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Identification and Characterization of Novel Biocontrol Bacterial Strains

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Because bacterial isolates from only a few genera have been developed commercially as biopesticides, discovery and characterization of novel bacterial strains will be a key to market expansion. Our previous screen using plant bioassays identified 24 novel biocontrol isolates representing 12 different genera. In this study, we characterized the 3 isolates showing the best biocontrol activities. The isolates were *Pantoea dispersa* WCU35, *Proteus myxofaciens* WCU244, and *Exiguobacterium* acetylicum WCU292 based on 16S rRNA sequence analysis. The isolates showed differential production of extracellular enzymes, antimicrobial activity against various fungal or bacterial plant pathogens, and induced systemic resistance activity against tomato gray mold disease caused by *Botrytis cinerea*. *E. acetylicum* WCU292 lacked strong *in vitro* antimicrobial activity against plant pathogens, but induced systemic resistance against tomato gray mold disease. These results confirm that the trait of biological control is found in a wide variety of bacterial genera.

Keywords : Biocontrol, Gray mold, Induced systemic resistance

Introduction

Plant diseases are major threat for healthy crop production and many diseases, and currently are controlled by chemical pesticides. Eco-friendly biopesticides are under development for the control of plant pathogenic fungi, bacteria, and viruses (Fravel, 2005). Current biopesticides include isolates of *Bacillus, Pseudomonas, Agrobacterium,* and *Streptomyces* selected initially because of their *in vitro* antimicrobial activity (Burgues, 1998; Fravel, 2005). One of the most commonly used biopesticides, the antibiotic-producing rhizobacterium *Bacillus subtilis* (Stein, 2005) offer advantages of ease of formulation and long-term survival characteristics (Emmert and Handelsman, 1999). *Pseudomonas* species also possess many traits that make them well suited as biocontrol against soilborne pathogens and growth-promoting agents (Hass and Keel, 2003; Kim *et al.,* 2011; Weller, 2007).

Biological control is control of disease utilizing another microorganism. Active control can be exerted through two mechanisms, (1) a direct inhibition of the pathogen's development and growth and (2) an indirect system where activation of the plant's defense mechanisms is involved. One direct mechanism for control of pathogen growth involves antimicrobials produced by the biocontrol agent. Many soil microbes produce such antibiotics by highly regulated processes (Haas and Keel, 2003; Kim *et al.,* 2011). Induced resistance has been documented for many nonpathogenic colonists (van Loon *et al.,* 1998).

For both types of protection, direct growth antagonism and induced plant defenses, the biological control agent must be in the right place and the right time during the infection process to be effective. With direct control the antimicrobial concentration and location are crucial. For induced resistance the plant must be activated before high pathogen pressure occurs raising the question of how to apply the microbe, how to maintain its population and stimulate the production of the microbial factors that activate plant defenses. The colonization potential of the microbe is significant in this respect (Lugtenberg *et al.,* 2001).

Relatively few bacterial genera have been systematically evaluated for their biocontrol activities. In a previous report, we screened a diverse collection of bacteria with the aim of identifying and developing novel biocontrol bacteria strains (Park *et al.*, 2013). In this study, we selected the most active strains for further genotypic and phenotypic characterization based on the level of activity and their novelty.

Materials and Method

Media and growth conditions. Bacterial isolates were cultivated at 25°C on solid and liquid media, King's Media B medium (KB, King *et al*., 1954), tryptic soy medium (TSB, Difco Inc., Detroit, MI, USA), and Luria-Bertani medium (LB, Difco). Plant pathogenic fungal strains were grown on potato dextrose agar medium at 25°C. Bacteria were stored in 20% glycerol and fungal spores in 10% dimethyl sulfoxide (DMSO) (Sigma Life Science, St. Louis, MO, USA) at –80°C.

Identification and phylogenic analysis. Total genomic DNAs from the selected bacteria were isolated by the CTAB-method (Ausubel *et al.,* 1989). The 16S rRNA full sequences of the selected isolates were amplified using polymerase chain reaction (PCR) using the primers: 5'-AGA GTT TGA TCC TGG CTC AG-3' (8F) and 5'-ACTCCAGTTACTGCACC ATT-3' (1492R) (Benitez and McSpadden Gardener, 2009) using a premix (Bioneer Inc., Daejeon, Korea) and a gradient thermo cycler (Bioneer). The PCR reaction was performed with 7 min predenaturation at 95°C, 35 cycles of 30 sec of denaturation at 95°C, 30 sec of annealing at 60°C, and 2 min of extension at 72°C, and a final extension for 7 min at 72°C The PCR products were purified using a purification kit (Bioneer) and cloned into a pGEM T-easy vector (Promega, Madison, WI). Nucleotide sequences of the PCR products were generated by Solgent Inc., (Daejeon, Korea) and analyzed using BLAST. Full length 16S rRNA sequences of type strains with highest homologous species were obtained from public GenBank (http://www.ncbi.nlm.nih.gov/). The multiple alignment of all 16S rRNA sequences, edited using BioEdit version 7.0 (Hall, 1977), were conducted using MEGA version 6 (Tamura *et al.,* 2013) based on the neighbor-joining method with the Kimura two parameter model (Kimura, 1980) with bootstrap values based on 1,000 replication.

Determination of production of extracellular enzymes and biosurfactant. Production of extracellular enzymes by the selected bacterial isolates was determined by formation of clear area around the colony on following agar media; extracellular protease on LB agar containing 0.7% skim milk, gelatinase on 1/2 LB agar with 4% gelatin, chitinase on 1/2 LB agar with 1% colloidal chitin, siderophore on chrome azurol S (CAS) agar (Alexander *et al.,* 1991). Production of amylase and lipase was determined by methods described elsewhere (Kaur *et al.,* 2008).

For these tests, the bacterial strains were streaked on TSA agar, incubated 2 days, and cells were re-suspended in sterile water and adjusted OD600nm = 0.1. Inoculum (10 μl) of each bacterial strain was transferred to the plates. After incubation at 28°C for 3 days, plates were examined for clear zones around the colonies.

Bio-surfactant activity measured in cell-free cultures by the drop-collapse method using 10W-40 Penzoil (Penzoil Co., Houston, TX, USA) as described by Burch *et al*. (2010). Three days old cultures grown in TSB were centrifuged, and filtered through a 0.2 μm Millipore filter (Billerica, MA, USA) to prepare the cell filtrate for assay.

Determination of antibacterial and antifungal activity. The bacterial strains were streaked on TSA agar plates, incubated 2 days, and suspended with sterile water and adjusted OD600nm = 0.1 to generate the suspension used in the bioassays.

Antagonism of four plant bacterial pathogens was assayed: *Pseudomonas syringae* pv. *tabaci*, *Pseudomonas syringae* pv. *tomato* DC3000, *Pectobacterium carotovorum* subsp. *carotovora* stain SCCI (Park *et al*., 2008), and *Xanthomonas campestris* pv. *vesicatoria* causing bacterial leaf spot disease on red pepper (a gift from Dr. Jae Soon Cha at ChungBuk National Univerity). *Escherichia coli* DH5α was used as a negative control. LB agar plates were spread with 100 μl of each pathogenic bacterial strain before adding a sterilized paper disc (8 mm diameter, Whatman 3 mm filter paper) at the center of the petri dish. The selected biocontrol bacterial strains were transferred to the discs. Two days after incubation at 28°C, inhibition zone of bacterial strain was measured.

To examine antifungal activity tests against *Colletotrichum coccodes*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Phytophthora capsici* obtained from Korean Agriculture Culture Collection (KACC, RDA, Suwon, Korea) were used. PDA agar plates were inoculated with plugs of 1 cm diameter. The bacterial suspension (10 μl) was inoculated 2 cm from the fungal plugs. This inoculum was omitted for the control plates. Inhibition of fungal growth was measured at 3 to 7 days after dual incubation at 25°C. Each experiment was repeated three times with three plates for each experiment.

Induced systemic resistance bioassay (ISR). Pepper (*Capsicum annuum*, Nogkwang variety, Hungnong Seed Com., South Korea) seeds were sown in a tray contained autoclaved nursery soil (Bio-Bed Soil, Hungnong Com.) and grown in a greenhouse maintained at 25°C. Two week-old seedlings were transplanted to pots (9 cm × 8 cm) containing sterile nursery soil and vermiculite (5:1, v:v). Suspensions of the bacterial strains prepared as above, 5 ml, were used to drench plants at the 3- to 4-leaf stage.

The fungal pathogen inoculum of *B. cinerea* KACC40573 was generated and applied 5 days after root drenching by foliar application at 1×10^5 spore/ml as described by Oh *et al.* (2013). Plants were placed in growth chamber (dark, 25°C, RH 100%) for 24 h before being returned to the greenhouse. Disease index for the upper four leaves excluding newly emerged leaves was rated and expressed on a 0–5 scale based on the percentage of infected area 5 days after inoculation with the pathogen. 0: no symptom 1: 1–20% diseased area, 2: 21–40%, 3: 41–60%, 4: 61–80%, 5: 81–100%. Statistical analysis of bioassay data (n = 3 for all tests) was performed to analysis of variance (ANOVA) using

IBM SPSS Statistics version 19 (IBM Corp., Somers, New York, USA).

Result and Discussion

Identification of the selected novel bacterial strains. Previously, a collection of approximately 3,000 strains were collected using multi-factor selection strategies from plant's phyllosphere (WCU244 and WCU292) or rhizosphere (WCU35) (Table 1, Park *et al.,* 2013). Twelve different bacterial genera were represented, some of which have been only rarely noted for their biocontrol activities such as *Lactococcus*, *Exiguobacterium*, *Novosphingobium*, *Roseateles* and *Proteus* species (Park *et al.,* 2013). Three of these strains were selected for further characterization in this paper: WCU35, WCU244 and WCU292.

Sequence analysis of the 16S rRNA genes showed WCU35 to have high sequence similarity with a type strain of *Pantoea dispersa* LMG2603 (Fig. 1), WCU244 with a type strain of *Proteus myxofaciens* NCIMB 13273 (Fig. 2), and WCU292 with a type strain of *Exiguobacterium acetylicum* DSM 20416 (Fig. 3).

Previously some *Pantoea* sp. were reported as biocontrol agents. Formulation of *P. agglomerans* strain CPA-2 has been studied for its efficacy (Costa *et al.,* 2001). Two strains that aggressively colonize fruit wounds offer potential in postharvest diseases of fruits (Bonaterra *et al.,* 2003). *Pantoea dispersa* strain SB1403 protects against leaf scald disease by *Xanthomonas albilineans* by inhibition of the albicidin toxin (Zhang and Birch, 1996).

Table 1. Source of the selected strains from primary screening (Park *et al.,* 2013)

Strain	Collection	Source	Media	Genus
WCU35	Rhizosphere	*Oryza sativa* L. cv. Dongjin	King's B agar	*Pantoea*
WCU244	Phyllosophere	*Geranium* sp.	Tryptic soy agar	*Proteus*
WCU292	Phyllosophere	*Trichosanthes kirilowii*	Tryptic soy agar	*Exiguobacterium*

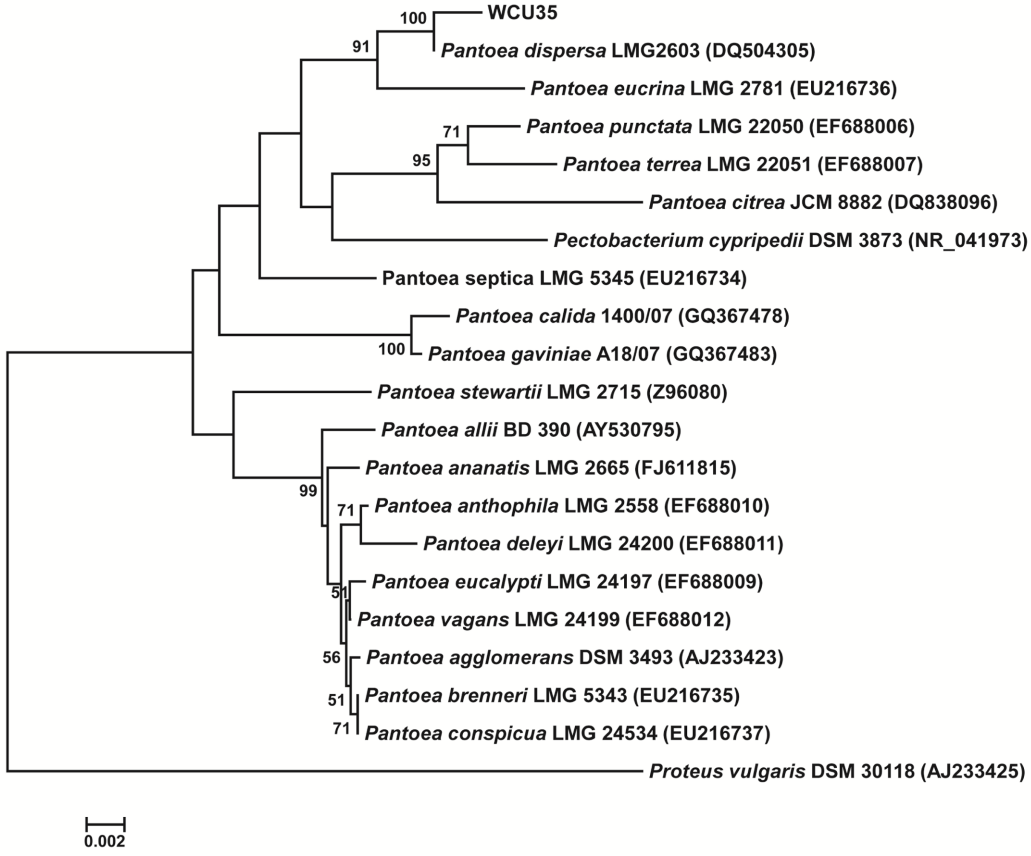

Fig. 1. Phylogenetic tree based on 16S rRNA sequences showing relationships among WCU35 and the *Pantoea* type strains by the neighbor-joining method and Kimura's two-parameter model. The numbers indicated at several nodes are consensus bootstrap values out of 1,000 replications. Scale bar 0.002 substitutions per nucleotide position. *Proteus vulgaris* DSM 30118 was used as the out-group. Accession numbers are given to end of the name.

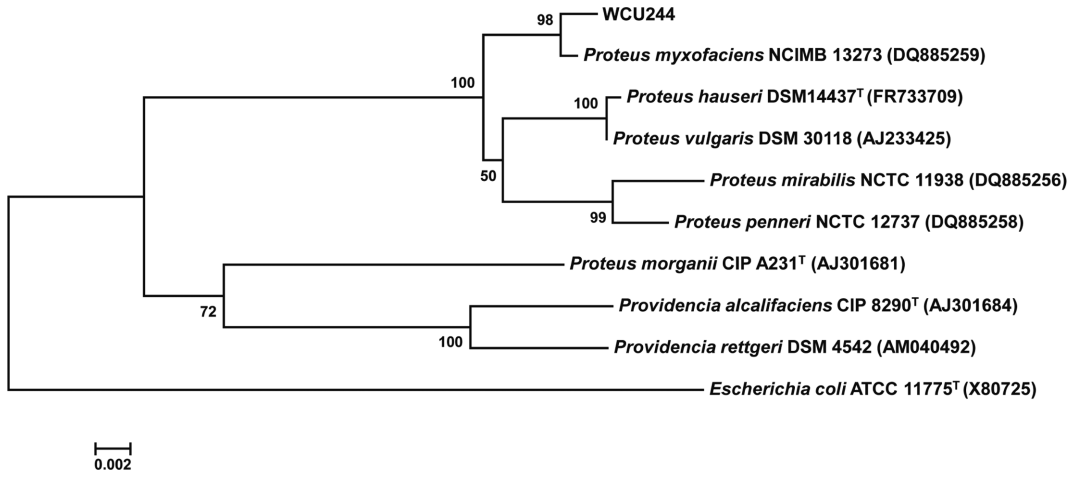

Fig. 2. Phylogenetic tree based on 16S rRNA sequences showing relationships among WCU244 and the *Proteus* type strains by the neighbor-joining method and Kimura's two-parameter model. The numbers indicated at several nodes are consensus bootstrap values out of 1,000 replications. Scale bar 0.002 substitutions per nucleotide position. *Escherichia coli* ATCC 11775^{T} was used as the out-group. Accession numbers are given to end of the name.

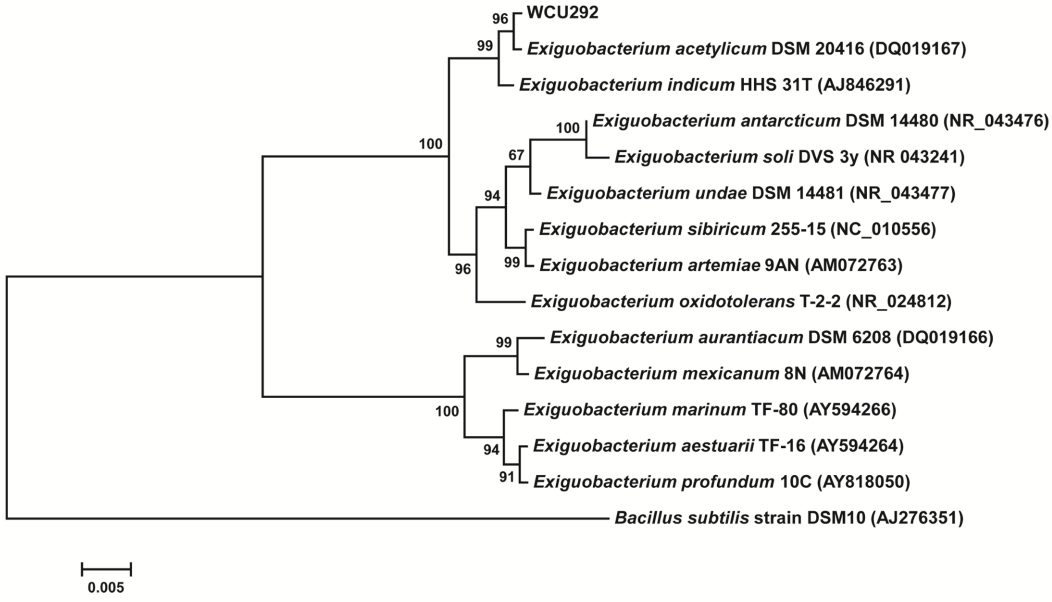

Fig. 3. Phylogenetic tree based on 16S rRNA sequences showing relationships among WCU292 and the *Exiguobacterium* type strains by the neighbor-joining method and Kimura's two-parameter model. The numbers indicated at several nodes are consensus bootstrap values out of 1,000 replications. Scale bar 0.005 substitutions per nucleotide position. *Bacillus subtillis* strain DSM10 was used as the out-group. Accession numbers are given to end of the name.

Proteus isolates have not been extensively studied as genus with biocontrol agents. *Proteus vulgaris* isolates have pathogenicity in humans and are associated with the gut. Therefore, WCU244 must be checked for human or animal pathogenicity (O'Hara *et al.,* 2000).

Some *Exiguobacterim* isolates were reported as biocontrol agents. *Exiguobacterium acetylicum* strain 1P (MTCC 8707) is a novel bacterial antagonist inhibiting growth and development of *Rhizoctonia solani*, *Sclerotium rolfsii*, *Pythium*, and *Fusarium oxysporum* (Selvakumar *et al.,* 2009). In addition, *Exiguobacterium* NII-0906 exhibited antifungal activity against phytopathogenic fungi *in vitro* assays and produced siderophore and hydrogen cyanide (Dastager *et al.,* 2010).

Expression of biocontrol traits of the selected bacterial isolates. Bacterial biocontrol agents typically produce a variety of metabolites that contribute to their biopesticidal activities (Kim *et al.,* 2011). Table 2 shows the *in vitro* production of secreted

Table 2. Production of biocontrol related enzymes and bio-surfactant in the selected bacterial strains[a]

Strain	Biocontrol related traits						
	Protease	Amylase	Lipase	Gelatinase	Chitinase	Biosurfactant[b]	Siderophore
Pantoea dispersa WCU35	–	–	–	–	–	–	+
Proteus myxofaciens WCU244	+	–	–	+	–	–	+
Exiguobacterium acetylicum WCU292	+	+	–	+	–	–	–

[a]Bacterial suspension (10 μl, OD600nm = 0.1) was spotted on to each tested medium, and halo or clear area was checked on each plate after 3– 7 days. +: halo or clear zone formation, –: no clear zone or halo formation.
[b]Cell free culture filtrate of each bacterial strain was tested using drop collapse assay. No water drop collapse was observed in the tested bacterial strains.

Table 3. *In vitro* antifungal activity of the selected bacterial isolates against plant pathogenic fungi[a]

Strain	Fungal pathogens[b]		
	Rhizoctonia solani	*Colletotrichum coccodes*	*Phytophthora capsici*
Pantoea dispersa WCU35	+	+	+
Proteus myxofaciens WCU244	–	–	–
Exiguobacterium acetylicum WCU292	–	–	–

[a]Inhibition of fungal growth were observed seven days after inoculation with the suspension of the selected bacterial isolates and fungal agar plugs on potato dextrose agar plates.
[b]–; no inhibition; +, <5 mm inhibition zone.

Table 4. *In vitro* antibacterial activity of the selected bacterial strains against plant pathogenic bacterial pathogens[a]

Strain	*Pseudomonas syringae* pv. *tomato* DC3000	*Pseudomonas syringae* pv. *tabaci*	*Xanthomonas campestris* pv. *vesicatoria*	*Pectobacterium carotovorum* subsp. *carotovora* SCC1
Pantoea dispersa WCU35	+[b]	–	+	–
Proteus myxofaciens WCU244	+	–	–	–
Exiguobacterium acetylicum WCU292	–	–	–	–

[a]Inhibition of bacterial pathogenic bacterial strains were observed seven days after inoculation with the suspension of the selected bacterial isolates and plant pathogenic bacterial suspension.
[b]–; no inhibition; +, <5 mm inhibition zone.

metabolites. *P. dispersa* WCU35 produced no enzymes but *E. acetylicum* WCU292 secreted differential arrays of three potentially biocontrol-related active metabolites. None showed chitinase activity or surfactant production under the conditions of testing.

The selected strains differed in their abilities to antagonize plant pathogenic bacteria and fungi as shown in Table 3. Only *P. dispersa* WCU35 inhibited all fungal pathogens as well as *P. syringae* pv. *tomato* DC3000 and *X. campestris* pv. *vesicatoria*. The selected *P. myxofaciens* WCU244 and *E. acetylicum* WCU292 had no *in vitro* fungal antagonism and only *P. myxofaciens* WCU244 inhibited one bacterial pathogen (Table 3 and Table 4).

Induced systemic resistance bioassay. Induction of systemic resistance was observed with *P. dispersa* WCU35 and *E. acetylicum* WCU292 activity against gray mold, but *P. myxofaciens* WCU244 did not stimulate induced resistance (Fig. 4). Since *E. acetylicum* WCU292 did not show any antifungal

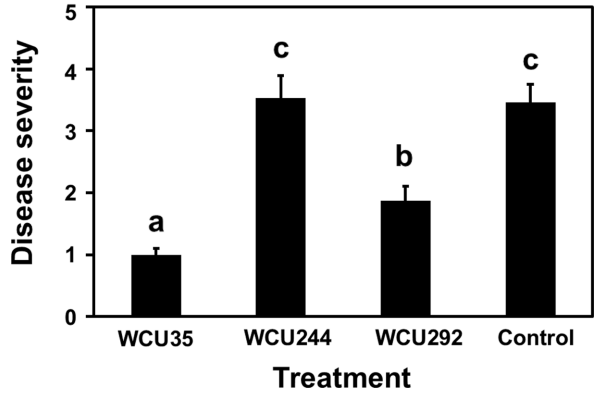

Fig. 4. Induced systemic resistance (ISR) activity of the selected bacterial strains against gray mold disease in red-pepper. Five days after root drench application of the selected bacterial suspension, a spore suspension of *B. cinerea* was sprayed and disease index was scored 5 days of challenge. Sterile water was used as negative control. The different letter indicated the value significantly differ based on Duncan's multiple range test ($p < 0.05$) of three independent experiment with three plants/each treatment.

activities against fungal pathogens, the major biocontrol mechanism for WCU292 could be resulted of activation of induced systemic resistance. Induced systemic resistance has been attributed to rhizosphere colonization with certain non-pathogenic microbes (van Loon *et al.,* 1998).

These analyses indicate that biocontrol activity could be resulted from different mechanisms because of the variability observed in the bioassays for different traits associated with disease control. Identification of the metabolites that directly inhibit pathogen growth or incite induced resistance in the plant is needed and further characterization of the isolates identified in this study will represent novel information. To date, there have been no commercial products developed containing isolates from these genera. Our findings illustrate that seeking novel biocontrol bacteria will be fruitful for extending the possibilities of control diverse plant diseases. The isolated strains from this study may offer new potentials for commercial development.

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