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Research article

Induction of systemic resistance in *Panax ginseng* against *Phytophthora cactorum* by native *Bacillus amyloliquefaciens* HK34

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ABSTRACT

Background: Korean ginseng (*Panax ginseng* Meyer) is a perennial herb prone to various root diseases, with *Phytophthora cactorum* being considered one of the most dreaded pathogens. *P. cactorum* causes foliar blight and root rot. Although chemical pesticides are available for disease control, attention has been shifted to viable, eco-friendly, and cost-effective biological means such as plant growth-promoting rhizobacteria (PGPR) for control of diseases.
Methods: Native *Bacillus amyloliquefaciens* strain HK34 was isolated from wild ginseng and assessed as a biological control agent for ginseng. Leaves from plants treated with HK34 were analyzed for induced systemic resistance (ISR) against *P. cactorum* in square plate assay. Treated plants were verified for differential expression of defense-related marker genes using quantitative reverse transcription polymerase chain reaction.
Results: A total of 78 native rhizosphere bacilli from wild *P. ginseng* were isolated. One of the root-associated bacteria identified as *B. amyloliquefaciens* strain HK34 effectively induced resistance against *P. cactorum* when applied as soil drench once (99.1% disease control) and as a priming treatment two times in the early stages (83.9% disease control). A similar result was observed in the leaf samples of plants under field conditions, where the percentage of disease control was 85.6%. Significant upregulation of the genes *PgPR10, PgPR5,* and *PgCAT* in the leaves of plants treated with HK34 was observed against *P. cactorum* compared with untreated controls and only pathogen-treated plants.
Conclusion: The results of this study indicate HK34 as a potential biocontrol agent eliciting ISR in ginseng against *P. cactorum*.

1. Introduction

Korean ginseng (*Panax ginseng* Meyer) is a perennial herb well-known for its medicinal value for over a thousand years [1,2]. It is regarded as a health tonic rich in antioxidants and anti-inflammatory properties [3]. Apart from improving vitality, longevity, mental state, and destressing capacity, ginseng is also prescribed for cardiovascular diseases, cancers, tumors, and chronic metabolic syndromes such as diabetes [4–6] with recent reports suggesting its effect against influenza A virus [7]. The roots are the storehouse of pharmacologically active chemicals in ginseng and Korean ginseng root products are known for their efficacy as functional foods or pharmaceuticals. However, the production of high-quality harvestable roots requires several years of cultivation in the same field under shady conditions. Long and successive cultivation depletes soil nutrition and physicochemical properties thereby facilitating diseases caused by soilborne pathogens [8]. Ginseng is prone to various root diseases caused by fungi and bacteria [9], with *Phytophthora cactorum* being one of the most dreaded pathogens that is responsible for foliar blight and root rot.

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Symptoms of foliar blight are dark green, water-soaked lesions on the leaves, and root rot leading to wilting and reddening of leaflets with brown spongy roots [10]. The disease spreads rapidly and destroys the entire cultivation zone within the span of a few weeks causing huge yield loss [11,12]. Although chemical pesticides are available for disease control in ginseng, increasing awareness of the hazardous effects of synthetic agrochemicals on the environment and the development of resistant species against fungicides are of serious concern for agriculturists. Intake of roots containing pesticide residues can cause serious health issues. Therefore, attention has been shifted to viable, eco-friendly, and cost-effective biological means for control of diseases.

Plant growth-promoting rhizobacteria (PGPR) are well established for growth promotion and induced systemic resistance (ISR) in plants against multiple pathogens. Various reports indicate the potential of beneficial bacteria in controlling diseases in ginseng [8,13,14]. *Pseudomonas* and *Bacillus* strains have been exploited for the management of plant diseases in many crops [15,16]. Various intricate mechanisms are involved in plant growth promotion and ISR, and these depend on many biotic and abiotic factors apart from the plant, pathogen, and bacteria. Whereas growth promotion is attributed to the production of phytohormones, siderophores, nutrient mobilization, nitrogen fixation, etc., ISR is mediated through triggering of the plant defense mechanism [17]. The *priming* effect of PGPR describes the potentiated activation of cellular defense responses on pathogen attack, which includes oxidative burst, cell wall reinforcement, activation of defense-related enzymes, and accumulation of secondary metabolites [18]. Priming prepares the plants for rapid and effective activation of the basal defense response upon pathogen attack. However, PGPR are yet to gain commercial success as inconsistency under field conditions is a major drawback. On application in the field, various factors such as acclimatization to the environment and competition with the native strains lead to the inability of potential strains to survive in the rhizosphere. Therefore, the search continues for potential bacterial candidates preferably of native origin to combat diseases and improve plant growth. *Bacillus* is the most abundant genus in the rhizosphere and is preferred for bioformulations due to advantageous traits such as a multilayered cell wall, endospore formation, the production of extracellular enzymes, antibiotic peptides, and peptide signal molecules, which contribute toward their survival under varied environmental conditions [19,20]. The present work evaluated native rhizosphere bacilli from *P. ginseng* for control of leaf blight and root rot disease caused by the fungus *P. cactorum*. One of the root-associated bacteria identified as *Bacillus amyloliquefaciens* strain HK34 effectively induced resistance against *P. cactorum* by activating the defense mechanism of the treated plants. The stimulation of defense-related pathways was confirmed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of marker genes. Our study analyzed and confirmed HK34 as a potential strain for ISR in ginseng.

2. Materials and methods

2.1. Isolation of native Bacillus from P. ginseng rhizosphere

Samples of ginseng were collected from 34 different wild ginseng roots provided by the Korean Association of Wild Ginseng. The ginseng roots were surface sterilized, crushed, and heated at 80°C for 15 minutes for isolation of *Bacillus* strains including endophytes. A total of 78 strains were isolated in tryptic soy agar (TSA) after serial dilution and each isolate was maintained at −80°C in TS broth with glycerol (20%) for long-term storage. Isolated bacteria were identified using 16S recombinant DNA analysis.

2.2. Isolation and identification of the pathogen P. cactorum

The fungal pathogen used in this study was isolated from leaves of 5-year-old ginseng with water-soaked symptoms. The infected leaf tissues (lesions) were cut into pieces and rinsed with 1% NaOCl for 30 seconds for surface sterilization. The leaf tissues were then placed on water agar media and incubated at 25°C. The hyphal tips grown out of the leaf tissues were transferred to V8 juice agar and incubated at 25°C for 10 days for pure culture. Taxonomical identification of the fungal pathogen was done based on previous studies [21–23].

2.3. Screening and selection of potential Bacillus isolates for ISR

All of the 78 isolates were screened for their efficacy as agents of ISR against *P. cactorum* in ginseng plants. For this purpose, germinated *P. ginseng* seedlings were sown in plastic plant trays with soil (5 replicates each). The plants were grown at 23°C for 1 month and then 5 mL of bacterial suspension (1×10^7 cfu/mL) was inoculated with the soil drench technique near the roots. After 1 week of bacterial treatment, plants were challenged with inoculum plugs (diameter, 5 mm) of 10-day-old *P. cactorum* grown in potato dextrose agar media. Estimation of disease occurrence was done after 48 hours of growth. Of the 11 isolates capable of controlling disease, the most potential strain designated as HK34 was selected for further studies.

2.4. Analysis of HK34 for ISR

The isolate HK34 was analyzed for ISR against *P. cactorum* under greenhouse and field conditions. For greenhouse studies, 1-year-old ginseng seedlings were planted in plastic plant trays. Distilled water and 0.1mM benzothiadiazole (BTH) treatments were used as negative and positive controls, respectively. For treatment of bacteria, the plants were divided into two sets. In the first set 1 month after transfer of plants, 10 mL of HK34 suspension (1×10^7 cfu/mL) was applied as soil drench and grown for 1 more week. Leaf samples were then collected from the plants to check for ISR against *P. cactorum* in square plate assay.

In the second set, as a priming treatment in the early stage, plants were treated two times with bacterial suspension (10 mL each of 1×10^7 cfu/mL). First, bacterial treatment was given at 1 week after transfer to pots and another treatment was carried out after an additional week of growth. The treated plants were grown for 2 additional weeks and then leaf samples were subjected to square plate assay against *P. cactorum* to analyze the priming effect.

For field studies, ginseng was sown in 0.64-m^2 area seed beds with a seedling gap of 3 cm × 3 cm in a completely randomized block design. A total of 700 replicates were maintained for this study. Ten liters of HK34 suspension (1×10^6 cfu/mL) was applied to each bed after 2 months of plant growth as soil drench. Leaf samples were collected for square plate assay after 1 week of bacterial treatment. Plants treated with distilled water were taken as negative control.

Whole leaves from the treated and control plants were tested after 1 week of treatment for ISR activity in a square plate (Nunc BioAssay dish; Apogent Company, Tåstrup, Denmark; 24 cm × 24 cm) [24]. Mycelial plug was deposited on each leaf and the leaves were kept with moist tissue papers in a square plate. The disease severity was calculated after incubation for 48 hours at 28°C using *Image J* (National Institute of Health, Bethesda, MD, USA).

2.5. Population of HK34 in soil

To estimate the population of HK34 in soil, two types of soil were selected, namely, greenhouse soil material and preplant field

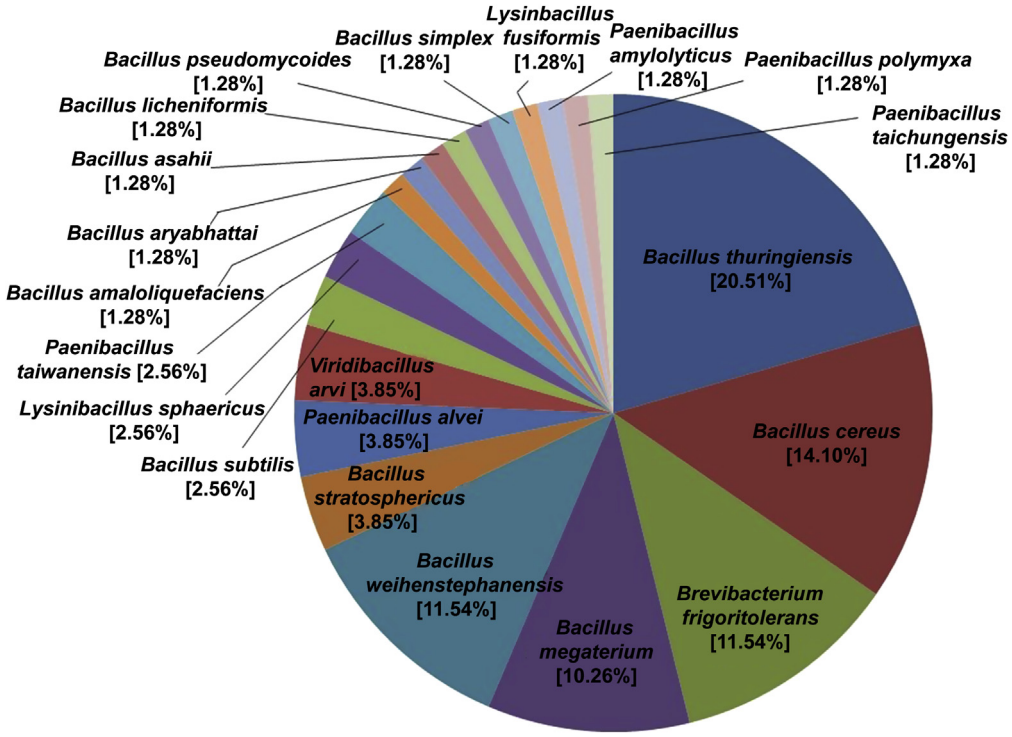

Fig. 1. Composition of rhizobacteria isolated from wild ginseng roots and percentage of each bacterium in the total population.

soil. Approximately 15 g of greenhouse soil and 50 g of field soil were autoclaved and treated with 5 mL or 10 mL of HK34 bacterial suspension (1×10^5 cfu/mL), respectively. The treated soil was incubated at 24°C and the bacterial population was estimated using the serial dilution technique at weekly intervals for up to 4 weeks in TSA media.

2.6. Root colonization of HK34 in P. ginseng

One-year-old *P. ginseng* plants were treated with 10 mL of HK34 suspension (1×10^7 cfu/mL). After 1 month of growth, roots were collected and the adherent soil was removed by shaking. Then the roots (1 g) were excised and ground from root tip to estimate the HK34 population using the serial dilution method. Plants treated with sterile distilled water (SDW) were taken as control.

2.7. Preparation of fungal pathogen inoculum for RT-PCR studies

The fungal pathogen *P. cactorum* was grown in V8 agar plates for 10 days at 25°C in the absence of light. The mycelia agar culture, cut into blocks using a sterile scalpel, was put into SDW and incubated at 23°C for 3—5 days under a wide-spectrum light. The plates were then chilled at 10°C for 30 minutes and returned to 23°C for another 1 hour to induce the release of zoospores. The zoospores were collected through four layers of cheesecloth and adjusted to a final concentration of 1×10^4 zoospores/mL using a hemocytometer.

2.8. RT-PCR analysis of selected defense-related marker genes in HK34-treated plant leaves

For RT-PCR studies, seeds of *P. ginseng* were surface sterilized with 1% NaOCl for 7 minutes after removal of the seed coat and allowed to germinate in water agar media for 1 week at 23°C. Seedlings were then transferred to SDW in 6-well cell culture plates (3 mL/cell) and kept for 2 weeks at 23°C under conditions of light (12,000 lux for 12 h/d). The SDW was then replaced with 3 mL of HK34 suspension (1×10^7 cfu/mL) and grown for another 1 week. The bacterial suspension was then removed and plants were challenge inoculated with zoospores of *P. cactorum* (1×10^4 zoospores/mL). Leaves were collected after 48 hours of incubation at 23°C for extraction of total RNA. Plants treated with only SDW and 0.1mM BTH were taken as negative and positive controls, respectively. Plants treated with SDW and challenged with *P. cactorum* were taken as pathogen control.

Total RNA was isolated from the ginseng plant using the easy-spin kit (iNtRON Biotechnology, Inc., Daejeon, South Korea) according to the manufacturer's instructions. The concentration of RNA was measured using a nanodrop spectrophotometer and it was adjusted to 1 μg. For complementary DNA (cDNA) synthesis, reverse transcription was performed using TOPscript RT DryMIX (*dt18plus*; Enzynomics, Daejeon, South Korea) according to the manufacturer's instructions. The cDNA synthesized was used as template for RT-PCR (Applied Biosystems) using the following gene-specific primers: 5′-GGC AGA AAA GCT GTT CAA GG-3′ and 5′-TTG CAT CTA TCC GGG TCT TC-3′ for *PgPR10* [25]. Specific primers used for *PgPR5* were 5′-AAC CGA CTG CAA CTT CGA CT-3′ and 5′-GGC ACA TTA AAC CCA TCC AC-3′ [26]. For the gene *PgCAT1*, the primers used were 5′-CAA GGA TGG GAA AGC ACA CT-3′ and 5′-TGG TTA CAT CGA GTG GGT CA-3′. The housekeeping gene that encoded β-actin (AY907207) was used as a control and was amplified with the primers 5′-CGT GAT CTT ACA GAT AGC TTG ATG A-3′ and 5′-AGA GAA GCT AAG ATT GAT CCT CC-3′. The thermal cycler conditions were as follows: 15 minutes at 95°C, followed by 40 cycles at 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 13 seconds.

2.9. Statistical analysis

The data were subjected to analysis of variance using *SAS JMP* software (SAS Institute, Cary, NC, USA). Significant differences in the treatment methods of each sample were determined using least significant difference at $p = 0.05$. All experiments were performed two times. For each experiment, the data were analyzed separately.

Table 1
Native *Bacillus* strains isolated from wild ginseng that completely suppressed *Phytophthora cactorum* in treated plants

Designation	Identification of isolates
HK1-4	*Bacillus weihenstephanensis*
HK3-1	*Bacillus weihenstephanensis*
HK4-1	*Bacillus thuringiensis*
HK9-2	*Bacillus megaterium*
HK10-2	*Bacillus megaterium*
HK10-3	*Bacillus stratosphericus*
HK11-3	*Bacillus simplex*
HK13-2	*Viridibacillus arvi*
HK14-2	*Bacillus weihenstephanensis*
HK16-4	*Paenibacillus taiwanensis*
HK34	*Bacillus amyloliquefaciens*

3. Results

3.1. Isolation of native Bacillus from P. ginseng rhizosphere and screening for disease control

A total of 78 different bacilli were isolated from the roots of wild ginseng plants (Fig. 1) with *Bacillus thuringiensis* (20.51%) being the bacteria with the highest percentage, followed by *Bacillus cereus* (14.10%) and *Bacillus weihenstephanensis* (11.54%) along with species of *Paenibacillus*, *Viridibacillus*, *Lysinibacillus,* and *Brevibacterium*. All the strains were studied for their efficacy in disease control in ginseng plants against *P. cactorum*. Of all the isolates tested, 11 strains (designated and identified as in Table 1) completely suppressed the disease. There was no disease symptoms in plants treated with these 11 isolates. The strain HK34 showed the best result when challenged with *P. cactorum*, and therefore, it was selected as a potential isolate for further studies of ISR.

3.2. Efficacy of HK34 against P. cactorum for ISR

To evaluate the efficacy of HK34 for ISR, plants were treated in two sets: one with single drench and the other was treated two times. In square plate assay for ISR in leaves of the first set of greenhouse experiments, one drench of HK34 significantly enhanced induced resistance against *P. cactorum* compared with the SDW-treated control (Fig. 2). The percentage disease control was 99.1% in HK34-treated plants compared with the control (Fig. 3). In the second set with two drenches of HK34 at weekly intervals, the percentage disease control was 83.9%, which signifies the priming effect of the bacterium against *P. cactorum* (Figs. 4 and 5). Although there is a slight decrease in disease control percentage with repeated drench of HK34, there was a significant effect compared with control and BTH. A result similar to that observed under greenhouse conditions was seen under field conditions, where percentage of disease control was 85.6% (Figs. 6 and 7) compared with the control. The consistent results of field studies as in greenhouse samples signify the ability of HK34 as a potential isolate against *P. cactorum*.

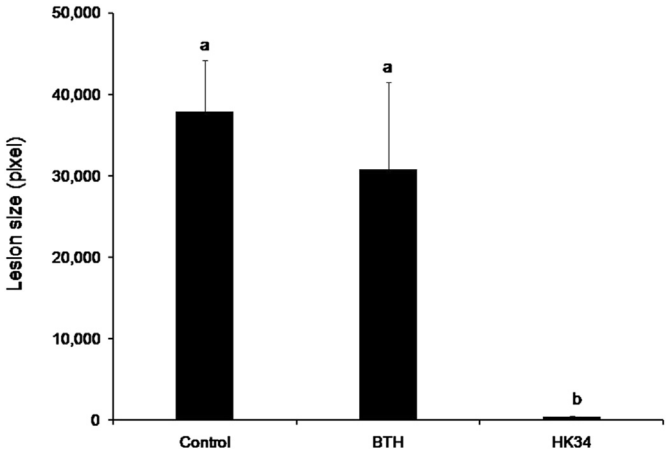

Fig. 3. Effect of HK34 on the lesion area of leaves challenged with *Phytophthora cactorum* compared with the sterile distilled water-treated control and benzothiadiazole (BTH). Different letters on each bar represent values that are statistically different ($p = 0.05$). The vertical bars indicate standard error.

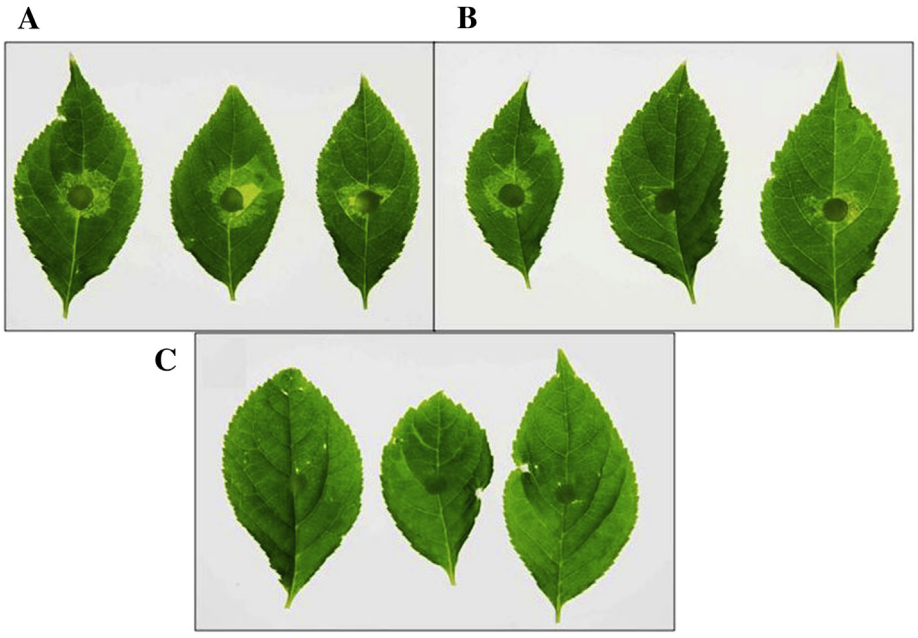

Fig. 2. Effect of HK34 on the induction of systemic resistance against *Phytophthora cactorum* in leaves obtained from plants treated once under greenhouse conditions. Leaves are from plants treated with (A) sterile distilled water (negative control), (B) 0.1mM benzothiadiazole (positive control), and (C) HK34.

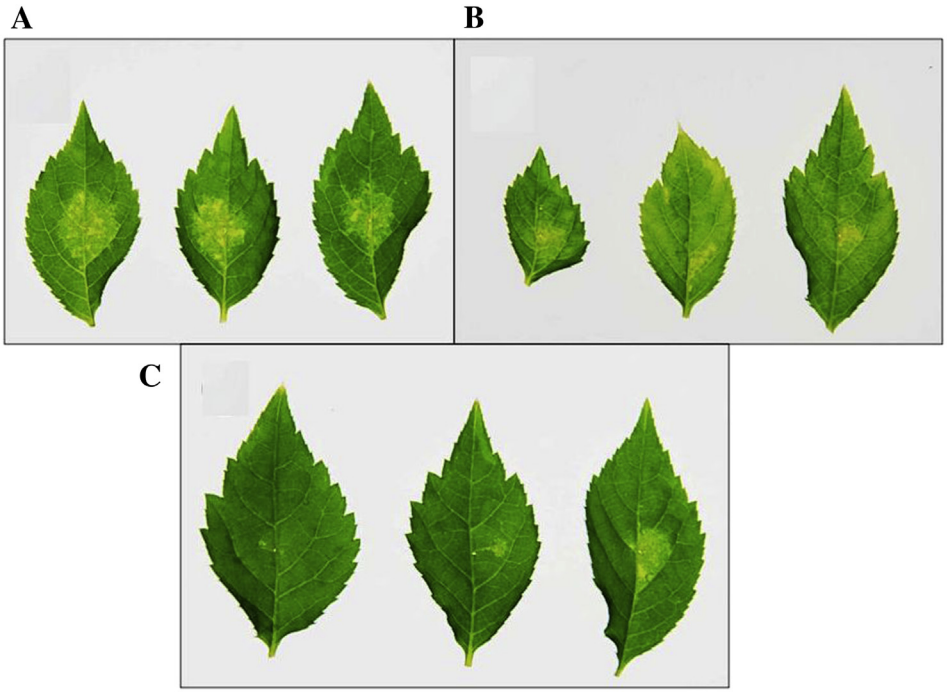

Fig. 4. Priming effect of HK34 on the induction of systemic resistance against *Phytophthora cactorum* in leaves obtained from plants treated two times in the early stage of growth under greenhouse conditions. (A) Sterile distilled water (negative control); (B) 0.1mM benzothiadiazole (positive control); and (C) HK34.

3.3. Population of HK34 in soil and root of P. ginseng

To assess the efficacy of HK34 to survive and establish in soil conditions, the population was estimated in greenhouse and field soil. When greenhouse soil material was treated with an initial inoculum of 10 mL HK34 suspension (1×10^5 cfu/mL), the population increased within 1 week (approximately 1×10^7 cfu/g of soil) and was maintained at approximately the same level until 4 weeks of incubation (Fig. 8). Similarly, the population of HK34 was estimated to be approximately 1×10^6 cfu/g of soil in a preplant field, which was also steadily maintained until 4 weeks. Root colonization studies with 1-year-old *P. ginseng* plants showed that when treated with an initial inoculum of 10 mL (1×10^5 cfu/mL) HK34 suspension, the population was maintained at 4.7×10^5 cfu/g root after 1 month of growth (Table 2).

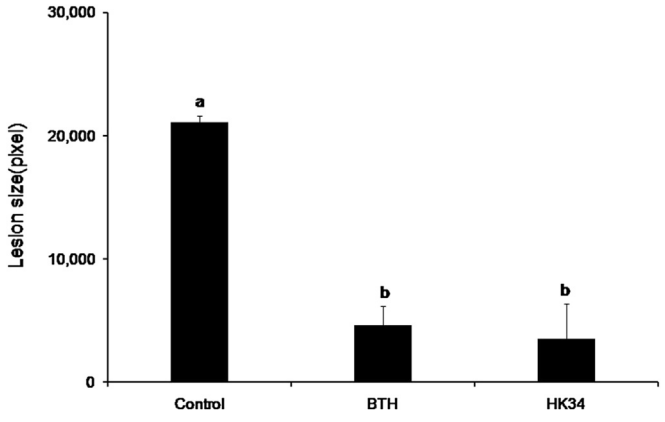

Fig. 5. Effect of HK34 as a priming treatment performed two times in the early stage of growth on the lesion area of leaves challenged with *Phytophthora cactorum* compared with the sterile distilled water-treated control and benzothiadiazole (BTH). Different letters on each bar represent values that are statistically different ($p = 0.05$). The vertical bars indicate standard error.

3.4. RT-PCR analysis of selected defense-related marker genes in HK34-treated plant leaves

The effect of HK34 treatment on the expression of selected defense-related markers genes of ISR was tested in leaves of treated plants. Analysis of defense-related genes for ISR against *P. cactorum* resulted in a significant upregulation of *PgPR5*, *PgPR10*, and *PgCAT* in leaves of plants treated with HK34 compared with untreated controls and only pathogen-treated plants (Fig. 9). Relative expression of *PgPR10* was highest (approximately 5-fold increase) in HK34-treated plants compared with the control followed by *PgPR5* (approximately 4-fold increase) and *PgCAT* (approximately 2-fold increase). In plants treated with BTH (positive control) expression of these genes was also greater compared with the control and pathogen treatment. However, the maximum increase in expression was evident in the HK34 treatment.

4. Discussion

Different species of bacilli ($n = 78$) were isolated from *P. ginseng* roots, indicating wild ginseng rhizosphere as a rich source of *Bacillus*. Studies have indicated an abundant distribution of *Bacillus* in the rhizosphere of *P. ginseng* than that in the nonrhizosphere soil [27]. Endophytic *Bacillus* sp. from ginseng has also been reported to have high antifungal activity against phytopathogens [28]. Study on the genetic diversity of ginseng rhizosphere revealed that populations of antimicrobial species of *Pseudomonas*, *Burkholderia*, and *Bacillus* were maximum in the early stages of plant growth and that the population gradually declined with increase in age of the plant, thereby facilitating infection by soilborne pathogens [29]. Soil bacterial communities are influenced by various factors including soil type, cropping pattern, and root exudates [30–33]. Because of

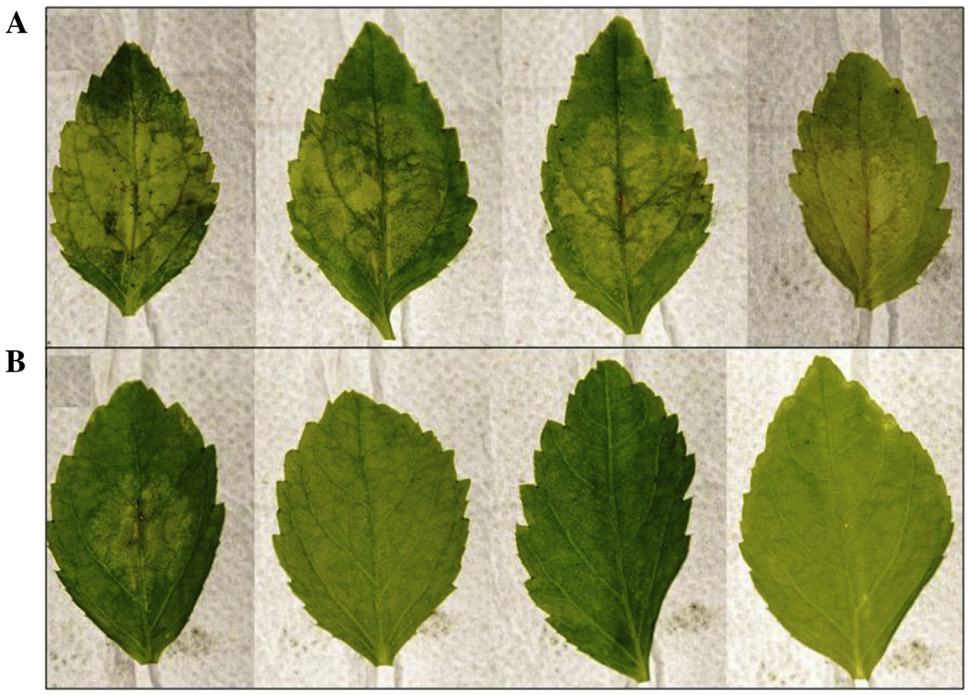

Fig. 6. Effect of HK34 against *Phytophthora cactorum* on ginseng plants under field conditions. (A) Sterile distilled water-treated control and (B) HK34.

the aforementioned factors, introduced PGPR may not be able to survive under field conditions, which thus provides inconsistent results. The present study evaluated a native rhizosphere *Bacillus* isolate of ginseng, which can adapt to the root exudates and cropping pattern of ginseng necessary for survival and establishment under field conditions. Estimation of HK34 population in greenhouse and field soils indicated the potency of this isolate to grow and multiply in different soil types. Root colonization study using field-grown ginseng plants showed the ability of HK34 to establish in ginseng roots.

Traditionally, several fungicides such as metalaxyl and mefenoxam were used to control *P. cactorum* and recent studies have indicated the efficacy of potassium phosphonate as a potential agent that induces resistance against the pathogen [34,35]. However, when chemical fungicides appied, they would be used repeatedly. Alternately, Bacilli used as biocontrol agents against fungal pathogens in ginseng have already been reported [8,13,14,28]. In this study, we identified a native isolate of *B. amyloliquefaciens* that induces resistance in ginseng plants against *Phytophthora* blight.

Fungal infection-induced activation of secondary messengers leads to transcriptional activation of defense-related *PR* genes. Application of HK34 induced expression of *PgPR10* in plants compared with the control. PR10 is a unique class of small acidic intracellular proteins induced by various biotic and abiotic stresses, which suggests their role in the defense mechanism of plants [36–38]. Studies report that challenge inoculation of ginseng with fungal pathogens such as *Botrytis* and *Alternaria* induced similar overexpression of PR10 transcripts [39]. Induction

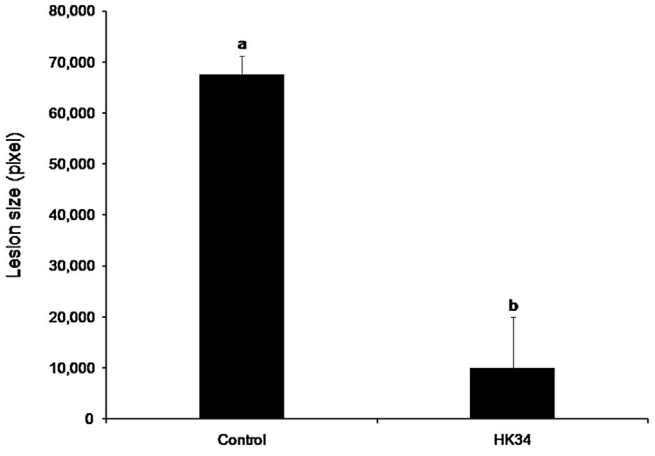

Fig. 7. Effect of the induction of systemic resistance on plants treated with HK34 under field conditions when challenged with *Phytophthora cactorum*. Different letters on each bar represent values that are statistically different ($p = 0.05$). The vertical bars indicate standard error.

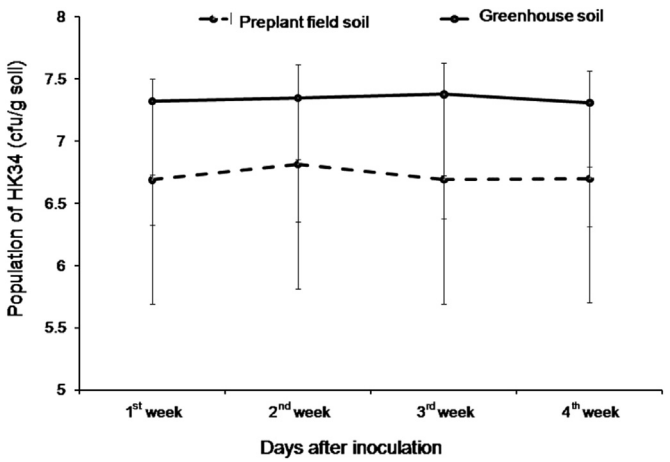

Fig. 8. Population of HK34 (cfu/g soil) in preplanting field soil and greenhouse soil at weekly intervals up to 1 month.

Table 2
Root colonization of HK34 in 2-year-old *Panax ginseng* plants after 1 month of treatment

Treatment	No. of colony (1×10^5 cfu/g)
Seedling drenched with10-mL suspension (1×10^5 cfu/mL)	4.7 ± 2.91

of *PR10* genes in response to biotic stress has been reported in other plants such as pepper and maize [40,41]. It has been reported that PgPR10 proteins from ginseng confer defense-related resistance against biotic and abiotic stresses through ribonuclease activity [25,39].

The PR5 family consists of thaumatin- and osmotin-like proteins involved in plant-defense responses [42]. Enhanced expressions of *PgPR5* in ginseng due to pathogen infection and abiotic stresses such as salinity, cold, heavy metal, and UV [26] signify its role in molecular defense mechanisms [2,42]. Abundant accumulation of *PgPR5* in vacuoles might protect against imbalance due to biotic and abiotic stresses, thereby inducing tolerance or resistance to the plants [26]. In the present study, increased expression of *PgPR5* in HK34-treated ginseng plants when challenged with *P. cactorum* indicated the induction of resistance.

Biotic and abiotic stresses induce the generation of reactive oxygen species (ROS), thus leading to the alteration of cellular redox homeostasis and oxidative stress [43]. Therefore, the plant system depends on the development of the ROS-scavenging system, which includes conversion of H_2O_2 to water and oxygen by various enzymes such as peroxidases and catalase [44,45]. Previous studies on ginseng reported a nonsignificant increase or slight downregulation of *PgCAT* during pathogen interaction [46]. A relative increase of *PgCAT* in HK34-treated plants may have helped in combating the ROS-mediated oxidative burst during interaction with *P. cactorum*.

In conclusion, it can be stated that native *B. amyloliquefaciens* isolate HK34 from ginseng is a potential candidate for biocontrol of *P. cactorum* because it upregulated the defense-related marker genes *PgPR5, PgPR10,* and *PgCAT* in treated ginseng plants, and thus elicits ISR. The efficacy was consistent under greenhouse and field-growing plants, indicating the potency to survive and act under varied conditions.

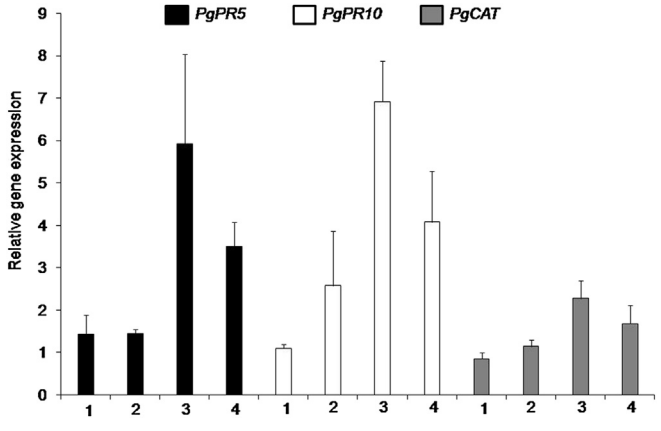

Fig. 9. Differential expression of defense-related marker genes in plants treated with HK34 analyzed through reverse transcription polymerase chain reaction. 1, Sterile distilled water (SDW) treated (negative control); 2, SDW treated and challenged with *Phytophthora cactorum* (pathogen control); 3, HK34 treated with challenge inoculation: 4, 0.1mM benzothiadiazole treated and challenged with *P. cactorum*. The vertical bars indicate standard error.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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