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Research article

Biological characteristics of *Paenibacillus polymyxa* GBR-1 involved in root rot of stored Korean ginseng

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ABSTRACT

Background: This study aims to describe the characterization of *Paenibacillus polymyxa* GBR-1 (GBR-1) with respect to its positive and negative effects on plants.
Methods: The morphological characteristics of GBR-1 were identified with microscopy, and subjected to Biolog analysis for identification. Bacterial population and media optimization were determined by a growth curve. The potential for GBR-1 as a growth promoting agent, to have antagonistic activity, and to have hydrolytic activity at different temperatures was assessed. The coinoculation of GBR-1 with other microorganisms and its pathogenicity on various stored plants, including ginseng, were assessed.
Results: Colony morphology, endospore-bearing cells, and cell division of GBR-1 were identified by microscopy; identification was performed by utilizing the Biolog system, gas chromatography of fatty acid methyl esters (GC-FAME). GBR-1 showed the strongest antagonistic activity against fungal and bacterial pathogens. GBR-1 cell numbers were relatively higher when the cells were cultured in brain heart infusion (BHI) medium when compared with other media. Furthermore, the starch-hydrolytic activity was influenced by GBR-1 at higher temperature compared to low temperatures. GBR-1 was pathogenic to some of the storage plants. Coinoculation of GBR-1 with other pathogens causes differences in rotting on ginseng roots. A significant growth promotion was observed in tobacco seedlings treated with GBR-1 suspensions under *in vitro* conditions, suggesting that its volatile organic compounds (VOCs) might play a role in growth promotion.
Conclusion: The results of this study indicate that GBR-1 has both positive and negative effects on ginseng root and other stored plants as a potential biocontrol agent and eliciting *in vitro* growth promotion.

1. Introduction

Paenibacillus polymyxa, formerly known as *Bacillus polymyxa*, is a plant growth promoting rhizobacterium (PGPR) used for the biocontrol of plant diseases [1–4]. The *Paenibacillus* genus, a Gram-positive, aerobic, rod-shaped, endospore-forming bacterium, is a PGPR [5,6]. A few commercially registered microbial fungicides in Korea mainly contain *Bacillus* spp. [7], possessing antifungal activity to control root rot disease in ginseng and other crops as well [8]. In our previous study [9], several bacterial isolates were obtained from the decaying ginseng roots, among which 20 were identified as *P. polymyxa*. This bacterium is a good biocontrol agent for plant-parasitic nematodes [4], and is also antagonistic to various plant pathogens including *Fusarium oxysporum* and *Phytophthora capsici*. In addition to these advantages, *P. polymyxa* serves as a causal agent for potato tuber rot and tomato seedling blight [10]. Ginseng root rot by this bacterium was dependent on the inoculum density level, causing root rotting at high inoculum density [9,11].

Korean ginseng (*Panax ginseng* Meyer) is one of the perennial herbs well known for its medicinal value in traditional herbal

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preparations for many years [12–14]. The roots were found to have pharmacologically active chemicals. However, long and successive cultivation practices will lead to over exploitation of the soil, which facilitates causing various soil borne diseases to the plants [15,16]. Various fungal and bacterial pathogens are responsible for causing diseases in ginseng; fungi are the major pathogens causing ginseng root diseases, among which is *Cylindrocarpon destructans* (Zins.) which causes root rot [17,18]. In order to develop an ecofriendly technology, use of pesticides is not recommended for soil-borne diseases because they may be toxic to living beings, and might lead to the development of fungicide-resistant pathogens [19]. The use of microorganisms for control of soil-borne diseases is an alternative to the chemical control of plant diseases, with no harmful effects on the environment [20]. in addition to the availability of many chemical pesticides in the market, attention has been diverted to an ecofriendly, nonpollutant, and efficient biological means including *P. polymyxa* GBR-1 (GBR-1) as a PGPR to control various diseases. The purpose of this study was to investigate the involvement of GBR-1 in stored ginseng roots, thereby improving the use of the bacterium as a biocontrol agent. Furthermore, both positive and negative effects on other storage plants, including ginseng, were evaluated.

2. Materials and methods

2.1. Isolation, identification, and culture conditions of GBR-1

The GBR-1 strain isolated from root rot of stored Korean ginseng [9] was cultured for 2 d on brain heart infusion (BHI) agar and suspended in sterilized distilled water (SDW). The pure colony was inoculated in BHI broth and stored at 4°C until further use. This bacterial suspension was diluted with SDW to adjust at different concentrations [colony forming units (CFU)] per mL ($OD_{600} = 0.8$). For microscopic observations, the bacterial colony was picked up with a spatula and placed in SDW on a Formvar-coated copper grid, dried, and stained with 2% uranyl acetate for negative staining. The preparation was examined under a JEM 1010 electron microscope (JEOL Ltd., Tokyo, Japan). The strain was maintained at −80°C in BHI broth with glycerol (20%) for long-term storage. For preparing bacterial suspensions, culture from −80°C was grown on BHI agar plates at 28°C for 24 h, and single colonies were transferred to BHI broth and incubated at 28°C for 24 h under shaking conditions at 250 rpm.

2.2. Biolog analysis and GC-Microbial Identification System

The putative *Paenibacillus* strain was tested for utilization of 95 carbon sources using the Biolog program [9]. Briefly, the bacterial cells cultured on BUG-M-T agar (bacterial universal growth (BUG) agar supplemented with 0.25% maltose and 0.9% thioglycolate) at 28°C for 48 h were suspended in inoculating fluid (0.4% NaCl, 0.03% Pluronic F-68, and 0.01% gellan gum), inoculated onto microplates (Biolog GP MicroPlate), and incubated at 28°C. After 24 h or 48 h of incubation, the plates were read with a MicroLog 3 automated Microstation system (BiOLOG, Hayward, CA, USA). The bacterium was identified based on the MicroLog Gram-positive database (version 4.0; BiOLOG, Hayward, CA, USA). Gas chromatography of fatty acid methyl esters (GC-FAME) was conducted to confirm the bacterial identification. The bacteria were cultured on tryptic soy agar (TSA; Difco, USA) plates at 28°C for 48 h. The colonies were harvested and placed in screwcap culture tubes, and 1 mL of saponification reagent (NaOH aqueous methanol) was added. A methylation reagent (hydrochloric acid in aqueous methanol) was added after heat treatment, and fatty acids were extracted with extraction solvent hexane/methyl-tert-butyl ether (MTBE), mild base (10.8 g NaOH in 900 mL), and a saturated NaOH solution. The fatty acid composition was analyzed by the Sherlock system, followed by the generation of a similarity index for isolates that corresponded to a microorganism in the database (MIDI Library version, TSBA 4.0, Library Generation system software version 4.0, MIDI Inc. Newark, USA).

2.3. Growth curve and optimal media for GBR-1

For the determination of growth of the strain GBR-1 in different growth media, a single colony of GBR-1 was inoculated to a test tube (20 mm diameter) containing 5 mL of growth medium and incubated for 24 h at 28°C under shaking conditions (250 rpm). The broth (5 mL) was transferred to a 500 mL baffled flask containing 100 mL growth medium and incubated at 28°C for 52 h with shaking at 250 rpm. The various tested media were BHI media, potato dextrose broth, nutrient broth, tryptic soy broth, and Luria Bertani broth. Samples were collected at 4 h intervals. Cell growth was examined as a measure of turbidity at 600 nm by a spectrophotometer (Ultraspec 4000 Spectrophotometer; Pharmacia Biotech Ltd, Little Chalfont, UK). For CFUs, the inoculum was serially diluted, and placed onto the various agar media plates. The viable cells were counted 48 h after incubation at 28°C.

2.4. Effect of different temperature conditions on starch hydrolytic activity by GBR-1

Starch hydrolytic activity of GBR-1 was tested using the method followed by Jeon et al [21]. For this, 10 μL of bacterial suspensions (1×10^8 CFU/mL) were spotted on modified starch agar plates (3 g beef extract, 5 g peptone, 2 g soluble starch, and 15 g agar/1 L), and incubated at different temperatures (4°C, 10°C, 15°C, 20°C, 25°C, and 30°C) for 3 d. The agar media were stained with Gram's iodine to examine starch hydrolysis by the formation of clear halos around bacterial colonies due to the enzyme activity. A definite clear zone formed around a bacterial colony was considered to be a positive (+) response to starch hydrolytic activity and no formation of the clear zone was considered as a negative (−) response.

2.5. Pathogenicity of GBR-1 on stored ginseng root at different temperature conditions

To assess the pathogenicity of GBR-1 on ginseng stored roots at various incubation temperatures, fresh 4-yr-old ginseng roots were purchased from a commercial market. A bacterial culture grown for 48 h on potato dextrose agar (PDA) medium supplemented with 0.5% peptone was diluted in SDW to form $\sim 5 \times 10^8$ CFU/mL. The cut surfaces of root discs of about 0.5 cm thick were inoculated at the center of the disc with 20 μL of the bacterial suspensions. Inoculated root discs were placed in Petri dishes with sufficient moisture, and incubated at 4°C, 10°C, 15°C, 20°C, 25°C, and 30°C. Nine discs were used per treatment, and the experiments were repeated at least once. SDW treatment was used as the control. Roots were examined daily for the development of root rot symptoms. This was measured as a decayed diameter zone: − no decayed zone, + weak (0–5 mm), ++ moderate (5–15 mm), and +++ strong (> 15 mm).

2.6. Pathogenicity of GBR-1 on other storage plants

Rot symptom development was examined on other postharvest plants and mushrooms which are listed in Table 3. Fresh plant materials and mushrooms were purchased from a commercial market. In the case of storage roots and tubers, the discs (5 mm in thickness) were made and inoculated with the bacterial suspension (20 μL, 5×10^8 CFU/mL) of GBR-1 on the center. Bulbs were

Table 1
Metabolic activities of *Paenibacillus polymyxa* GBR-1 at 48 h and 72 h in the Biolog GP microplate assay

Substrates	Incubation time		Substrates	Incubation time	
	48 h	72 h		48 h	72 h
Tween 80	+	+	Salicin	−	−
Adonitol	−	−	Stachyose	−	+
Amygdalin	−	−	Sucrose	+	+
L−Arabinose	+	+	D−Trehalose	−	−
D−Arabitol	−	−	Turanose	w	+
Arbutin	+	+	Xylitol	−	−
D−Cellobiose	−	−	D−Xylitol	+	+
Dextrin	+	+	Fumaric acid	−	−
D−Fucose	+	+	L−Proline	−	−
L−Fucose	−	−	Adenosine	−	−
D−Galactose	w	+	α−Keto−glutaric acid	+	+
D−Galacturonic acid	−	+	β−Methyl−D−glucoside	w	+
Gentiobiose	−	+	Palatinose	−	+
α−D−Glucose	+	+	D−Psicose	−	−
D−Melibiose	−	−	D−Raffinose	+	+
Glycogen	+	+	Succinic acid	−	−
L−Serine	−	−	Alaninamide	+	+
α−D−Lactose	−	−	L−Alanine	+	+
Lactulose	−	+	L−Alanyl−glycine	−	−
Maltitol	−	−	L−Asparagine	w	+
Maltose	+	+	L−Aspartic acid	−	−
Maltotriose	+	+	L−Glutamic acid	−	−
D−Mannose	+	+	L−Ornithine	−	−
D−Melezitose	+	+	L−Phenylalanine	−	−

+, positive; −, negative; w, weak reaction

inoculated on the flesh scale leaf tissues with wounding. In the case of mushrooms, the bacterial suspension was inoculated on the caps after wounding with a needle. Rot and lesion development was examined 3 d after inoculation, and the disease severity was scored as ++: severe brown rot, +: mild brown rot, ±: yellowish discoloration, and −: no discoloration.

2.7. In vitro antagonistic activity of GBR-1

For the *in vitro* antibiosis assay, PDA and nutrient agar (NA) plates were used to test for antibiosis against fungal and bacterial pathogens, respectively. The tested plant pathogenic fungi and bacteria are listed in Table 4. For the antifungal assay, the fungal mycelia plugs (5 mm) from the edge of a precultured colony, were taken using a cork borer and placed onto the center of each PDA plate. One wk after incubation, inhibition of the fungal mycelial growth by bacterial colonies was measured to determine the antifungal activity of the GBR-1. The other *P. polymyxa* strains (M27 and M3109) which possess antagonism were also used in comparison with our target strain GBR-1. For the antibacterial assay, the plant pathogenic bacteria cultured in nutrient broth were swabbed onto the NA plates. The bacterial suspensions (10 μL) of GBR-1 (1×10^8 CFU/mL) cultured from BHI broth were loaded on the sterile paper disk (8 mm diameter, Advantec Co. Tokyo, Japan), and placed onto the middle of PDA or NA plates. The inhibition zone was observed in diameter 7 d after incubation at 25°C for the antifungal assay, and 3 d after incubation at 28°C for the antibacterial assay. There were three replications per treatment, and the assay was repeated at least once. Antimicrobial activity was defined in terms of size of the inhibition zone and denoted as follows: − no inhibition zone, + weak (0−5 mm), ++ moderate (5−15 mm), and +++ strong (> 15 mm).

Table 2
Cellular fatty acid profiles of *Paenibacillus polymyxa* GBR-1

Fatty acid shorthand name	Percent of fatty acid (%)[1)]
14:0 ISO	1.89
14:00	1.96
15:0 ISO	6.01
15:0 ANTEISO	62.15
15:0	0.79
16:0 ISO	9.17
16:1 w11c	1.07
16:00	7.54
17:0 ISO	2.57
17:0 ANTEISO	6.85

[1)] Based on the results of GC of fatty acid methyl esters analysis, GBR-1 had 10 fatty acids

Table 3
Pathogenicity of the GBR-1 isolate on storage plants

Storage plant and mushroom	Organ	Disease severity[1)]
Panax ginseng	Storage root	++
Capsicum annuum	Fruit	++
Platycodon grandiflorum	Storage root	++
Codonopsis lanceolata	Storage root	++
Ligusticum acutilobum	Storage root	++
Ipomoea batatas	Storage root	++
Solanum tuberosum	Tuber	++
Daucus carota var. *sativa*	Storage root	++
Cucurbita pepo	Fruit	+
Chamaecereus silvestrii	Storage root	++
Allium sativum	Bulb	−
Allium sepa	Bulb	−
Flammulina velutipes	Cap	+
Agaricus bisporus	Cap	−
Pleurotus ostreatus	Cap	+
Lentinus edodes	Cap	++

[1)] Disease severity was examined 3 d after inoculation with 5×10^8 CFU/mL of GBR-1
++, brown rot; +, mild brown rot; ±, yellowish discoloration; −, no discoloration (no symptom).

2.8. In vitro plant growth promotion by GBR-1

For *in vitro* plant growth promotion assays, surface-sterilized wild type tobacco (*Nicotiana tabacum* L. cv. Nc-82) seeds were placed in Petri plates containing half-strength Murashige and Skoog (MS) basal medium (Duchefa, Haarlem, The Netherlands) with agar (0.8%). The plates were incubated in a growth chamber at 25°C with a 14 h light and 10 h dark regime for 1 wk to induce seed germination. The seedlings (15−20/plate) were transferred to Petri plates containing freshly prepared MS medium. After 3 d, a suspension of GBR-1 (20 μL, 1×10^8 CFU/mL) was dropped onto a sterile paper disc, which was placed at the edge of the Petri plate.

Table 4
Antimicrobial activity of *Paenibacillus polymyxa* GBR-1

	Microorganism	Host[1)]	GBR-1[2)]
Fungi	*Colletotrichum gloeosporioides*	Apple	+++
	Colletotrichum acutatum	Pepper	+++
	Cylindrocarpon destructans	Ginseng	+++
	Alternaria mali	Apple	++
	Botrytis cinerea	Apple	++
Bacteria	*Erwinia carotovora* subsp. *carotovora*	Chinese cabbage	++
	Xanthomonas axonopodis pv. *glycines*	Bean	+++
	Xanthomonas axonopodis pv. *vesicatoria*	Pepper	++
	Pseudomonas syringae	Apple	++
	Ralstonia solanacearum	Tobacco	+

[1)] Different host plants from which the pathogen was isolated
[2)] Antimicrobial activity was defined in terms of size of the inhibition zone and denoted as follows: −, no inhibition zone; +, weak (0−5 mm); ++, moderate (5−15 mm); and +++, strong (> 15 mm)

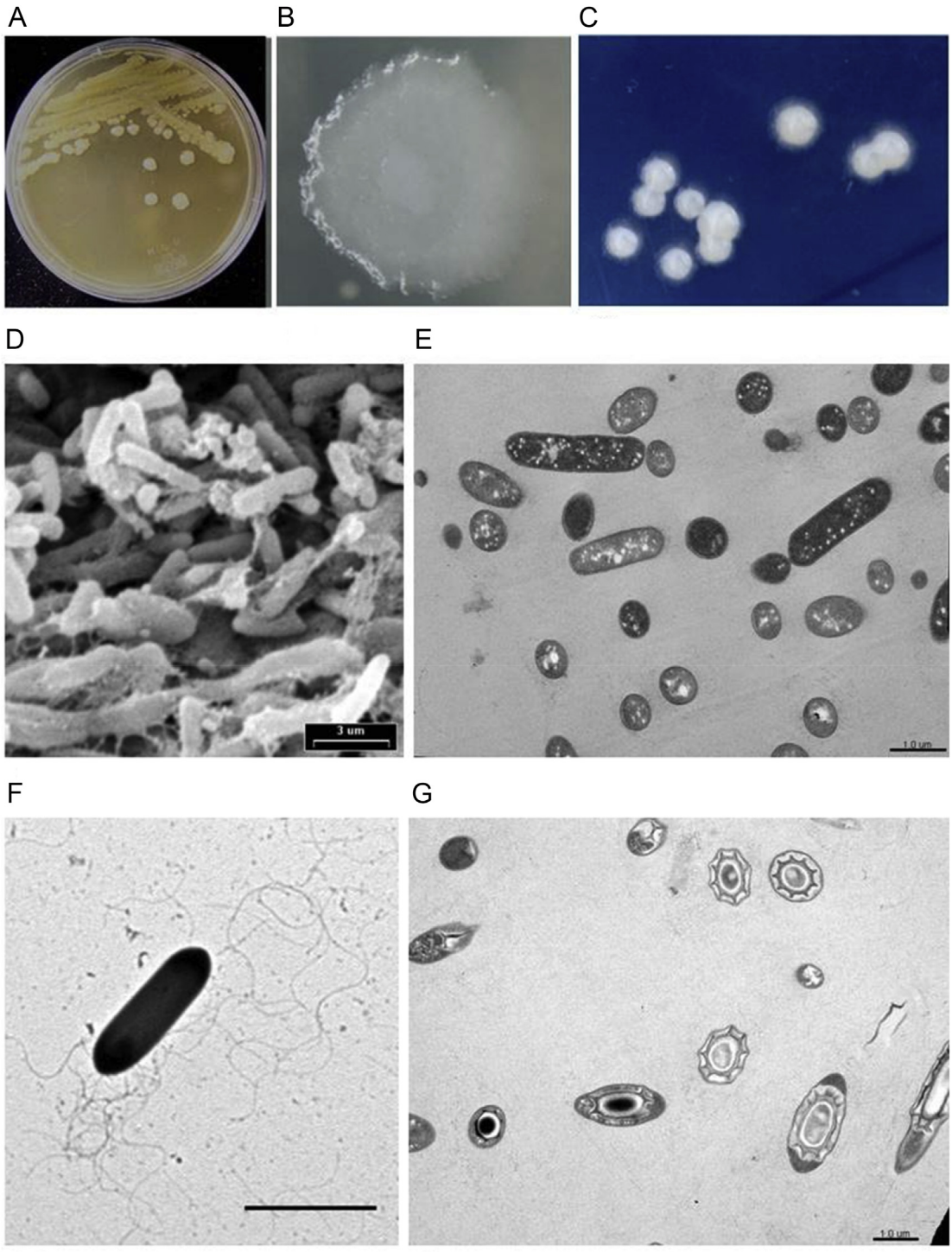

Fig. 1. Observation of *Paenibacillus polymyxa* GBR-1 grown for 2 d. (A) Brain heart infusion (BHI) agar, (B) modified starch agar, and (C) and potato dextrose agar (PDA). GBR-1 is viewed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) for its morphology. SEM reveals that the bacteria are composed of ovoid-rod-shaped cells scattered (D). The colonies are mostly vegetative cells, some of which are in the process of cell division (E). The colonies are densely populated with rectangular rod-like bacterial cells with flagella (F). The bacterial cells viewed by TEM are mostly endospore-bearing cells (G).

The plates were completely sealed with parafilm, arranged in a completely randomized design, and incubated at 25°C under a 12 h light/12 h dark photoperiod. Two wk after treatment, the total leaf surface area was measured by an image analysis system (LI-3100 area meter; LI-COR, Lincoln, USA).

2.9. Coinoculation of GBR-1 with other microorganisms

Interactions between GBR-1 and other pathogens for ginseng root rots were examined by coinoculation tests. *Pectobacterium carotovorum* subsp. *carotovorum*, *Bacillus subtilis*, and *Fusarium*

solani isolates were coinoculated with GBR-1 on 4-yr-old ginseng root discs. An amount of 20 μL of each bacterial or conidial suspensions combination (1×10^5 and 1×10^8 CFU/mL for bacteria, and 1×10^4 and 1×10^6 spores/mL for *F. solani*) was dropped over the surface of the root discs. The inoculated root discs were placed on Whatman No. 1 filter paper soaked with SDW in Petri plates, and incubated in a chamber at 25°C. Three plates containing three root discs each were used for each treatment. Symptom developments on root discs were examined 3 d after inoculation. The disease severity scoring was given as follows: +++: early rot (rotted 2 d after inoculation), ++: brown rot, +: mild brown rot, ±: yellowish discoloration, and −: no discoloration.

3. Results

3.1. Isolation, morphological identification, Biolog, and GC-MIDI of Paenibacillus polymyxa GBR-1

Out of 36 bacterial isolates related to the genus *Paenibacillus*, 20 isolates were related to the species *P. polymyxa*, based on their products amplified from genomic DNA with primers specific to the genus and species (data not shown). The putative *P. polymyxa* isolates from ginseng root rots, were rod-shaped, Gram-positive, and had peritrichous flagella (Fig. 1A–1C). For morphological identification of GBR-1 colonies by microscopy, scanning electron microscopy revealed that bacteria were composed of ovoid-rod-shaped bacterial cells scattered (Fig. 1D). The bacterial cells from the colonies were mostly vegetative cells, some of which were in the process of cell division (Fig. 1E). The bacterial cells viewed by transmission electron microscopy (TEM) revealed that they were of rectangular rod-like with flagella (Fig. 1F) and bearing endospores (Fig. 1G). The Biolog results of the GBR-1 showed that it could be classified as *P. polymyxa*, as its closest match because it was able to utilize various carbohydrates (Table 1). Comparing these traits to the Biolog database revealed this strain with a match probability of 100% to *P. polymyxa*. Based on the results of GC-FAME analysis (Table 2), the GBR-1 strain was identified as *P. polymyxa*, with 10 fatty acids.

3.2. Growth curve and optimal media for GBR-1

In the present study, we determined that GBR-1 cell numbers were relatively higher when cultured in BHI broth as compared in other media (Fig. 2). The number of GBR-1 bacterial cells increased drastically to 4.6×10^9 CFU/mL after 16 h of incubation and started to decline at 28 h in BHI broth. The bacterial growth rate was found to be similar between 16 h and 28 h. The next optimum growth medium was found to be tryptic soy broth, where the bacterial cells were found to be 7.9×10^8 CFU/mL at 16–24 h. The smallest number of GBR-1 occurred when the cells were cultured in potato dextrose broth medium. The results clearly showed that the optimal medium was BHI for the most efficient proliferation of GBR-1 cells. The results for the time required for bacterial cell growth revealed that maximum cell growth was noticed 20–28 h after incubation in all the tested media and then started to decline its growth.

3.3. Effects of temperature conditions on starch-hydrolytic activity by GBR-1

In the assay of starch-hydrolytic activity by GBR-1 treatment at different temperature conditions, strong starch-hydrolytic activity was observed only at 30°C after 48 h of incubation at high density inoculum treatment, followed by 25°C as 4.2 mm of clear zone was observed (Fig. 3). Starch-hydrolytic activity was not seen at the low temperature (4°C), while at temperatures of 10°C, 15°C, and 20°C, the clear halo zones were 0.5 mm, 1.5 mm, and 3.6 mm, respectively. The visual observations indicate that bacterial growth increased significantly at higher temperatures compared to the low temperatures.

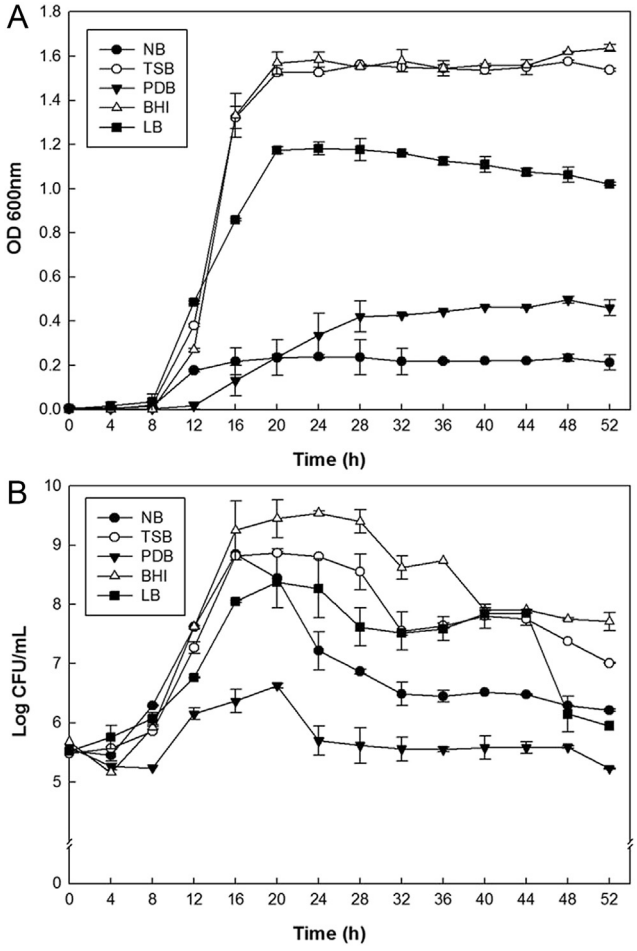

Fig. 2. The population changes of GBR-1 at different incubation temperatures (0–52 h) on different growth media such as nutrient broth (NB), tryptic soy broth (TSB), potato dextrose broth (PDB), brain heart infusion broth (BHI), and Luria Bertani broth (LB). At every 4 h, (A) the optical density (OD) is read at 600 nm and (B) the viable cells are counted at the same time by plating the culture onto the agar plates after serial dilution. The GBR-1 population shows a high number in BHI after 20 h. The experiment was repeated at least once with three replicates/treatment producing similar results. Means and standard deviations of the three replicates are shown.

3.4. Pathogenicity of GBR-1 on the stored ginseng root at different temperature conditions and pathogenicity on other storage plants

The pathogenicity of GBR-1 was tested on stored ginseng roots at various incubation temperature conditions (Fig. 4). The 4-yr-old ginseng root discs (5 mm thick) were inoculated at the center, with a 20 μL bacterial suspension at the concentration of 5×10^8 CFU/mL. GBR-1 started to induce rot symptoms on the ginseng root discs inoculated at a high concentration (5×10^8 CFU/mL) from the increasing temperatures. Under incubation at different temperature conditions (4–30°C), the highest decayed diameter was observed at 30°C on the 7th d, followed by 25°C. At low temperatures such as 20°C, 15°C, and 10°C, the decayed diameter was moderate. Additionally, the pathogenicity of GBR-1 on other storage plants was determined in addition to *P. ginseng* at high

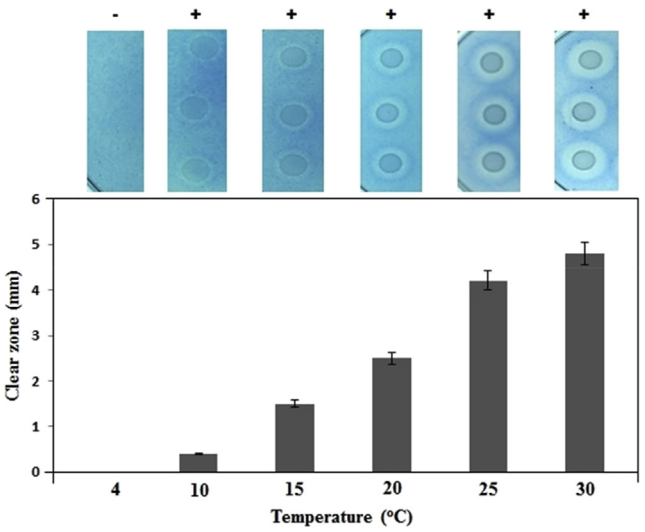

Fig. 3. Starch hydrolytic activity of the GBR-1 strain at different incubation temperatures (4–30°C) under *in vitro* conditions. Strong starch hydrolytic activity is observed in bacterial colonies from the high-density inoculum 72 h after inoculation at 30°C. A clear zone formed around a bacterial colony is considered to be a positive (+) response for starch hydrolytic activity and no formation of the clear zone is considered as a negative (−) response. The experiment was repeated at least once producing similar results.

inoculum density (5×10^8 CFU/mL). The bacterial suspension was inoculated on the center of the discs of all the tested storage plants. Three d later, the disease severity was recorded. The bacterium caused rot symptoms on all the storage plants except *Allium sativum* and *Allium sepa* where there was no damage of the scaly leaves. There was no disease severity in *Agaricus bisporus*, whereas the fungi *Flammulina velutipes* and *Pleurotus ostreatus* were prone to mild brown rot symptoms by GBR-1 inoculum (Table 3). Root, tuber, or storage organ discs of several plant species and fresh mushrooms were inoculated with GBR-1 suspensions. With the exceptions of *Allium* spp. and *A. bisporus*, all plants and mushrooms were rotten by GBR-1 inoculation (Table 3). A weak rotting occurred on the fresh tissues of *Cucurbita pepo*, *F. velutipes*, and *P. ostreatus*. Symptoms in these organs were similar to those of ginseng roots, showing brown rots.

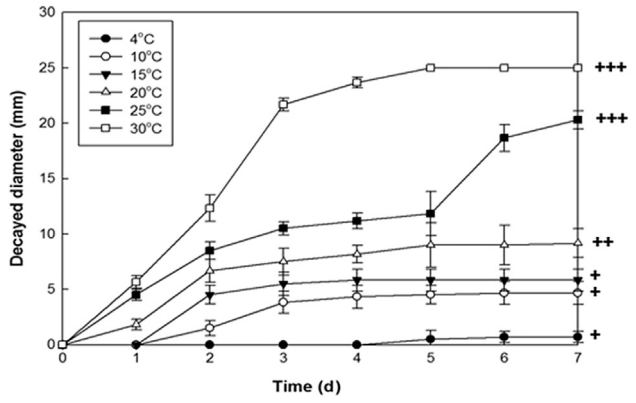

Fig. 4. Assessment of pathogenicity of GBR-1 on stored ginseng root at various temperatures (4–30°C). The higher decayed tissues are observed at high temperature (30°C) on the 7th d when compared to lower temperatures. Roots are examined daily for the development of root rot symptoms. This is measured as a decayed diameter zone and represented as follows: − no decayed zone, + weak (0–5 mm), ++ moderate (5–15 mm), and +++ strong (> 15 mm). The experiment was repeated at least once with three replications/treatment producing similar results.

3.5. *In vitro antagonistic activity of GBR-1*

In the present study, the *in vitro* antagonistic activity of GBR-1 was tested against fungal and bacterial plant pathogens on PDA and NA plates, respectively, that cause severe diseases in various crops (Table 4). A considerable variation was observed between fungal and bacterial antagonists with regard to the hyphal interaction and subsequent events to the inhibition of the growth of fungal pathogens. There was a greater growth inhibition in three pathogenic fungi, *Colletotrichum gloeosporioides*, *Colletotrichum acutatum*, and *C. destructans* (Fig. 5) when compared to the other two fungi, *Alternaria mari* and *Botrytis cinerea*, where there was only a moderate inhibition. In the case of bacterial pathogens, growth of *Xanthomonas axonopodis* pv. *glycines* was strongly inhibited (> 15 mm), while the growth of other bacteria was moderately inhibited and there was only a weak inhibition (0–5 mm) in *Ralstonia solanacearum*. The formation of an inhibition zone against fungal and bacterial pathogens is considered as an antibiosis, whereby the antibiotic metabolites from GBR-1 might have inhibited the mycelial growth of the fungal pathogens.

3.6. *In vitro plant growth promoting effect by GBR-1*

To investigate the effect of GBR-1 on plant growth promotion, 1-wk-old tobacco seedlings grown in Petri plates containing half MS medium were coincubated with a suspension of GBR-1 at the edge of the Petri plate for 3 wks at 25°C. Biomass production was stimulated in plants exposed to GBR-1 (Fig. 6). Following exposure to GBR-1, the leaf surface area increased significantly by 2.75 cm^2 when compared to the control (1.8 cm^2). The growth promotion effect might be due to the release of volatile organic compounds (VOCs) from GBR-1. By contrast, growth of the seedlings was suppressed near to the bacterial inoculation; this might be due to some other metabolites released by bacteria, while other seedlings apart from the inoculation site were found to have vigorous growth, which might be due to the release of VOCs from the bacteria suspensions. The growth promotion by GBR-1 treatment was found to be higher as compared to water-treated controls. The demonstration of volatile detection was not done as the VOCs were the major regulators of this enhancement of plant growth and development.

3.7. *Coinoculation of GBR-1 with other microorganisms*

In the coinoculation test, the GBR-1 strain was mixed with other bacterial suspensions at high (1×10^8 CFU/mL) and low (1×10^5 CFU/mL) concentrations, and GBR-1 was mixed with fungal spores of *F. solani* at high (1×10^6 spores/mL) and low (1×10^4 spores/mL) concentrations prior to inoculation onto the ginseng root discs. When *P. carotovorum* suspension alone at a low concentration was inoculated onto the root discs without GBR-1 suspensions, there was a strong disease severity where rotting occurred 2 d after inoculation, whereas with root disc inoculation by *P. carotovorum* along with GBR-1 at a low concentration, the root rot was induced moderately and was visualized as mild brown rot. This is because of the suppression of soft rot pathogens by GBR-1 (Table 5). When *P. carotovorum* and GBR-1 were inoculated at high concentrations on the root discs, root rot was strongly induced. In the case of *B. subtilis*, inoculation at low and high concentrations without coinoculation with GBR-1 or GBR-1 alone at low concentrations showed no root rot symptoms on the discs, which might be due to the non-pathogenicity of *B. subtilis*, whereas the low concentration of *B. subtilis* and high concentration of GBR-1 induced root rot at moderate levels, appearing as brown rot by visualization. In the case of *F. solani*, the fungal spore inoculum at low concentration

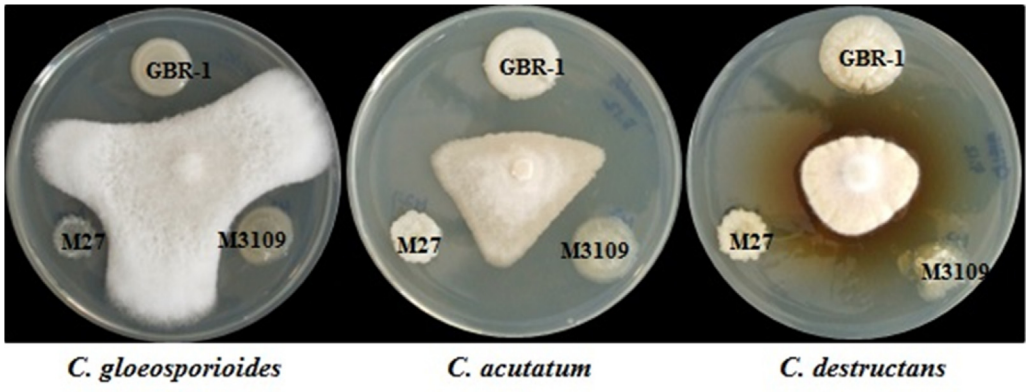

Fig. 5. *In vitro* antagonistic activity of bacteria GBR-1, M27, and M3109 against major fungal pathogens *Colletotrichum gloeosporioides*, *Colletotrichum coccodes*, and *Cylindrocarpon destructans*. Antifungal activity is defined in terms of the size of the inhibition zone and denoted as follows: − no inhibition zone, + weak (0–5 mm), ++ moderate (5–15 mm), and +++ strong (> 15 mm). The experiment was repeated at least once with three replications/treatment producing similar results.

without GBR-1 showed moderate root rot symptoms appearing as brown rot, but coinoculation with GBR-1 suspensions showed no root rot symptoms because of inhibitory activity of GBR-1 against *F. solani*. The coinoculation of fungal spores and GBR-1 at high concentrations induced root rot symptoms on the root discs moderately. These results suggest that GBR-1 would possess pathogenicity at high inoculum density and had no inhibitory effect against *P. carotovorum* and *F. solani*.

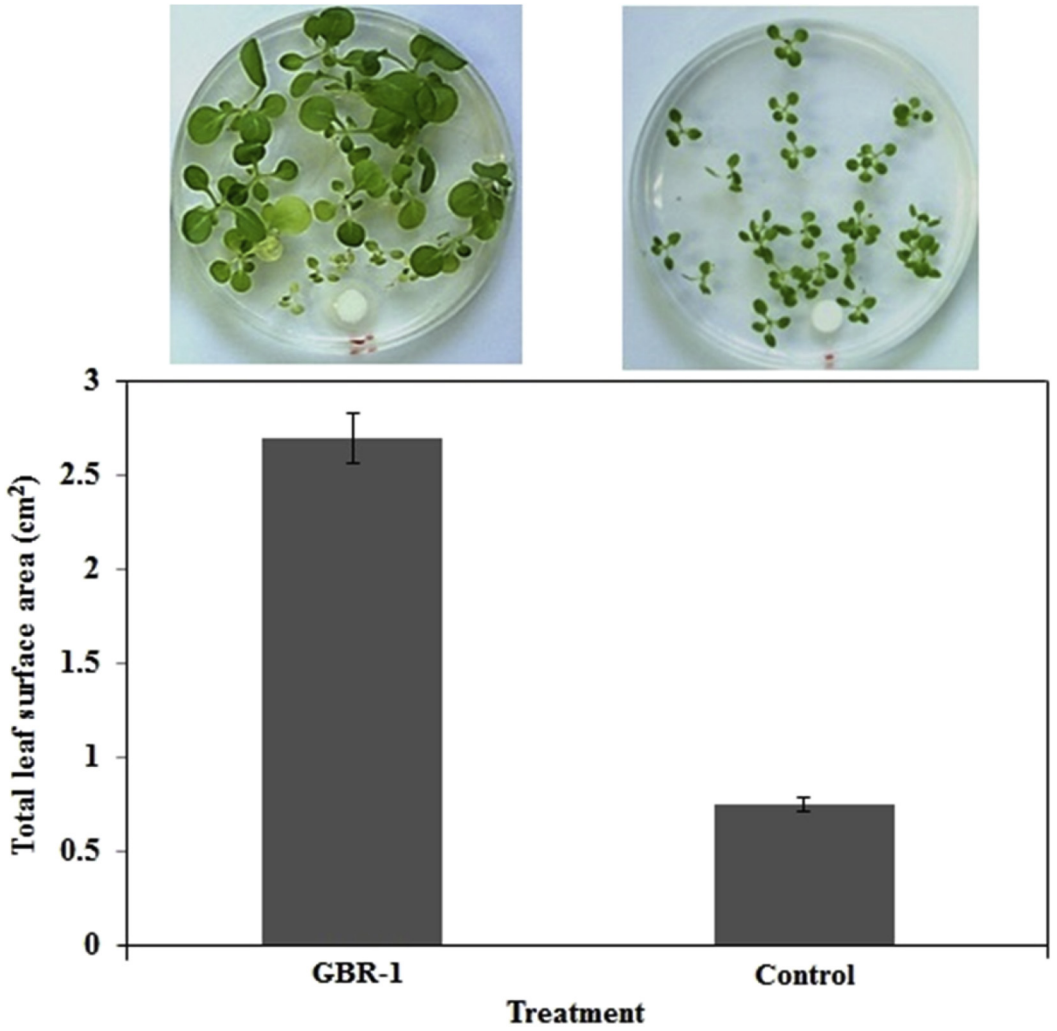

Fig. 6. Plant growth promoting effect by GBR-1 on tobacco seedlings under *in vitro* conditions in comparison with water-treated controls. The total leaf surface area is measured 3 wk after incubating the Petri dishes at 25°C. The experiment was repeated at least once with three replications/treatment producing similar results.

Table 5
Root rot severity caused by co-inoculation of *Paenibacillus polymyxa* GBR-1 with other microorganisms at different inoculum concentrations

Microorganisms	Inoculum Concentration of GBR1 (CFU/m)	Disease severity[1)]		
		0	10^5	10^8
Control		–	–	++
Pectobacterium carotovorum	1×10^5	+++	+	+++
	1×10^8	+++	+++	+++
Bacillus subtilis	1×10^5	–	–	++
	1×10^8	–	–	±
Fusarium solani	1×10^4	++	–	++
	1×10^6	++	++	++

[1)] Disease severity scoring was recorded 3 d after inoculation as: +++, early rot (rotted 2 d after inoculation); ++, brown rot; +, mild brown rot; ±, yellowish discoloration; and –, no discoloration

4. Discussion

The present study was undertaken to investigate the role of bacterial strain, GBR-1, as one of the root rotting bacteria in ginseng with beneficial effects. GBR-1, isolated from ginseng root rot, was confirmed as a *P. polymyxa* based on morphology, the Biolog program, and GC-FAME. GBR-1 is involved in plant growth promotion and potential biocontrol agents against phytopathogens that cause severe diseases in various crops in Korea. In our previous study, the effect of inoculum density level on ginseng root rot was studied [11]. This result might have been attributed to differentiate colonies from the high concentration of inoculum that were filled with exopolysaccharide. The ovoid-rod-shaped bacterial (endospore-bearing) cells were distributed (Fig. 1C–1E). Therefore, the activation of starch hydrolysis, tissue rot in ginseng, and colony formation may be influenced mainly by the concentration of the inoculum. Additionally, these biological characteristics might be interrelated. In our previous study, *P. polymyxa* strains causing root rot in ginseng at high inoculum concentrations also showed strong starch-hydrolytic activity [9]. By contrast, the present study showed that the starch hydrolysis was induced at high temperature incubation. These results suggest that GBR-1 may primarily act as a rotting agent for stored ginseng roots in the inoculation test.

In population changes of GBR-1, there was a greater population in BHI media when compared to other media used in this study. Cell wall degrading enzymes secreted from plant pathogens play a role in pathogenicity [22], and are correlated with the starch hydrolysis, which has been directly related to pathogenicity, although amylase of *Xylella fastidiosa* was reported as one of the pathogenicity factors [23]. These facts indicate that starch hydrolytic activity may not be directly related to tissue rotting, but involved in enhancing other activities in the disintegration of the cellular components in ginseng. By contrast, the temperature conditions may also play an important role in rotting of roots. Plant growth promotion by rhizobacteria can occur directly or indirectly [24]. No rotting symptoms were observed on ginseng root discs inoculated with GBR-1 cell suspensions of 5×10^8 CFU/mL at 4–15°C, but there was a substantial increase of root rot at 30°C after 7 d. This indicates that GBR-1 is not an exact root rot pathogen as it is influenced by incubation temperature. This result is associated with the ability of the strain on starch-hydrolytic activity [21]. Brown rot was observed in most of the storage plants at a high inoculum density level.

With regards to the antagonistic activity, there was a strong inhibition of mycelial growth by GBR-1, in addition to inhibition of the growth of the bacteria *X. axonopodis* pv. *glycines*. These *in vitro* antagonistic activity results suggest that GBR-1 could be a potential biocontrol agent against three fungal pathogens, *C. gloeosporioides*, *Colletotrichum coccodes*, and *C. destructans*, because the GBR-1 might have inhibitory capacities on mycelial growth and reproduction. A few previous reports showed that *P. polymyxa* strains had a broad spectrum activity against bacteria, fungal, and nematode species [5,25,26]. This provides an advantage to the GBR-1 in its antagonistic effect on the pathogens. The toxic substance released by *Colletotrichum* spp. was evident in various alternations of its hyphae together with other *Bacillus* spp. [27]. Several studies have been focused on biocontrol activities of *Bacillus vallismortis* to control various plant diseases during the past several years [28].

GBR-1 stimulated plant growth promotion *in vitro*, as this study describes the relationship between GBR-1 and plants *in vitro*. In this study, we provide evidence that VOCs released by GBR-1 might regulate plant growth promotion. Biochemical analysis of GBR-1 was not yet identified. Our results provide new insight into plant growth promotion for identification of a broad range of VOCs in plant–microbe interactions. Several reports indicate that VOCs released by common PGPR can be of various chemical groups containing inorganic compounds [29–31]. GBR-1 strains were also involved in induced systemic resistance in tomatoes against *Meloidogyne incognita* that cause root-knot nematode [32]. Further, in a coinoculation study, GBR-1 with other microorganisms such as *P. carotovorum*, *B. subtilis*, and *F. solani* on ginseng root discs for the development of root rot severity, individual inoculation of GBR-1 was shown to positively affect the root rot severity and no disease was found at low inoculum, but there was a moderate rot occurrence at a high inoculum density of GBR-1. When they were coinoculated with GBR-1, there was a reduction of root rot severity. Our study is supported by a previous report [33], where the bacteria on the ginseng rhizosphere were found to be greater when both bacteria and pathogens were coinoculated than when the bacterial isolate alone was inoculated.

In conclusion, it can be stated that the native GBR-1 isolate from ginseng root rot is a potential candidate for growth promotion and biocontrol of various fungal pathogens and a causative agent at higher inoculum densities, as it possesses dosage dependency characteristic for its activity in the environment, indicating the potency to survive and act under varied conditions. It is recommended that the concentration of GBR-1 should be monitored when it is used as a biocontrol agent of ginseng, especially in the treatment of stored roots. As the disease causing characteristics of this bacterium are harmful to its regular usage as a biocontrol agent, these characteristics first must be determined to minimize negative impacts.

Conflicts of interest

We declare no conflicts of interest.

Acknowledgments

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