PCR products was performed using 0.8% agarose in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 100 V for 20 min at room temperature. In a preliminary PCR using the primers 19F and 407R, an *nifH* fragment of approximately 390 bp was amplified from *Paenibacillus azotofixans* KCTC3740. For the Southern blotting, an *nifH* DNA probe was prepared by PCR using the 19F and 407R primers and chromosomal DNA of *P. azotofixans* KCTC3740 as a template. The PCR fragment was labeled with digoxigenin using a DIG labeling kit (Roche Co., Mannheim, Germany). The chromosomal DNA for the *Paenibacillus* isolates was blotted from gels to positively-charged nylon membranes as described by Sambrook *et al.* [32], and the prehybridization and hybridization conditions for the DIG-labeled probe were the same as described in the manual for the DIG nucleic acid detection kit from Roche Co.

BOX-PCR and Genomic Fingerprinting

The BOX-PCR was carried out as described by Versalovic *et al.* [38]. The single primer BOXA1R (5'-ACGTGGTTT-GAAGAGATTTTCG-3') was used to detect BOX regions in the genomic DNA of the *Paenibacillus* isolates. The amplification reaction was conducted in 25 μl of mixtures containing 20 pmol of the primer, 1.25 mM of each deoxynucleoside triphosphate, 2.5 μl of 10× PCR buffer with 4 mM $MgCl_2$, 10% DMSO, and 2 units of *Taq* DNA polymerase. The GeneAmp PCR system 9700 (Applied Biosystems Co., Foster City, CA, U.S.A.) was used, based on the following temperature profile: 1 cycle (94°C for 90 sec, 53°C for 3 min, 72°C for 1 min), 34 cycles (94°C for 30 sec, 53°C for 30 sec, 72°C for 1 min), and final extension at 72°C for 1 min. The BOX-PCR products were separated by electrophoresis on 1.6% agarose gel in TBE buffer (Tris-borate-EDTA buffer) at 10 V/cm for 90 min at room temperature. The gel was stained with ethidium bromide, and the image was stored as a JPG computer file using Gel Manager (Prime-tech Co., Daejeon, Korea).

Results and Discussion

Isolation of Endospore-Forming Rhizobacteria from Winter Crops

A total of 450 heat-stable bacterial strains were isolated from the rhizosphere of winter crops, such as barley, wheat, onion, green onion, and Chinese cabbage. Heat treatment at 80°C for 30 min enabled the spore-forming bacteria to be efficiently screened from the plant roots. The bacterial strains were collected from 11 different locations in Gyeongsangnamdo and Jeollanamdo Provinces, South Korea, from 1995 to 2002. Among the 450 spore-forming isolates, 104 isolates were screened that exhibited general characteristics of species from the genus *Paenibacillus*, such as colony shape, odor, color, and morphology of the endospores.

Identification of Isolates Based on PCR and 16S rDNA Sequencing

Among the 104 candidate species for the genus *Paenibacillus*, 41 isolates were selected based on the presence of a 759 bp DNA fragment in the PCR amplification of the 16S rDNA, using specific primers, PPG1 and PPG2, and then identified as *Paenibacillus* spp. by sequencing the 16S rDNA fragment. *P. polymyxa* E681 strain, which was previously reported as a PGPR with a broad spectrum of antifungal activity [30], was used as a positive control in this study. All the *Paenibacillus* isolates and *P. polymyxa* E681 exhibited common colony features, such as translucent to white, convex, mucoid, and bright yellow, when the strains were grown in TSA.

In a control PCR experiment, *Bacillus thuringiensis* serovar kurstaki strain 4D11 and *Bacillus subtilis* 168 did not exhibit a 759 bp band, even though they are Gram-positive endospore-forming bacilli (Fig. 1). In addition, the band patterns for *Burkholderia* spp. and *E. coli* were totally different from those for the *Paenibacillus* isolates. Therefore, as shown in Fig. 1, the PCR amplification using the PPG1 and PPG2 primers successfully differentiated *Paenibacillus* spp. from the other *Bacillus* species and Gram-negative bacteria. PCR products corresponding to the 759 bp region between 113 bp and 871 bp of the 16S

rDNA for *Paenibacillus* species were consistently generated from all the *Paenibacillus* spp. examined, such as *P. polymyxa* E681, *P. azotofixans* KCTC 3740, *P. macerans* KCTC 1822, *P. polymyxa* ATCC 15970, *P. polymyxa* ATCC 39564, and *P. polymyxa* ATCC 842, as shown in Fig. 1.

PCR-amplified 16S rDNA fragments of about 759 bp were detected in all 41 *Paenibacillus* isolates and directly sequenced. The sequences were aligned and a total 450 bp nucleotide positions were analyzed by computing the genetic distances, and then distance trees were generated to establish the genetic relationship between the isolates and the reference microorganisms. The homology of the 16S rDNA among the 41 isolates was more than 99% with the genus *Paenibacillus*. Figure 2 shows a representative phylogram that reveals the genetic affiliations of all the *Paenibacillus* isolates and their relationship with 32 reference *Paenibacillus* spp. in the GenBank database. The phylogeny presented in this study revealed that all the known *Paenibacillus* species phylogenetically form a coherent cluster. Twenty-three isolates among the 41 isolates appeared to be closely clustered with *Paenibacillus polymyxa*, whereas 3 isolates matched with *P. kribbensis*. Nonetheless, all the 41 isolates were identified as *Paenibacillus* spp. (Fig. 2), indicating the effectiveness of the initial selection based on certain general characteristics, such as colony shape and morphology of the endospores.

In Vitro Antagonistic Activity of *Paenibacillus* Isolates Towards Pathogenic Fungi and Bacteria (Table 1 & Table 2)

In the antagonistic capacity assay using the 41 *Paenibacillus* isolates and based on an inhibition zone greater than 16 mm in diameter between the fungus and the bacteria, 3 isolates exhibited antagonism towards *R. solani* (isolates 46, 102, and 104), *E. coli* DH10B isolates (31, 35, and 92), and *X. campestris* pv. *vesicatoria* (isolates 65, 69, and 103), 35 towards *Alternaria* sp., *P. cactorum*, *Botrytis cinerea*, and *Sclerotinia* sp. (isolates 1, 14, 17, 18, 21, 31, 33, 34, 35, 36, 37, 38, 41, 43, 44, 46, 50, 64, 65, 66, 67, 69, 70, 71, 72, 82, 89, 90, 92, 102, 103, 104, G157, H210, and E681), 2 towards *A. tumefaciens* (isolates 44 and 103), one towards *P. syringae* pv. *tomato* DC3000 (isolate 103), and 4 towards *E. carotovora* subsp. *carotovora* (isolates 18, 38, 44, and 69).

Meanwhile, based on a diameter range of 11 mm to 15 mm, 31 isolates exhibited antagonism towards *R. solani* (isolates 1, 14, 17, 18, 21, 31, 33, 34, 35, 36, 37, 38, 41, 43, 44, 64, 65, 66, 67, 69, 70, 71, 72, 82, 89, 90, 92, 103, G157, H210, and E681), 26 towards *P. aphanidermatum* (isolates 1, 17, 21, 31, 33, 34, 35, 36, 37, 41, 43, 44, 46, 64, 66, 67, 69, 82, 89, 91, 102, 103, 104, G157, H210, and E681), one towards *F. oxysporum* (isolate 102) and *B. glumae* (isolate 91), 14 towards *Penicillium* sp. (isolates 31, 34, 36, 37, 38, 43, 44, 46, 66, 67, 69, 70, 102, and 104), 3 towards *E. coli* DH10B (isolates 38, 44, and 103), 18 towards *A. tumefaciens* (isolates 1, 17, 18, 38, 65, 66, 67, 69, 70, 71, 72, 82, 90, 92, 104, G157, H210, and E681), 13 towards *P. syringae* pv. *tomato* DC3000 (isolates 1, 38, 65, 70, 71, 89, 90, 91, 92, 104, G157, H210, and E681), 23 towards *E. carotovora* subsp. *carotovora* (isolates 17, 21, 31, 33, 34, 37, 46, 64, 65, 66, 67, 70, 71, 72, 90, 91, 92, 104, G157, H210, and E681), 11 towards *X. campestris* pv. *vesicatoria* (isolates 1, 41, 44, 70, 71, 72, 89, 90, G157, H210, and E681), and 4 towards *B. subtilis* (isolates 36, 37, 102, and 104).

Then, for a diameter ranged from 6 mm to 10 mm, one isolate exhibited antagonism towards *R. solani* (isolate 91) and *X. campestris* pv. *vesicatoria* (isolate 64), 8 towards *P. aphanidermatum* (isolates 18, 38, 65, 70, 71, 72, 90, and 92) and *E. carotovora* subsp. *carotovora* (isolates 14, 35, 36, 41, 43, 82, 89, and 108), 34 towards *F. oxysporum* (isolates 1, 14, 17, 18, 21, 31, 33, 34, 35, 36, 37, 38, 41, 43, 44, 46, 64, 65, 66, 67, 69, 70, 71, 72, 82, 89, 90, 91, 92, 103, 104, G157, H210, and E681), 31 towards *F. solani* (isolates 1, 14, 17, 18, 33, 34, 35, 36, 37, 38, 41, 44, 46, 64, 65, 66, 67, 69, 70, 71, 72, 89, 90, 91, 92, 102, 103, 104, G157, H210, and E681), 21 towards *Penicillium* sp. (isolates 1, 14, 17, 18, 21, 33, 35, 41, 64, 65, 71, 72, 82, 89, 90, 91, 92, 103, G157, H210, and E681), 35 towards *Aspergillus* sp. (isolates 1, 14, 17, 18, 21, 31, 33, 34, 35, 36, 37, 38, 41, 43, 44, 46, 64, 65, 66, 67, 69, 70, 71, 72, 82, 89, 90 , 92, 95, 102, 103, 104, G157, H210, and E681), 26 towards *E. coli* DH10B (isolates 1, 14, 17, 18, 21, 33, 34, 37, 41, 43, 64, 65, 66, 67, 69, 70, 71, 72, 82, 89, 90, 91,104, G157, H210, and E681), 5 towards *A. tumefaciens* (isolates 14, 33, 41, 64, and 89), and 23 towards *B. subtilis* (isolates 1, 14, 17, 31, 34, 35, 38, 41, 43, 44, 46, 65, 66, 67, 70, 71, 72, 82, 89, 90, G157, H210, and E681).

The isolates 9, 10, 12, and 83 did not exhibit any antagonistic activity towards any of the fungi and bacteria tested, whereas the isolate 50 exhibited antagonism only towards *Alternaria* sp. Furthermore, the isolate 14 did not exhibit any activity against *P. aphanidermatum*, *Alternaria* sp., *B. glumae*, *P. syringae* pv. *tomato* DC3000, and *X. campestris* pv. *vesicatoria*, and none of the *Paenibacillus* isolates exhibited any significant antagonism towards *Sclerotium rolfsii*, *Mucor ambigus*, *Rhizopus oryzae*, and *Saccharomyces cerevisiae*.

In previous studies, certain members of the genus *P. polymyxa* and *P. thiaminolyticus* have been reported to exhibit antagonistic activities towards several soilborne fungi [21, 25]. These *Paenibacillus* strains are also known to produce antimicrobial compounds, such as polymyxin, fusaricidin, octopytin, and baciphelacin [21, 25, 36], and Chung *et al.* [10] reported that *Paenibacillus koreensis* sp. nov. produces an iturin-like antifungal compound. In this study, the *Paenibacillus* isolates examined also showed

Table 1. *In vitro* antagonism of selected *Paenibacillus* isolates towards diverse fungal plant pathogens.

Microorganisms	Antifungal activity of *Paenibacillus* isolates[a]																			
	1	9	10	12	14	17	18	21	31	33	34	35	36	37	38	41	43	44	46	50
Rhizoctonia solani	++	–	–	–	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+++	–
Sclerotium rolfsii	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium aphanidermatum	++	–	–	–	-	++	+	++	++	++	++	++	++	++	+	++	++	++	++	–
Mucor ambigus	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Rhizopus oryzae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Alternaria sp.	+++	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Fusarium oxysporum	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
Fusarium solani	+	–	–	–	+	+	+	–	–	+	+	+	+	+	+	+	–	+	+	–
Phytophthora cactorum	+++	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–
Botrytis cinerea	+++	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–
Penicillium sp.	+	–	–	–	+	+	+	+	++	+	++	+	++	++	++	+	++	++	++	–
Aspergillus sp.	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
Sclerotinia sp.	+++	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–

Microorganisms	64	65	66	67	69	70	71	72	82	83	89	90	91	92	95	102	103	104	G157	H210	E681
Rhizoctonia solani	++	++	++	++	++	++	++	++	++	–	++	++	+	++	-	+++	++	+++	++	++	++
Sclerotium rolfsii	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium aphanidermatum	++	+	++	++	++	+	+	+	++	–	++	+	++	+	-	++	++	++	++	++	++
Mucor ambigus	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Rhizopus oryzae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Alternaria sp.	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	+++	+++	+++	+++	–	+++	+++	+++	+++	+++	+++
Fusarium oxysporum	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	++	+	+	+	+	+
Fusarium solani	+	+	+	+	+	+	+	+	-	–	+	+	+	+	–	+	+	+	+	+	+
Phytophthora cactorum	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	+++	+++	+++	+++	–	+++	+++	+++	+++	+++	+++
Botrytis cinerea	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	+++	+++	+++	+++	–	+++	+++	+++	+++	+++	+++
Penicillium sp.	+	+	++	++	++	++	+	+	+	–	+	+	+	+	–	++	+	++	+	+	+
Aspergillus sp.	+	+	+	+	+	+	+	+	+	–	+	+	-	+	+	+	+	+	+	+	+
Sclerotinia sp.	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	+++	+++	+++	+++	–	+++	+++	+++	+++	+++	+++

[a]The antifungal activity of the *Paenibacillus* isolates was expressed based on the diameter of the zone of inhibition.
–: No inhibition; +: 6–10 mm; ++: 11–15 mm; +++: >16 mm.

Table 2. *In vitro* antagonism of selected *Paenibacillus* isolates towards diverse bacterial plant pathogens and non-pathogens.

Microorganisms	Antibacterial activity of *Paenibacillus* isolates[a]																				
	1	9	10	12	14	17	18	21	31	33	34	35	36	37	38	41	43	44	46	50	
E. coli	+	-	-	-	+	+	+	+	+++	+	+	+++	-	+	++	+	+	++	-	-	
Agrobacterium tumefaciens	++	-	-	-	+	++	++	-	-	+	-	-	-	-	++	+	-	+++	-	-	
Burkholderia glumae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	
Pseudomonas syringae pv. *tomato* DC3000	++	-	-	-	-	-	-	-	-	+	-	-	-	-	++	+	-	-	+	-	
Erwinia carotovora subsp. *carotovora*	-	-	-	-	+	++	+++	++	++	++	++	+	+	++	+++	+	+	+++	++	-	
Xanthomonas campestris pv. *vesicatoria*	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	++	-	-	
Bacillus subtilis	+	-	-	-	+	+	-	-	+	-	+	+	++	++	+	+	+	+	+	-	
Saccharomyces cerevisiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Microorganisms	64	65	66	67	69	70	71	72	82	83	89	90	91	92	95	102	103	104	G157	H210	E681
E. coli	+	+	+	+	+	+	+	+	+	-	+	+	+	+++	-	-	++	+	+	+	+
Agrobacterium tumefaciens	+	++	++	++	++	++	++	++	++	-	+	++	-	++	-	-	++	-	++	++	++
Burkholderia glumae	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	+	-	-	-	-
Pseudomonas syringae pv. *tomato* DC3000	+	++	-	+	-	++	++	-	-	-	++	++	++	++	-	-	+++	++	++	++	++
Erwinia carotovora subsp. *carotovora*	++	++	++	++	+++	++	++	++	+	-	+	++	++	++	-	-	+	++	++	++	++
Xanthomonas campestris pv. *vesicatoria*	+	+++	-	-	+++	++	++	++	-	-	++	++	-	-	-	-	+++	-	++	++	++
Bacillus subtilis	-	+	+	+	-	+	+	+	+	-	+	+	-	-	-	++	-	++	+	+	+
Saccharomyces cerevisiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]The antibacterial activity of *Paenibacillus* isolates was expressed based on the diameter of the zone of inhibition.
-: No inhibition; +: 6–10 mm; ++: 11–15 mm; +++: >16 mm.

antagonism towards diverse plant pathogenic fungi and bacteria (Table 1 and Table 2), However, the *Paenibacillus* isolates were also highly resistant to kanamycin, rifampicin, and streptomycin (data not shown), which is important in order to colonize and populate the rhizosphere. Huddleston *et al.* [13] reported that streptomycin-resistant isolates are effective competitors in soil with a large number of streptomycin-producing *Streptomyces* spp. The endospore-forming capability of *Paenibacillus* species may also be a key mechanism to resist and survive in the diverse conditions usually encountered in soil [14].

Plant Growth-Promoting and Root-Colonizing Abilities of *Paenibacillus* Isolates

The plant growth-promoting ability of the *Paenibacillus* isolates was determined using a known PGPR strain, *P. polymyxa* E681 [30], as a reference (Fig. 3). Twenty-four isolates (isolates 1, 10, 12, 14, 17, 18, 33, 36, 37, 41, 44, 46, 50, 64, 65, 66, 69, 71, 82, 83, 92, 103, G157, and E681) had a growth-promoting effect on the cucumber seedlings, and 7 isolates (isolates 9, 34, 38, 67, 72, 90, and H210) had no effect.

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring root-colonizing bacteria [31]. Such bacteria have recently been applied to increase plant growth and control plant diseases [31]. Previously, *Paenibacillus* strains have been reported to promote the growth of diverse crops [30]. The root-colonizing ability of PGPR is an important trait that has a consistent effect on growth promotion and biological control, when PGPR are applied to fields or greenhouses [4, 30]. In the present study, the 41 isolates exhibited a colonizing ability on cucumber seedling roots when the DLF method was used (data not shown). The bacterial populations of the 41 isolates retrieved from the tips of cucumber roots were similar to that of *P. polymyxa* E681 used as the reference strain [30].

P. polymyxa and various other *Paenibacillus* spp. have been shown to produce chitinase, amylase, and protease [3, 21]. These enzymes are capable of digesting insoluble nutrient materials, such as chitin, starch, and protein, and the digestion products are then transported into the plant cells, where they are used as nutrients for plant growth [3, 21]. In this study, 41 strains were tested for their secretion of extracellular enzymes, such as amylase, cellulase, and protease, and the result showed that 37 isolates exhibited high amylase activity, 36 hydrolyzed carboxymethyl cellulose very effectively, and 34 exhibited strong protease activity, indicating that the synthesis of extracellular hydrolytic enzymes would seem to be very common among *Paenibacillus* spp. isolated from plant roots, and that these enzymes are apparently necessary for adaptation to a rhizosphere soil environment.

Detection of the *nifH* Gene in Genomic DNA of *Paenibacillus* Isolates

To detect the *nifH* gene in the 41 *Paenibacillus* isolates, the method developed by Ueda *et al.* [37] was used. The isolates were tested for the presence of sequences

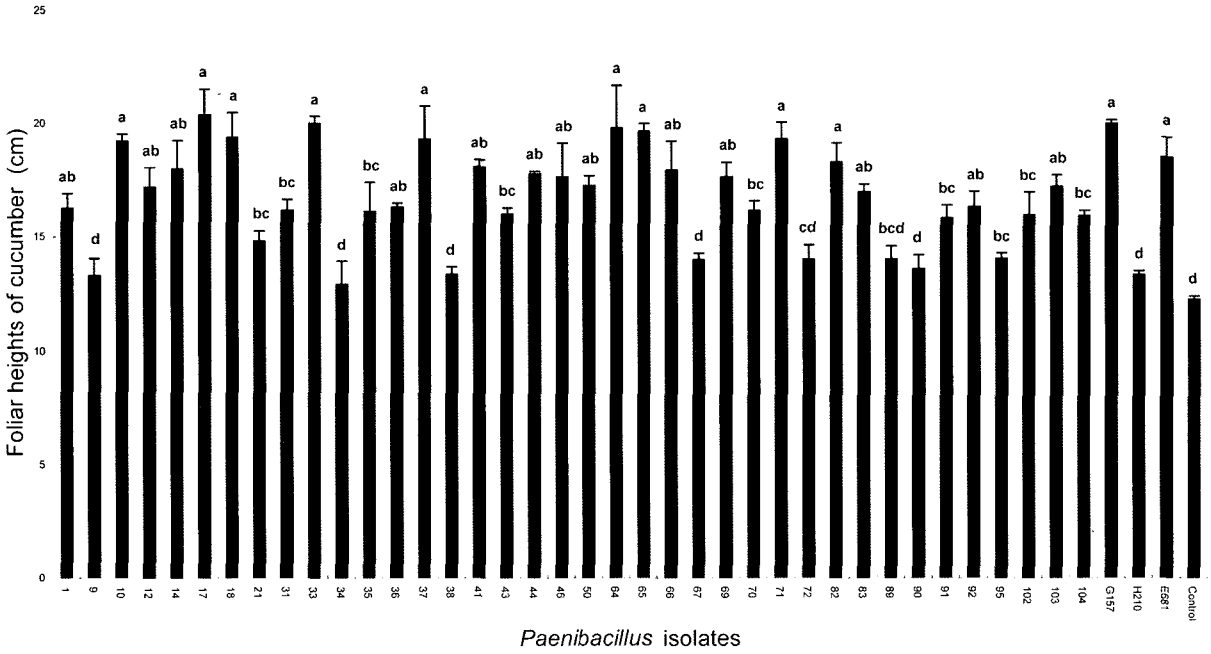

Fig. 3. Effect of *Paenibacillus* isolates on growth promotion of cucumber seedlings.
The cucumber seeds were inoculated with bacterial suspensions (10^7 cfu/ml) of 41 *Paenibacillus* isolates, and the plant heights were measured 35 days after sowing. Means with the same letter are not significantly different at P=0.05 according to Fisher's protected least significant difference.

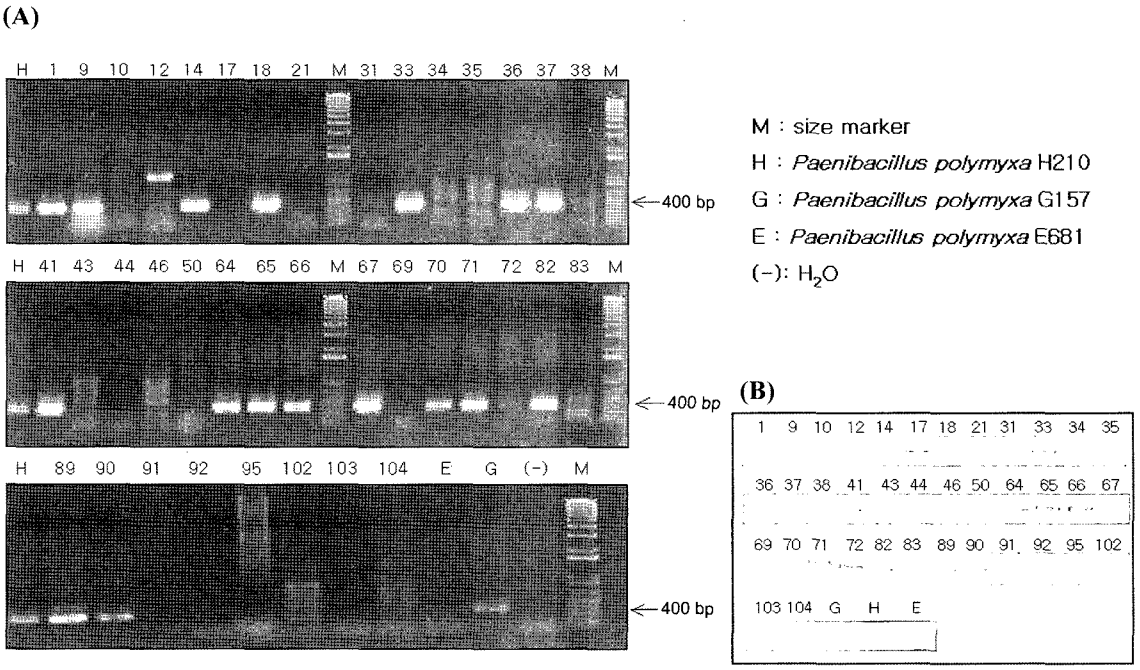

Fig. 4. Detection of the *nifH* gene in *Paenibacillus* isolates using 19F and 407R primers (A) and results of Southern hybridization of the *nifH* gene (B).

homologous to the *P. azotofixans nifH* gene using PCR and Southern hybridization. From the PCR amplification using specific *nifH* primers and Southern hybridization, 21 isolates (isolates 1, 9, 14, 17, 18, 33, 36, 37, 41, 64, 65, 66, 67, 70, 71, 72, 82, 89, 90, G157, and H210) were shown to contain the *nifH* gene, whereas the other 20 isolates (isolates 10, 12, 21, 31, 34, 35, 38, 43, 44, 46, 50, 69, 83, 91, 92, 95, 102, 103, 104, and E681) did not (Fig. 4); that is, 49% of the *Paenibacillus* isolates did not contain the *nifH* gene.

It has been reported that the N_2-fixation of *P. polymyxa* and *P. azotofixans* plays an important role in plant-growth promotion [34]. However, in the present study, the *nifH* gene was detected in only 14 isolates (isolates 1, 14, 17, 18, 33, 36, 37, 41, 64, 65, 66, 71, 82, and G157) out of the 24 isolates that exhibited a PGP effect in the cucumber seedlings. Conversely, 5 isolates (isolates 9, 67, 72, 90, and H210) out of 7 non-PGPR strains were found to contain the *nifH* gene.

Grouping of *Paenibacillus* Isolates Based on BOX-PCR DNA Fingerprint

The genetic diversity of the *Paenibacillus* isolates was investigated using a BOX-PCR [34, 40]. To generate reproducible patterns, the method described by Versalovic *et al.* [38] was applied to prepare the primer (BOXA1R) and for the PCR amplification. A high level of genetic diversity was found among the 41 *Paenibacillus* isolates, allowing them to be divided into 3 groups (Fig. 5). The size of the amplified DNA fragments for the *Paenibacillus* isolates ranged from 200 bp to 2 kb, and the differences between the isolates were assessed visually based on the band migration patterns of the PCR products. The *Paenibacillus* isolates belonging to Group A (isolates 1, 17, 18, 33, 64, 65, 66, 67, 70, 71, 72, 90, 82, 89, G157, and H210) revealed a common band pattern, including 310 bp, 410 bp, 700 bp, and 1,800 bp, whereas the *Paenibacillus* isolates belonging to Group B (isolates 34, 44, 102, 104, 36, 37, 38, 69, 92, 43, and 46) had a common band of approximately 600 bp. The other *Paenibacillus* isolates exhibiting various band patterns (isolates 9, 12, 14, 21, 31, 35, 41, 50, 83, 91, 95, 103, 10, and E681) were assigned to Group C.

Interestingly, all 11 isolates in Group B (isolates 34, 44, 102, 104, 36, 37, 38, 92, 43, 46, and 69) were isolated from gramineous plants (10 isolates from barley and one isolate from wheat), whereas the isolates in Groups A and C were from diverse crops. However, no relationship was found between the location of the crop sampling and the groups of *Paenibacillus* isolates.

Among the 23 isolates identified as *P. polymyxa* strains, based on their 16S rDNA sequences, 16 isolates (isolates 1, 17, 18, 33, 64, 65, 66, 67, 70, 71, 72, 82, 89, 90, G157, and H210) were assigned to Group A, and 10 of them (isolates 1, 17, 18, 33, 64, 65, 66, 71, 82, and G157) exhibited a plant growth-promoting effect in the cucumber seedlings. Meanwhile, the isolate 104, the only *P. polymyxa* strain assigned to Group B, showed no growth-promoting

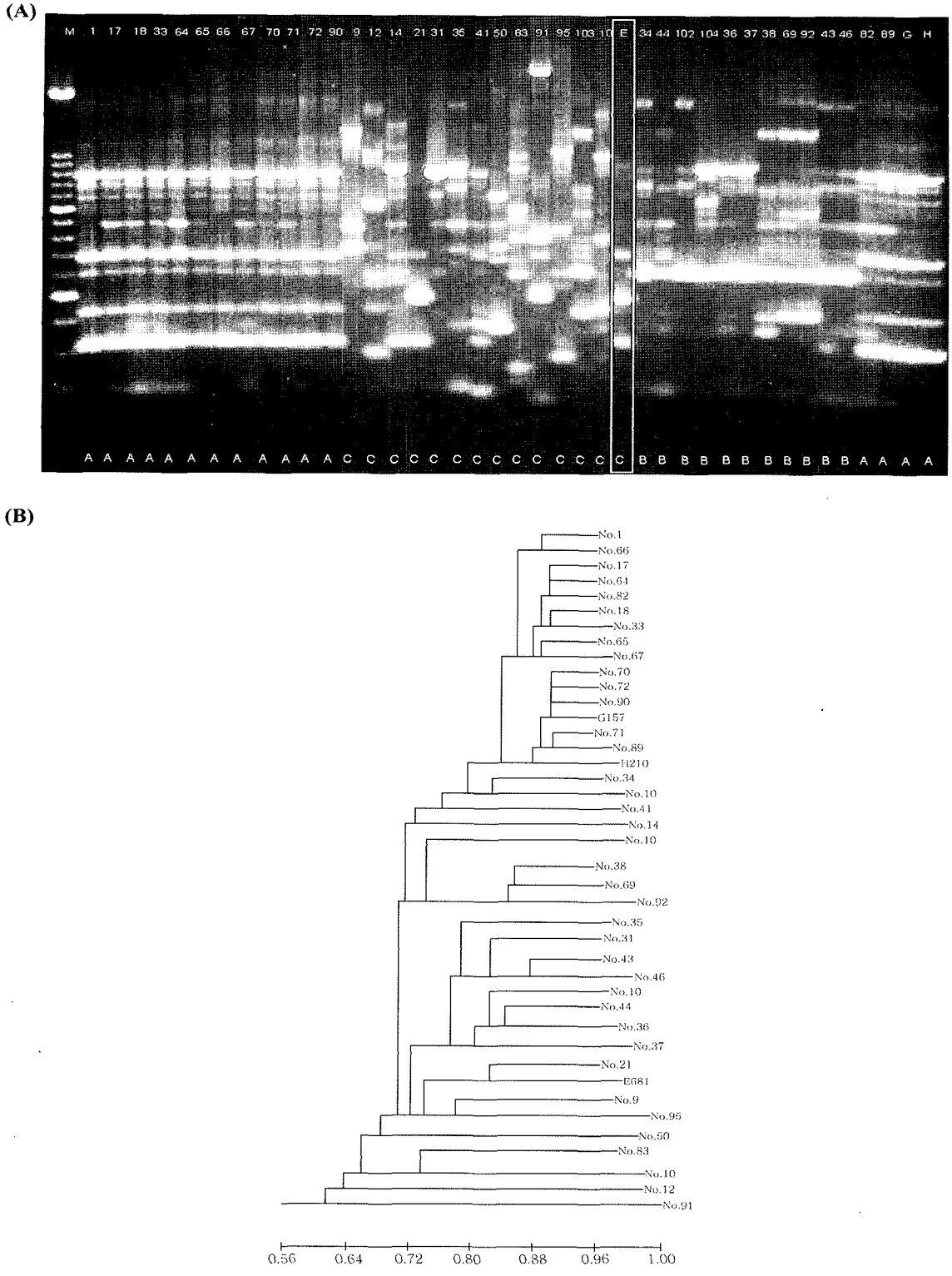

Fig. 5. (A) BOX-PCR fingerprint patterns of 41 *Paenibacillus* isolates collected from the rhizosphere of winter crops.
The chromosomal DNA of the isolates was amplified with the BOXA1R primer [38]. The isolates were divided into 3 groups; one (letter A) group (1, 17, 18, 33, 64, 65, 66, 67, 70, 71, 72, 90, 82, 89, G, and H) showed a common band pattern, including ca. 310 bp, 410 bp, 700 bp, and 1,800 bp, whereas another (letter B) group (34, 44, 102, 104, 36, 37, 38, 69, 92, 43, and 46) showed a common band of approximately 600 bp. However, the third (letter C) group (9, 12, 14, 21, 31, 35, 41, 50, 83, 91, 95, 103, 10, and E) did not show any common band pattern in the agarose gel. (B) Dendrograms (UPGMA) based on data from PCR amplification using the BOXA1R primer, showing similarities among 41 *Paenibacillus* isolates collected from the rhizosphere of winter crops.

effect. Among 6 *P. polymyxa* strains assigned to group C (isolates 14, 21, 41, 91, 95, and E681), 3 isolates (isolates 14, 41, and E681) increased the growth of the cucumber seedlings. Therefore, these results indicate the absence of any close relationship between the BOX-PCR patterns of the *P. polymyxa* isolates and the ability to promote plant growth.

Seldin *et al.* [34] earlier demonstrated a homogeneity among *P. azotofixans* strains isolated from maize grown in Cerrado soil. However, in the present study, the 23 *P. polymyxa* strains among the total 41 isolates were shown to be heterogeneous.

In conclusion, 41 spore-forming bacilli strains isolated from winter crops grown in the southern part of Korea were identified as *Paenibacillus* spp. by 16S rDNA sequencing, and 23 (56%) were classified as *P. polymyxa*, a type species for the genus, implying that *P. polymyxa* is the dominant species among root-associated *Paenibacillus* spp. in winter crops. The majority of the *Paenibacillus* isolates also exhibited antagonistic activities towards various plant pathogens, suggesting that *Paenibacillus* spp. may be effective as biological control agents. However, the present study failed to reveal any significant relationship between root colonization ability, the presence of the *nifH* gene, and plant growth-promoting effect. Nonetheless, the present results provide a better understanding of the nature of *Paenibacillus* isolates from the rhizosphere, and provide resources for identifying novel genes related to plant growth promotion and inhibition of plant pathogens.

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