J Ginseng Res 45 (2021) 218–227

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research Article

Siderophore-producing rhizobacteria reduce heavy metal-induced oxidative stress in *Panax ginseng* Meyer

Yue Huo [1,3], Jong Pyo Kang [2], Jong Chan Ahn [2], Yeon Ju Kim [1,2,**], Chun Hong Piao [3], Dong Uk Yang [1], Deok Chun Yang [1,2,*]

[1] Department of Oriental Medicinal Biotechnology, College of Life Sciences, Kyung Hee University, Yongin, 17104, Republic of Korea
[2] Graduate School of Biotechnology, College of Life Sciences, Kyung Hee University, Yongin, 17104, Republic of Korea
[3] College of Food Science and Engineering, Jilin Agricultural University, Changchun, 130118, PR China

ARTICLE INFO

Article history:
Received 29 July 2019
Received in Revised form
27 December 2019
Accepted 30 December 2019
Available online 7 January 2020

Keywords:
Heavy metal resistance
Oxidative stress
Panax ginseng
Siderophore-producing rhizobacteria

ABSTRACT

Background: *Panax ginseng* is one of the most important medicinal plants and is usually harvested after 5 to 6 years of cultivation in Korea. Heavy metal (HM) exposure is a type of abiotic stress that can induce oxidative stress and decrease the quality of the ginseng crop. Siderophore-producing rhizobacteria (SPR) may be capable of bioremediating HM contamination.
Methods: Several isolates from ginseng rhizosphere were evaluated by *in vitro* screening of their plant growth-promoting traits and HM resistance. Subsequently, *in planta* (pot tests) and *in vitro* (medium tests) were designed to investigate the SPR ability to reduce oxidative stress and enhance HM resistance in *P. ginseng* inoculated with the SPR candidate.
Results: *In vitro* tests revealed that the siderophore-producing *Mesorhizobium panacihumi* DCY119^T had higher HM resistance than the other tested isolates and was selected as the SPR candidate. In the *planta* experiments, 2-year-old ginseng seedlings exposed to 25 mL (500 mM) Fe solution had lower biomass and higher reactive oxygen species level than control seedlings. In contrast, seedlings treated with 10^8 CFU/mL DCY119^T for 10 minutes had higher biomass and higher levels of antioxidant genes and nonenzymatic antioxidant chemicals than untreated seedlings. When Fe concentration in the medium was increased, DCY119^T can produce siderophores and scavenge reactive oxygen species to reduce Fe toxicity in addition to providing indole-3-acetic acid to promote seedling growth, thereby conferring inoculated ginseng with HM resistance.
Conclusions: It was confirmed that SPR DCY119^T can potentially be used for bioremediation of HM contamination.

1. Introduction

Panax ginseng, also called Asian or Korean ginseng, is one of the most important and commonly used of the ginsengs [1]. Ginseng is used to maintain body homeostasis and has been found to contain several active compounds including ginsenosides, acid polysaccharides, phenols, and polyethylene compounds that confer many of its pharmacological effects including antioxidative, anticancer, antistress effects, and enhancement of immune system and liver function [2,3]. *P. ginseng* Meyer cultivated in Korea is usually harvested after 4 to 6 years of cultivation; the soil it is grown in is loamy, deep, and well-drained and one of the best habitats for rhizospheric microorganisms [4]. There are a variety of microbes in the ginseng rhizosphere soil that benefit plant growth and ginseng metabolism. Rhizosphere bacteria (rhizobacteria) may influence plants in a direct or indirect manner. For example, rhizobacteria can increase plant growth by supplying nutrients and hormones, producing siderophores to reduce heavy metal (HM) toxicity, and raising induced systemic resistance [5]. Several Korean research groups have isolated novel bacteria strains from the soils in which

* Corresponding author. Graduate School of Biotechnology, College of Life Sciences, Kyung Hee University, Seocheon-dong, Giheung-gu, Yongin-si, Gyeonggi-do, 17104, Republic of Korea.
** Corresponding author. Graduate School of Biotechnology, College of Life Sciences, Kyung Hee University, Seocheon-dong, Giheung-gu, Yongin-si, Gyeonggi-do, 17104, Republic of Korea.
E-mail addresses: yeonjukim@khu.ac.kr (Y.J. Kim), dcyang@khu.ac.kr (D.C. Yang).

https://doi.org/10.1016/j.jgr.2019.12.008
p1226-8453 e2093-4947/$ – see front matter

ginseng has been cultivated and screened these strains for their ability to promote plant growth [6,7]. Cultivated ginseng is very vulnerable to abiotic stresses (such as salt, drought, and HM stress) over its long cultivation period, and these stressors can reduce ginseng quality over its 4–6 years of cultivation [8].

HM contamination of soil is an important abiotic stress to plants in addition to being an environmental, ecological, and nutritional problem. HM pollution is the important problem for environmentalists because HMs are not degraded by chemical or biological methods, making them difficult to be removed from the environment. Compared with other pollutants, the toxic effects of HMs last longer [9,10]. Iron is an essential micronutrient for plants and is involved in several fundamental processes such as photosynthesis, respiration, and DNA and hormone syntheses. However, when absorbed in excess, this metal can shift the cell redox balance to a pro-oxidant state, causing alterations in the morphologic, biochemical, and physiological characteristics of plants and generating oxidative stress. Plants are greatly affected by HM stress, especially in polluted soils. Plant survival becomes difficult, and the health of plants is impaired under HM stress. Remediation of HM contamination in soil has been achieved using physical and chemical processes; however, these processes are costly, non-sustainable, and time-consuming [11]. Plant growth-promoting rhizobacteria (PGPR) are plant-associated microorganisms that have been researched for their abilities to bioremediate HM pollution of soils and to promote plant growth in HM-contaminated soils [12]. Among various PGPRs, siderophore-producing rhizobacteria (SPR) can improve nutrient uptake and promote plant growth under conditions of HM stress.

Our goals in the study were to isolate and characterize several potential novel rhizobacteria as SPR candidates and to investigate if the chosen candidate enhanced the resistance of *P. ginseng* seedlings to HM stress. After identification of a candidate, namely *Mesorhizobium panacihumi* DCY119^T, ginseng seedlings were inoculated with or without this candidate and cultivated under Fe stress. Reactive oxygen species (ROS) scavenging activity and antioxidant chemical production by the plants were then assessed. Additionally, microbial antioxidant activity, indole-3-acetic acid (IAA) production, and siderophore production of this strain were assessed.

2. Materials and methods

2.1. Isolation and characterization of isolates

Bacterial strains were isolated from rhizospheric soil samples from fields in which ginseng are cultivated in Gochang-gun (35° 26′ 89″ N 126° 42′ 740″ E) and Gyeonggi province (37° 14′ 45″ N 127° 05′ 00.6″ E), Republic of Korea. Genomic DNA was extracted and purified using a DNA isolation kit (Gene All Biotechnology, Seoul, Republic of Korea). 16S rRNA gene sequences were amplified by Genotech (Daejeon, Republic of Korea) using the previously described methods [13]. 16S rRNA gene sequences were compiled using Seq-Man software (DNASTAR) and compared with 16S rRNA gene sequences available in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net).

2.2. In vitro screening of plant growth promotion

Siderophore production was assessed using the chrome azurol S agar assay [14]. Siderophore production was confirmed by a change in color of the halo around colonies in blue–green medium from yellow to red. Phosphate solubilizing ability was verified by the plate screening method developed by Pikovskaya [15]. A clear halo region around the colonies in an opaque medium indicated a positive phosphate solubilization result. Quantitative testing of IAA production was investigated [16] with slight modifications; each strain was cultured by shaking in modified King B broth with and without L-tryptophan (3 mg/mL), after mixing with Salkowski reagent, and optical density (OD) values at 535 nm were read to analyze the production of IAA after 7 days of incubation.

2.3. Determination of heavy metal resistance of the isolated bacterial strains

Strains were grown in Luria Bertani (LB) broth medium supplemented with different metal salts ($FeCl_3$, $CdCl_2 \cdot 2.5H_2O$, $HgCl_2$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, and $AlCl_3 \cdot 6H_2O$) as previously described [17,18]. An 1% (v/v) overnight culture solution inoculated with a single colony was added to 5 mL of LB broth supplemented with different concentrations (25 μM to 300 μM) of each tested metal salts solution. Strains cultured in the same media without metals served as a control. Metal resistance was evaluated according to growth measured at 600 nm within 0-54 h. If growth was detected during inoculation, the concentration of the selected metal was increased to 50 mM. Inhibitory concentrations were measured using a spectrophotometer at an absorbance of 600 nm against an LB broth (blank) containing the same amount of HM [19]. The resistance of the novel strains isolated from the ginseng rhizosphere was evaluated by calculating the concentration at which there was 50% inhibition of growth (IC_{50}) [20,21].

2.4. Correlation of optical density at 600 nm and colony forming units

M. panacihumi DCY119^T was incubated in Yeast Mannitol Agar at 30°C for 24 h. After centrifugation at 3000 g for 10 min, precipitated cells were dissolved using sterilized distilled water (SDW) to create serial dilutions with different OD_{600} values. The different OD values suspensions were smeared on agar plates in triplicate. Colony forming units (CFUs) were counted after incubation at 30°C for 2 days [22].

2.5. Compatibility of strain DCY119^T with 2-year-old ginseng seedlings

Based on the correlation between OD_{600} and CFU/mL determined in Section 2.4, 10^4–10^{12} CFU/mL of DCY119^T suspensions were prepared. Two-year-old seedlings were first dipped into the different bacterial suspensions for 10 min and then planted in pots (11 cm high and 11 cm diameter) filled with a sterilized soil mixture (vermiculite, perlite, and peat moss at a 3:1:1 ratio to which 25% SDW (v/v) had been added). Seedlings dipped into water without bacteria were the negative control. Each treatment group was replicated in three pots, and each pot contained five seedlings. The temperature was controlled at 25±2°C, and moisture levels were maintained at 60 ± 5%. The photoperiod was adjusted to 16 h of day-time and 8 h of night-time with lamps (Philips TLD-RS-FLR32SSEX-D 865K) that provided 9500 lux for each covered area within the chambers. Seedlings were watered once weekly with sterilized tap water, which was applied to the bottom of the pots. The symptom development was observed 10 days after inoculation (when leaves of seedlings were opened), sampling and morphological observations were conducted. The biomass (fresh weight [FW] and dry weight [DW]) of each group was also measured for compatibility analysis.

2.6. Assessment of the iron tolerance level of 2-year-old ginseng seedlings

Based on previous reports of the Fe concentration in multi-contaminated soil (139.045 g/kg) and the 0.3–0.8 (3.483–11.098 g/kg) rusty root index of ginseng rhizosphere soil [23], 25 mL $FeCl_3$ solution (250 mM, 500 mM, or 1000 mM) was added to each sprouted (after 10 days) seedling pot as same as watering method. For the control, SDW was added instead of the $FeCl_3$ solution. Cultivation conditions of pots assay are described in Section 2.5. After symptoms appeared, seedlings were collected to record their morphological appearance and for further analysis. Each treatment was replicated in three pots with five seedlings per pot.

2.7. In planta pot tests of resistance of P. ginseng inoculated with DCY119^T to Fe stress

2.7.1. DCY119^T inoculation and Fe stress treatment

Strain DCY119^T was identified as the most promising candidate for enhancing ginseng growth (at concentrations up to 10^8 CFU/mL) and was therefore selected for *P. ginseng* inoculation. Seedlings were inoculated with DCY119^T as mentioned in Section 2.5. Twenty-five milliliters of 500 mM $FeCl_3$ solution was added to each pot to induce Fe stress. After symptoms appeared, plants were harvested and analyzed. Four different experimental groups were analyzed: control (non-inoculation and no Fe stress); bacterial treatment (DCY119^T inoculation without Fe stress); Fe stress treatment (non-inoculation with Fe stress); and bacteria + Fe stress treatment (DCY119^T inoculation with Fe stress). Each treatment was replicated in five pots with five seedlings per pot.

2.7.2. Sampling and morphological observation

On the day of sampling, all seedlings were compared and assigned different grades based on their symptoms, and the disease severity index was then calculated. Some parts of the seedlings were immediately immersed in a liquid nitrogen box and stored at −70°C in a deep freezer for RNA isolation and H_2O_2 and malondialdehyde (MDA) determination. Other parts of the seedlings were used for morphological observation and measurement of growth parameters such as the FW and DW of the samples and the total phenolic, total flavonoid, total soluble sugar (TSS), and reducing sugar contents. The ginseng samples were first dried at 50°C to obtain a constant weight for biomass determination.

2.7.3. Quantitative H_2O_2 and MDA measurements

H_2O_2 content was detected using the previous protocol [24] with modification; 0.2 g of fresh tissue was homogenized at 4°C in 2 mL 0.1% trichloroacetic acid (TCA, *w/v*). The supernatant was collected after centrifugation at 12,000 *g* for 15 min at 4°C. Reaction mixes (1 mL) comprised 250 μL sample supernatant, 250 μL phosphate buffer (10 mM, pH 7.0), and 500 μL KI (1 M). Absorbance values at 390 nm of each sample were measured. In this study, an H_2O_2 standard curve with a concentration ranging from 0.05 to 0.1 mM was used to calculate H_2O_2 content. Experiments were carried out in triplicate.

MDA was measured to assess lipid peroxidation following the method described in [25] with modification. Briefly, 250 μL of the supernatant homogenized in 0.1% TCA was mixed with 500 μL thiobarbituric acid agent (0.5% thiobarbituric acid in 20% TCA). The reaction solution was incubated for 30 min in boiling water and cooled rapidly in an ice box. After centrifugation at 10,000 g for 5 min, the absorbance at 532 nm and 600 nm was determined. After subtracting nonspecific absorbance at 600 nm, MDA content in each group was calculated according to the molar extinction coefficient of MDA (155 mM^{-1} cm^{-1}) [26] and expressed as nmol MDA g^{-1} FW.

2.7.4. Determination of total phenolic and total flavonoid contents

Total phenolics and total flavonoids in each group were detected using a previously described method [27] with modification. Briefly, 0.5 g of dried material was extracted by 10 mL 80% methanol for 1 h, and this was repeated three times, after which the filtrate was combined for evaporation. The crude extract was redissolved in DW for further compound analysis. For total phenolics analysis, 0.3 mL of extract solution was added to 1.5 mL Folin-Ciocalteu reagent (10× dilution of 2 N) and allowed to incubate for 5 min after shaking thoroughly. One milliliter of 7.5% Na_2CO_3 solution was added, and the mixture was allowed to sit for 30 min in the dark, and then, the absorbance of the samples was measured at 715 nm. Total phenolic content was calculated from a standard curve using gallic acid as the standard. For total flavonoid measurement, the reaction mixture contained 0.3 mL extract solution, 0.3 mL 5% $NaNO_2$, and 0.3 mL 10% $AlCl_3$. After mixing well, the mixture was allowed to sit for 6 min, and then 0.5 mL 1 N NaOH was added. The solution was mixed well, and absorbance was measured immediately at 510 nm. Total flavonoid content was calculated with a calibration curve based on rutin.

2.7.5. Determination of total soluble sugar and reducing sugar contents

The content of TSS in the dried material was detected using Irigoyen's method [28]. One milliliter of extract solution from Section 2.7.4 was mixed with 5 mL anthrone reagent and incubated in boiling water. The absorbance value of the mixture was measured at 625 nm. Reducing sugar content was determined following the 3,5-dinitrosalicylic acid (DNS) method [29]. One milliliter of extract solution was mixed with 1 mL DNS reagent. The mixture was incubated in boiling water for 5 min and cooled at room temperature. The absorbance was recorded at 550 nm. TSS and reducing sugars contents were calculated from standard curves prepared using several concentrations of glucose.

2.7.6. RNA extraction and RT-PCR analysis

Total RNA was isolated from frozen samples using TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH), and 1 μg of isolated RNA from each sample was reverse transcribed with RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas, Waltham, MA) following the manufacturer's instructions. RT-PCR was performed in a reaction volume of 20 μL consisting of 2 μL of the synthesized cDNA, 10 pmol of forward and reverse primers of each target gene (Supplementary Table S1), 10 μL of Premix Taq™ DNA Polymerase (Takara Bio USA, Inc., Mountain View, CA), and water up to 20 μL using a MyCycler™ thermal cycler (BioRad, Hercules, CA). An initial denaturation step was performed at 95°C for 5 min, followed by 30–35 cycles of 95°C for 45 s, 56–62°C for 60 s, and 72°C for 90 s. A final elongation step was performed at 72°C for 5 min. PCR products were electrophoresed through 1.5% agarose gels for visual analysis. Glyceraldehyde 3-phosphate dehydrogenase was used as a control [30].

2.8. In vitro medium assay of the Fe resistance of DCY119^T

2.8.1. Growth of DCY119^T in Fe-medium

Two types of Fe media were assessed: normal culture medium supplemented with different concentrations (0 to 32 mM) of $FeCl_3$ as described in Section 2.3 and Fe-contaminated soil (see Section 2.7.1) that was extracted using DW. After filter-sterilizing the media through a 0.22 μm filter, a series of soil extract media mixed with the normal medium was prepared (0, 25%, 50%, 75%, 100%). Strain

Table 1
In vitro assessment of plant growthpromoting traits and antagonism of the novel strains

Stain	Siderophore production	Phosphate solubilization	Antifungal activity		IAA Concentration (μg/mL)	
			C. destructans KACC 44660^T	*F. solani* KACC 44891^T	Without L-tryptophan	With L-tryptophan
DCY112^T	w	+	w	+	1.5 ± 0.08	1.6 ± 0.04
DCY115^T	+	+	+	+	-	-
DCY117^T	+	-	-	+	-	-
DCY118^T	w	-	+	+	-	3.0 ± 0.14
DCY119^T	++	+	-	-	4.7 ± 0.74	17.6 ± 1.63

IAA, indole-3-acetic acid
+, positive; -, negative; w, weak activity; ++, stronger activity

DCCY119^T was inoculated in the different growth media, and OD value at 600 nm was detected and used to express the growth of bacteria after shake culture at 30°C for 48 h–54 h.

2.8.2. Production of IAA and siderophores and 2,2-diphenyl-1-picrylhydrazyl scavenging activity of isolated bacterial strains

IAA and siderophore assays were performed as described in Section 2.2. Antioxidant activity was detected using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method of Liu and Pan [31]. The supernatant of each bacteria culture suspension was centrifuged at 10,000 rpm for 10 min, passed through a sterile membrane filter (0.45 μm), and then 0.5 mL of the filtrate was mixed with 1 mL 1 mM DPPH solution and incubated for 20 min at room temperature. The absorbance value of each sample was measured at 517 nm. DPPH solution without sample was used as a control, and ascorbic acid was used as the standard. DPPH free radical scavenging activity of each sample was calculated by the following formula:

$$\%\,\mathrm{DPPH\,scavenging\,activity} = \left(A_{\mathrm{Control}} - A_{\mathrm{Sample}}\right) \Big/ A_{\mathrm{Control}} \times 100$$

3. Results and discussion

3.1. Molecular identification of bacteria

The following five PGPR strains candidate for SPR were isolated from the ginseng rhizosphere: DCY112^T, DCY115^T, DCY117^T, DCY118^T, and DCY119^T. Based on 16S rRNA gene sequence analysis, the isolates belonged to the following genera: *Rhodanobacter*, *Paraburkholderia*, *Lysobacter*, *Ornithinimicrobium*, and *Mesorhizobium*. Strain information is provided in Supplementary Table S2.

3.2. In vitro assessment of plant growth promotion

The plant growth–promoting activities of the novel strains are shown in Table 1. After 7 days of incubation, strain DCY112^T produced 1.5 ± 0.08 μg/mL (without L-tryptophan) and 1.6 ± 0.04 μg/mL (with L-tryptophan) IAA, strain DCY119^T produced 4.7 ± 0.74 μg/mL (without L-tryptophan) and 17.6 ± 1.63 μg/mL (with L-tryptophan) IAA. Strains DCY115^T, DCY117^T, and DCY118^T did not produce IAA. Strain DCY119^T, which produced IAA when supplied with L-tryptophan, was identified as a potential producer of plant growth regulating hormones. Strains DCY112^T, DCY115^T, and DCY119^T were able to solubilize phosphate as evidenced by a clear halo around the colonies. All novel strains were able to produce siderophores, but strain DCY119^T was the strongest producer among the tested strains. Previous studies have demonstrated that the siderophores production activity of strains is directly correlated with enhanced HM resistance and indirectly promotion of plant growth by IAA production ability against HM toxicity [32,33].

3.3. In vitro assessment of heavy metal–resistant bacteria

HM resistance of the bacteria was next determined. Microbial turbidity decreased with an increased concentration of HM. The IC_{50} values (Fig. 1) of each bacterial strain were calculated [21]. Among all the tested strains, DCY119^T was the most resistant to HM in the order of Fe > Al > Cu > Cd > Co > Hg. Given these results, we

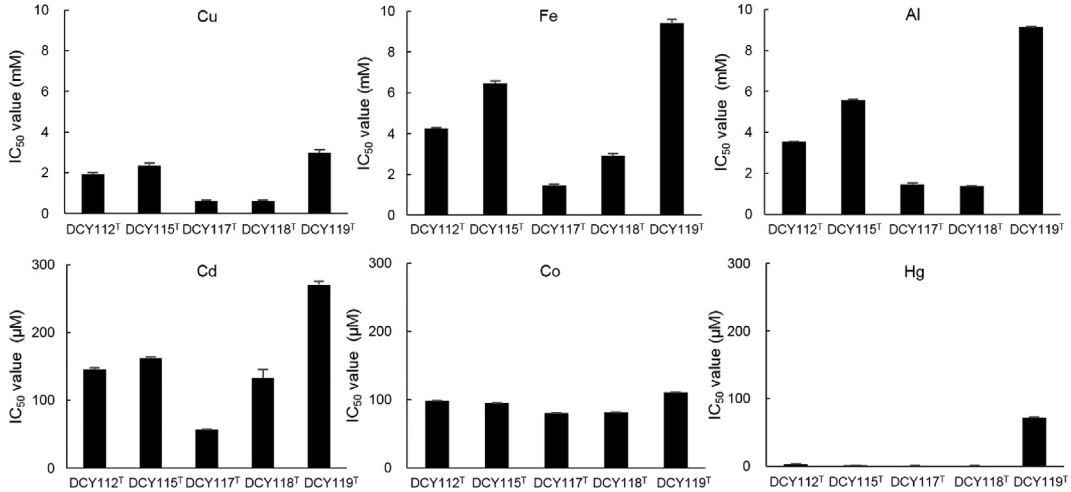

Fig. 1. Concentrations of heavy metals at which a 50% (IC_{50}) decrease in microbial growth was observed. After 54 hours of shaking incubation of DCY119^T in different metal concentration mediums, the OD values at 600 nm of each group were measured for calculation of IC_{50}. OD, optical density.

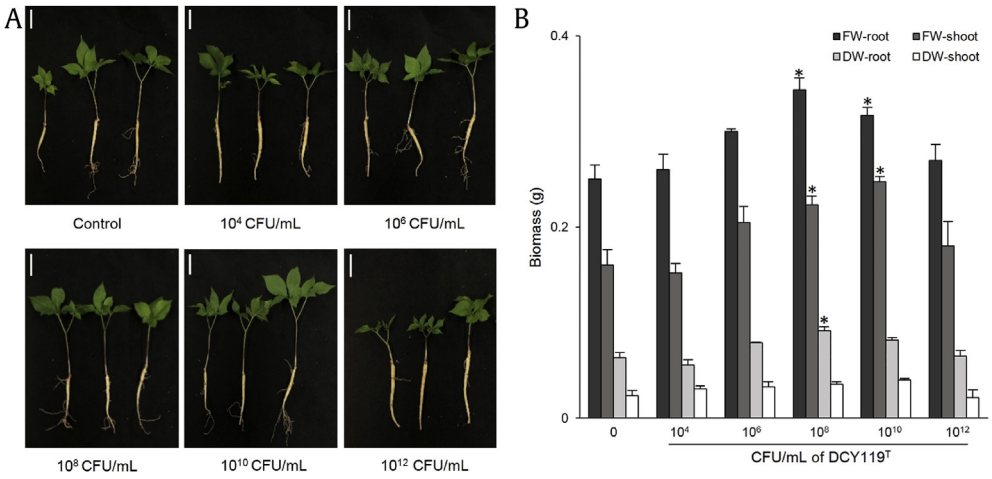

Fig. 2. (A)*In planta* compatibility of DCY119^T with 2-year-old *P. ginseng* seedlings. Each group included three pots, each with five seedlings. Bar, 2 cm. (B) Biomass (fresh and dry weights) of *P. ginseng* seedlings inoculated with various concentrations of DCY119^T. Error bars indicate the standard deviation (n = 3). Statistical significance using Student *t* test was assigned at "*" for $p < 0.05$. FW, fresh weight; DW, dry weight.

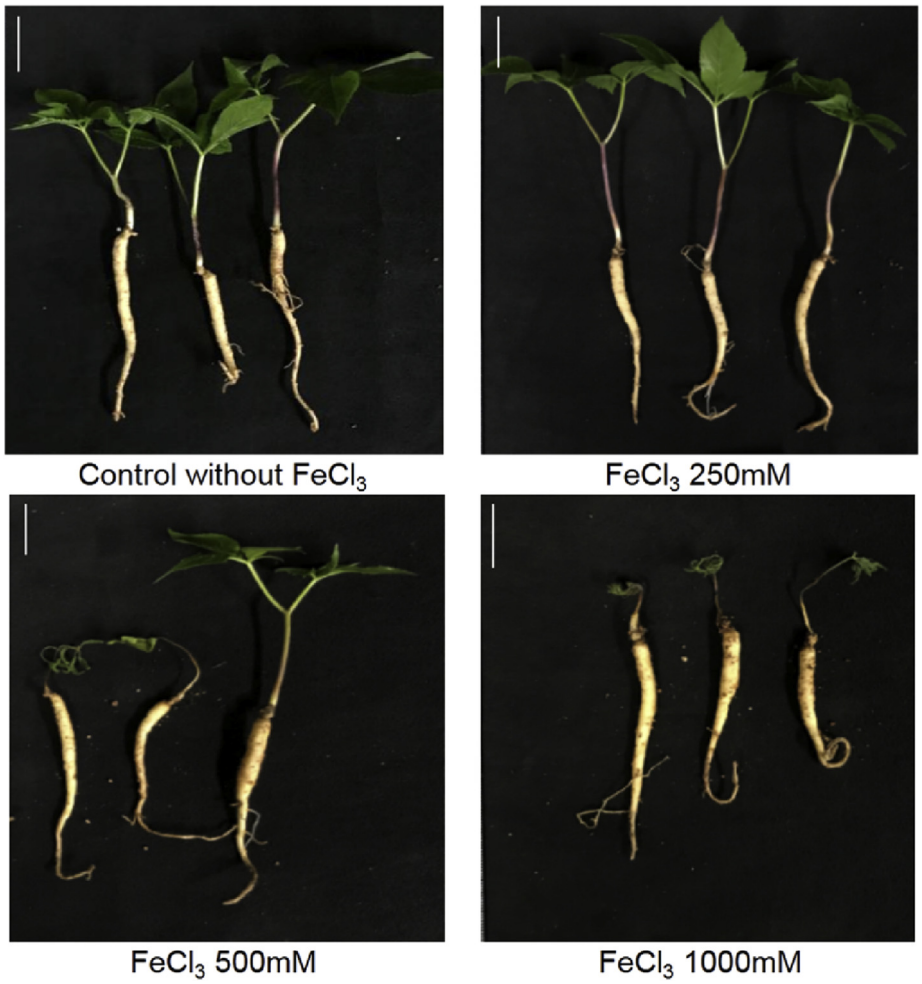

Fig. 3. Morphological appearance of *P. ginseng* in response to different Fe stress levels. Each group included three pots with five seedlings per pot. After symptoms appeared, the morphological appearance of ginseng seedlings in each group was assessed. Bar, 2 cm.

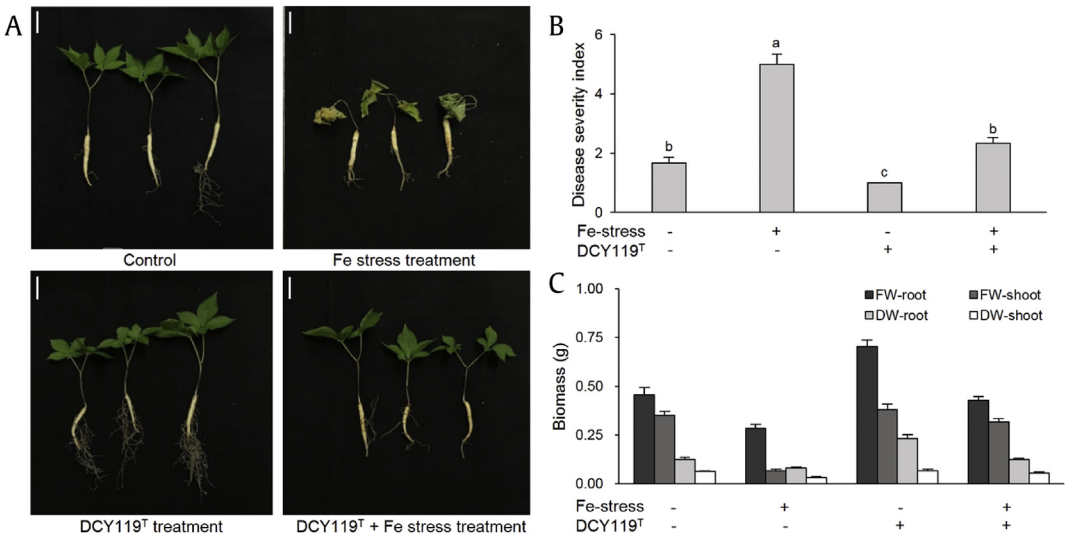

Fig. 4. (A) Morphological observation of *P. ginseng* seedlings inoculated with or without bacteria and then exposed to Fe. Each group included five pots with five seedlings per pot. Bar, 2 cm. (B) Disease severity index values of roots with or without inoculated bacteria then exposed to Fe stress were determined. Bars with the same letters are not significantly different at $p < 0.05$. Scale bar: 2 cm. (C) Root and shoot biomass analysis of *P. ginseng* seedlings inoculated with bacteria and then exposed to Fe. FW, fresh weight; DW, dry weight. Values represent the mean ± SD from three independent experiments. Fe-stress, 500 mM $FeCl_3$ treatment.

selected the siderophore-producing, HM-resistant bacterium DCY119^T for further study.

3.4. Compatibility of *Mesorhizobium panacihumi* DCY119^T with *P. ginseng*

By identifying the correlation between OD_{600} and CFU/mL, the required bacterial inoculant amount can be prepared relatively effectively (i.e., there is no need to wait for colony counts on an agar plate). The relationship between OD_{600} values and CFU/mL is shown in Table S3.

After bacterial inoculation for 10–14 days in a pot, the compatibility of DCY119^T with *P. ginseng* was assessed by investigating morphological alterations according to the concentration of bacterial suspension (approximately 0, 10^4, 10^6, 10^8, 10^{10}, 10^{12} CFU/mL) as shown in Fig. 2A. No disease symptoms were observed in any group. However, seedlings in the 10^{12} CFU/mL treatment showed inhibited growth compared with the control seedlings. After sampling, the biomass of fresh and dry root and shoots were determined (Fig. 2B). DCY119^T at 10^8 CFU/mL promoted seedling growth (largest biomass). For this reason, 10^8 CFU/mL of strain DCY119^T was selected for further experiments.

3.5. Assessment of the Fe tolerance level of 2-year-old ginseng seedlings

Seedlings were stressed with different concentrations of Fe (0, 250, 500, and 1000 mM) based on previous reports. After symptoms appeared, the morphological appearance of ginseng seedlings in each group was assessed and is shown in Fig. 3. The growth of ginseng seedlings was fully hindered when they were exposed to 1000 mM Fe. Disease symptoms appeared consistent with those seen in the plants suffering from iron toxicity [34,35], and root and shoot growth was inhibited as the previous description that HM stress inhibited growth and decreased the biomass of plants [34,36]. At 500 mM Fe, ginseng seedlings gradually developed

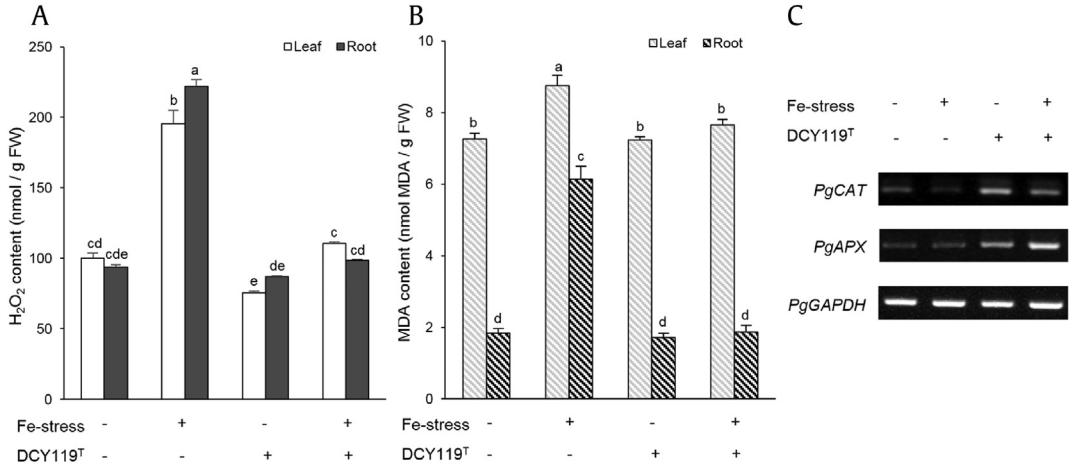

Fig. 5. Estimation of H_2O_2 (A) and MDA (B) contents of each group. Values represent mean ± SD from three independent experiments. Bars with the same letters are not significantly different at $p < 0.05$. (C) Expression of ROS scavenging-related genes and a housekeeping gene (C). *CAT*, catalase; *APX*, ascorbate peroxidase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Fe-stress, 500 mM $FeCl_3$ treatment; FW, fresh weight; MDA, malondialdehyde; ROS, reactive oxygen species.

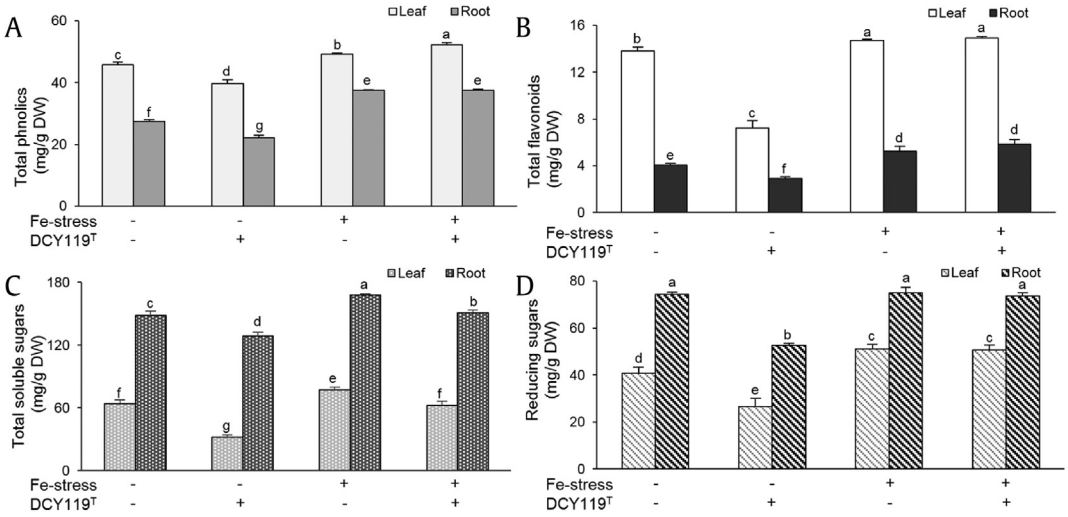

Fig. 6. Estimation of total phenolic (A), total flavonoid (B), total soluble sugars (C), and reducing sugars (D) contents. Values represent the mean ± SD from three independent experiments. Bars with the same letters are not significantly different at $p < 0.05$. DW, dry weight. Fe-stress, 500 mM $FeCl_3$ treatment.

yellowing on the leaves, and eventually, there was complete wilting of the foliage. Rusty root was also observed, which supports the hypothesis that Fe can induce rusty disease in ginseng roots [23]. We used 500 mM Fe as the HM stress for further study.

3.6. Induction of Fe stress resistance in *P. ginseng* by strain DCY119^T

3.6.1. Morphological observations and evaluation of the disease severity index of seedlings under Fe stress

The morphological appearance of seedlings after sampling is shown in Fig. 4A. Fe toxicity directly inhibited ginseng seedling growth, while seedlings inoculated with DCY119^T showed significantly reduced Fe stress and grew to a similar extent to control (unstressed) seedlings. To calculate disease severity index (DSI), all roots were graded from 1-6 using the scale described in Supplementary Table S4. DSI was normalized for each isolate using the following equation: $DSI = [(X_1 \times 1) + (X_2 \times 2) + (X_3 \times 3) + (X_4 \times 4) + (X_5 \times 5) + (X_6 \times 6)] / (X_1 + X_2 + X_3 + X_4 + X_5 + X_6)$, where X_1, X_2, X_3, X_4, X_5, and X_6 are the number of plants with disease severity scores of 1, 2, 3, 4, 5, and 6, respectively. Based on the described disease symptoms in Table S4, the DSI of each group was calculated and is shown in Fig. 4B. As expected, DCY119^T-inoculated groups had some of the lowest DSI values. Several reports have indicated that SPR-like DCY119^T can stimulate plant growth, leading to healthy plants and increased plant yields [37,38]. The previous reports indicated that Korean ginseng inoculated with *P. simiae* N3, *B. ginsengiterrae* N11-2, and *C. polytrichastri* N10 were able to support seedlings growth and increase biomass under aluminum stress, which was similar to the results in this study [39]. Stressed seedlings inoculated with DCY119^T did not show any symptoms and grew significantly better than the Fe-treated noninoculated group, with no significant difference in growth from the control group. These results demonstrated that DCY119^T promotes ginseng growth and protects seedlings against Fe stress.

3.6.2. Determination of the shoot and root biomass of *P. ginseng* seedlings under Fe stress

Shoot and root biomass (FW and DW) are shown in Fig. 4C. Under nonstressful conditions, seedlings inoculated with DCY119^T had higher seedling biomass than control seedlings. This result suggests that SPR-DCY119^T promotes ginseng growth as a potential PGPR, consistent with the results presented in Section 3.2 and 3.4. HM stress as an abiotic stress can decrease plant yields, which we confirmed in this study. However, strain DCY119^T can protect seedlings against Fe toxicity, thereby preventing a decrease in the biomass of ginseng. These results in this study were supported by the published report that plant growth–promoting bacteria as the inoculant of stressed plant could prevent the biomass decrease and improve the plant productivity under stressful environments [40,41].

3.6.3. Effects of H_2O_2, lipid peroxidation accumulation, and induction of oxidative stress

Various abiotic stressors, especially HM stress, can lead to overaccumulation of ROS (such as hydrogen peroxide, H_2O_2) in plants. ROS are highly reactive and toxic as they oxidize proteins, lipids, and carbohydrates and damage DNA [42,43]. H_2O_2 was useful as the secondary messenger in plant signaling networks and could trigger response to various environmental stresses in plants [44]. However, an excessive amount of H_2O_2 might have activated cell-to-cell signaling as a warning message to other cells, furthermore, high H_2O_2 accumulation was found in stressed plants and caused oxidative stress. Sukweenadhi et al. [40] reported that excess H_2O_2 level was found in salt-stressed ginseng seedlings compared with control seedlings, and it could be decreased by antioxidant enzyme expression in bacteria-inoculated stressed seedlings. In this study, H_2O_2 production was investigated (Fig. 5A). Fe stress increased the H_2O_2 content of seedlings, indicating that Fe stress induced ROS accumulation and inhibited growth. However, seedlings inoculated with DCY119^T exhibited a significant decrease in H_2O_2 production relative to those not inoculated with this strain, indicating that DCY119^T protected the seedlings against ROS accumulation in response to Fe toxicity. Lipid peroxidation is a damaging process that occurs in plants in response to various stresses. The content of MDA, widely used to determine lipid peroxidation level, was also analyzed in this study, and the results are shown in Fig. 5B. Fe-stressed seedlings produced higher amounts of MDA, while inoculation with DCY119^T decreased MDA levels in seedlings.

These results demonstrated that Fe stress increased the H_2O_2 and MDA contents of seedlings and decreased the biomass of the roots and shoots, resulting in growth inhibition of the seedlings. Seedlings inoculated with DCY119^T were significantly protected

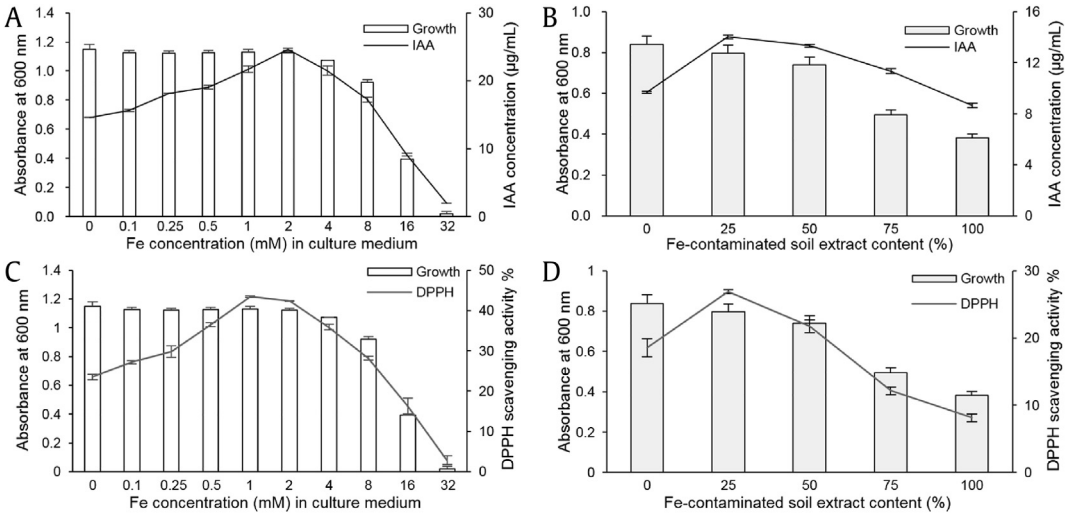

Fig. 7. Growth and IAA production of DCY119^T in media containing different amounts of Fe. (A) Bacteria grew in culture medium supplemented with different concentrations of Fe solution. (B) Bacteria grew in different Fe-contaminated soil extract medium diluted with culture medium. Growth and DPPH scavenging activities of DCY119^T in media containing different amounts of Fe. (C) Bacteria grew in culture medium supplemented with different concentrations of Fe solution. (D) Bacteria grew in different Fe-contaminated soil extract medium diluted with culture medium. Values represent mean ± SD from three independent experiments. OD value at 600 nm was used to express the growth of bacteria. IAA, indole-3-acetic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; OD, optical density.

against oxidative stress in response to Fe exposure, indicating that DCY119^T induced antioxidant defenses in the seedlings.

3.6.4. *ROS scavenging antioxidant defenses against Fe stress*

To analyze the ROS scavenging activity of seedlings, the expression of two ROS scavenging-related genes, namely *PgAPX* and *PgCAT*, was determined, and results are shown in Fig. 5C. These genes were overexpressed in seedlings inoculated with DCY119^T compared with noninoculated groups. Higher expression of ROS scavenging-related genes would increase antioxidant activity, thereby enhancing Fe resistance [45].

Nonenzymatic antioxidants react rapidly with free radical intermediates in an autoxidation chain and prevent their progression when added in small amounts to a material. Plants with high levels of antioxidants have been reported to have greater resistance to oxidative damage than those with low levels of antioxidants. Several studies have indicated that the level of oxidative cellular damage in plants exposed to abiotic or biotic stress can be controlled by their antioxidative defense systems. Phenolic and flavonoid compounds are the primary nonenzymatic components of the antioxidant system [42]. Therefore, in addition to enzymatic antioxidant gene expression, we evaluated the level of nonenzymatic antioxidant chemicals (total phenolic and total flavonoid contents); results are shown in Fig. 6A and B. Compared with Fe-stressed seedlings, which had lower phenolic and flavonoid contents than control seedlings, seedlings inoculated with DCY119^T had significantly higher total phenolic and flavonoid contents. Therefore, DCY119^T helped activate the antioxidant system by increasing the production of nonenzymatic antioxidants, ultimately enhancing seedling resistance to Fe toxicity.

These results suggest that both the increase in expression of enzymatic antioxidant genes and the increase in the production of nonenzymatic antioxidant chemicals induced by DCY119^T protected the ginseng seedlings from oxidative stress and enhanced their resistance to Fe.

3.6.5. *Estimation of total soluble sugar and reducing sugar contents*

Sugars play an important role in plants. Sugar contents of stressed plants can be decreased in response to iron or other HM stresses [34,46]. Several reports also indicated that sugars are key osmolytes that contribute to osmotic adjustment and relate to the stress response [40,47]. Accordingly, TSS and reducing sugar contents of each group seedlings were detected (Fig. 6C and D). These results showed that sugar contents of Fe-stressed seedlings were decreased compared with control seedlings, while seedlings treated with DCY119^T showed higher sugar production than stressed seedlings without bacteria to prevent the sugar decrease

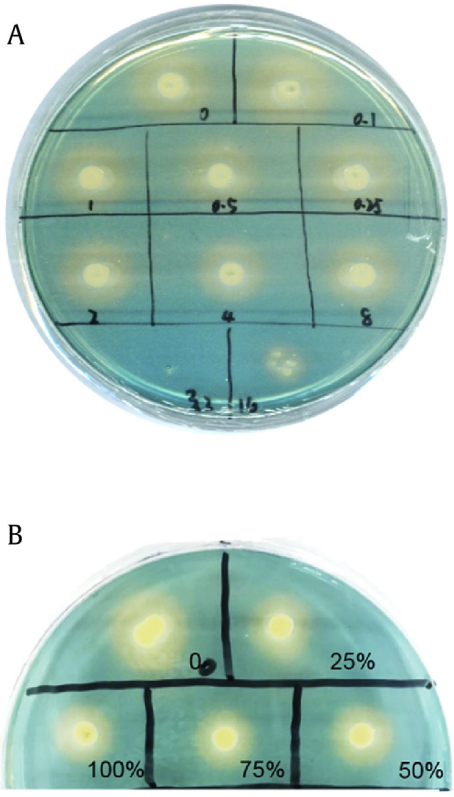

Fig. 8. Siderophores production activities of DCY119^T in different Fe medium. (A) Bacteria grew in culture medium supplemented with different concentrations of Fe solution. (B) Bacteria grew in different Fe-contaminated soil extract medium diluted with culture medium (B).

against Fe toxicity, which were indicated again that DCY119^T have the HM resistant potential to reduce Fe toxicity in ginseng.

3.7. *In vitro medium analysis of the heavy metal resistance of DCY119^T*

The growth of DCY119^T was also evaluated by culturing in culture medium supplemented with different Fe concentrations or different concentrations of extracts from Fe-contaminated soil. OD values at 600 nm were used in this study to express the growth of bacteria (Fig. 7), and DCY119^T grew in the culture medium supplemented with Fe and showed the potential Fe resistance. At 16 mM, the growth of DCY119^T was significantly starting to decrease; the MIC_{Fe} was 32 mM. Meanwhile, the growth of DCY119^T was also observed in Fe-contaminated soil extract medium, which indicates that DCY119^T can survive in Fe-contaminated soil.

Siderophores production, IAA production activity, and DPPH scavenging activity in the different growth media were evaluated, and results are shown in Figs. 7 and 8. When Fe stress in the culture medium was increased, DCY119^T could still produce IAA and siderophores and scavenge DPPH radicals. Similar results were obtained for growth in Fe-contaminated soil extract media. SPR can decrease free metal ions though binding toxic metals with siderophores and reduce oxidative stress, thereby increasing the biomass of plants exposed to HMs [48,49]. IAA produced by SPR can increase plant growth in HM-contaminated soils by inducing root proliferation and promoting nutrient and metal uptake [50].

Together, our results indicate that the siderophore-producing bacterium DCY119^T is a potential bioremediating agent that can promote ginseng growth and enhance its HM resistance.

4. Conclusions

Five PGPR-isolated strains as SPR candidates were isolated from the ginseng-rhizospheres of Gochang-gun and Gyeonggi provinces, Republic of Korea. Among the tested bacteria, the siderophore-producing bacterium *M. panacihumi* DCY119^T showed HM resistance, especially to Fe and was selected for further study. Fe-500 mM was sufficient to increase the H_2O_2 and MDA contents and induce ROS accumulation and oxidative stress in ginseng seedlings. However, preincubation of the seedlings with 10^8 CFU/mL of DCY119^T could directly and indirectly promote ginseng growth against Fe stress. When DCY119^T-inoculated seedlings exposed to Fe stress, DCY119^T produced siderophores and performed ROS scavenging to directly decrease the toxicity of seedlings and induce ROS scavenging gene overexpression and nonenzymatic antioxidant chemicals, thereby decreasing ROS accumulation and preventing oxidative stress in ginseng seedlings. In addition, DCY119^T produced IAA to enhance the nutrient uptake of seedlings, protect them from Fe-induced oxidative stress, and promote plant growth to indirectly reduce Fe stress. In conclusion, strain DCY119^T is a siderophore-producing rhizobacterium that shows the potential for bioremediation of HM contamination of ginseng.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported by a grant from the Korea Institute of Planning & Evaluation for Technology in Food, Agriculture, Forestry, & Fisheries (KIPET NO: 317007-3), Republic of Korea.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2019.12.008.

References

[1] Kim JH. Pharmacological and medical applications of *Panax ginseng* and ginsenosides: a review for use in cardiovascular diseases. J Ginseng Res 2018;42:264–9.
[2] Lee JI, Park KS, Cho IH. *Panax ginseng*: a candidate herbal medicine for autoimmune disease. J Ginseng Res 2019;43:342–8.
[3] Kim YJ, Zhang D, Yang DC. Biosynthesis and biotechnological production of ginsenosides. Biotechnol Adv 2015;33:717–35.
[4] Nguyen NL, Kim YJ, Hoang VA, Subramaniyam S, Kang JP, Kang CH, Yang DC. Bacterial diversity and community structure in Korean ginseng field soil are shifted by cultivation Time. PloS One 2016;11:e0155055.
[5] Etesami H, Maheshwari DK. Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: action mechanisms and future prospects. Ecotox Environ Safe 2018;156:225–46.
[6] Huo Y, Kang JP, Kim YJ, Yang DC. *Paraburkholderia panacihumi* sp. nov., an isolate from ginseng-cultivated soil, is antagonistic against root rot fungal pathogen. Arch Microbiol 2018;200:1151–8.
[7] Sukweenadhi J, Kim YJ, Lee KJ, Koh SC, Hoang VA, Nguyen NL, Yang DC. *Paenibacillus yonginensis* sp. nov., a potential plant growth promoting bacterium isolated from humus soil of Yongin forest. Antonie Van Leeuwenhoek 2014;106:935–45.
[8] Farh MEA, Kim YJ, Hoang VA, Sukweenadhi J, Singh P, Huq MA, Yang DC. *Burkholderia ginsengiterrae* sp. nov. and *Burkholderia panaciterrae* sp. nov., antagonistic bacteria against root rot pathogen *Cylindrocarpon destructans*, isolated from ginseng soil. Arch Microbiol 2015;197:439–47.
[9] Ali H, Khan E, Ilahi I. Environmental chemistry and ecotoxicology of hazardous heavy metals: environmental persistence, toxicity, and bioaccumulation. J Chem 2019;2019:1–14.
[10] Gautam PK, Gautam RK, Banerjee S, Chattopadhyaya MC, Pandey JD. Heavy metals in the environment: fate, transport, toxicity and remediation technologies. Nova Sci Publishers 2016;60:101–30.
[11] Mishra J, Singh R, Arora NK. Alleviation of heavy metal stress in plants and remediation of soil by rhizosphere microorganisms. Front Microbiol 2017;8:1706.
[12] Ojuederie OB, Babalola OO. Microbial and plant-assisted bioremediation of heavy metal polluted environments: a review. Int J Environ Res Public Health 2017;14:1504.
[13] Huo Y, Kang JP, Park JK, Li J, Chen L, Yang DC. *Rhodanobacter ginsengiterrae* sp. nov., an antagonistic bacterium against root rot fungal pathogen *Fusarium solani*, isolated from ginseng rhizospheric soil. Arch Microbiol 2018;200:1457–63.
[14] Maheshwari R, Bhutani N, Suneja P. Screening and characterization of siderophore producing endophytic bacteria from *Cicer arietinum* and *Pisum sativum* plants. J Appl Biol Biotechnol 2019;7:7–14.
[15] Wang Z, Xu G, Ma P, Lin Y, Yang X, Cao C. Isolation and characterization of a phosphorus-solubilizing bacterium from rhizosphere soils and its colonization of Chinese cabbage (*Brassica campestris* ssp. chinensis). Front Microbiol 2017;8:1270.
[16] Kang JP, Huo Y, Kim YJ, Ahn JC, Hurh J, Yang DU, Yang DC. *Rhizobium panacihumi* sp. nov., an isolate from ginseng-cultivated soil, as a potential plant growth promoting bacterium. Arch Microbiol 2019;201:99–105.
[17] Gupta K, Chatterjee C, Gupta B. Isolation and characterization of heavy metal tolerant Gram-positive bacteria with bioremedial properties from municipal waste rich soil of Kestopur canal (Kolkata), West Bengal, India. Biologia 2012;67:827–36.
[18] Paudyal S, Aryal RR, Chauhan S, Maheshwari D. Effect of heavy metals on growth of *Rhizobium* strains and symbiotic efficiency of two species of tropical legumes. Sci World 2007;5:27–32.
[19] Oaikhena EE, Makaije DB, Denwe SD, Namadi MM, Haroun AA. Bioremediation potentials of heavy metal tolerant bacteria isolated from petroleum refinery effluent. Am J Environ Protect 2016;5:29–34.
[20] Egler M, Grosse C, Grass G, Nies DH. Role of the extracytoplasmic function protein family sigma factor *RpoE* in metal resistance of *Escherichia coli*. J Bacteriol 2005;187:2297–307.
[21] Fones HN, McCurrach H, Mithani A, Smith JAC, Preston GM. Local adaptation is associated with zinc tolerance in *Pseudomonas* endophytes of the metal-hyperaccumulator plant *Noccaea caerulescens*. Proc R Soc B-Biol Sci 2016;283:20160648.
[22] Jeon YH, Kim YH. Differential structural responses of ginseng root tissues to different initial inoculum levels of *Paenibacillus polymyxa* GBR-1. Plant Pathol J 2008;24:352–6.
[23] Wang QX, Xu CL, Sun H, Ma L, Li L, Zhang DD, Zhang YY. Analysis of the relationship between rusty root incidences and soil properties in *Panax ginseng*. In: IOP conference series: earth and environmental science, vol. 41. IOP Publishing; 2016, 012001.

[24] Nxele X, Klein A, Ndimba BK. Drought and salinity stress alters ROS accumulation, water retention, and osmolyte content in sorghum plants. S Afr J Bot 2017;108:261–6.
[25] Wang CJ, Yang W, Wang C, Gu C, Niu DD, Liu HX, Wang YP, Guo JH. Induction of drought tolerance in cucumber plants by a consortium of three plant growth-promoting rhizobacterium strains. PLoS One 2012;7:e52565.
[26] Rejeb KB, Benzarti M, Debez A, Bailly C, Savoure A, Abdelly C. NADPH oxidase-dependent H_2O_2 production is required for salt-induced antioxidant defense in *Arabidopsis thaliana*. J Plant Physiol 2015;174:5–15.
[27] Ali MB, Hahn EJ, Paek KY. CO_2-induced total phenolics in suspension cultures of *Panax ginseng* CA Mayer roots: role of antioxidants and enzymes. Plant Physiol Biochem 2005;43:449–57.
[28] Farokhzad A, Nobakht S, Alahveran A, Sarkhosh A, Mohseniazar M. Biochemical changes in terminal buds of three different walnut (*Juglans regia* L.) genotypes during dormancy break. Biochem Syst Ecol 2018;76:52–7.
[29] Jin Y, Kim YJ, Jeon JN, Wang C, Min JW, Noh HY, Yang DC. Effect of white, red and black ginseng on physicochemical properties and ginsenosides. Plant Food Hum Nutr 2015;70:141–5.
[30] Liu J, Wang Q, Sun M, Zhu L, Yang M, Zhao Y. Selection of reference genes for quantitative real-time PCR normalization in *Panax ginseng* at different stages of growth and in different organs. PLoS One 2014;9:e112177.
[31] Liu C, Pan TM. *In vitro* effects of lactic acid bacteria on cancer cell viability and antioxidant activity. J Food Drug Anal 2010;18:77–86.
[32] Hansda A, Kumar V, Anshumali A, Usmani Z. Phytoremediation of heavy metals contaminated soil using plant growth promoting rhizobacteria (PGPR): a current perspective. Recent Res Sci Technol 2014;6:131–4.
[33] Tirry N, Joutey NT, Sayel H, Kouchou A, Bahafid W, Asri M, El Ghachtouli N. Screening of plant growth promoting traits in heavy metals resistant bacteria: prospects in phytoremediation. J Genet Eng Biotechnol 2018;16:613–9.
[34] Chatterjee C, Gopal R, Dube BK. Impact of iron stress on biomass, yield, metabolism and quality of potato (*Solanum tuberosum* L.). Sci Hortic 2006;108:1–6.
[35] Rahman M, Punja ZK. Biochemistry of ginseng root tissues affected by rusty root symptoms. Plant Physiol Biochem 2005;43:1103–14.
[36] Ghani A. Toxic effects of heavy metals on plant growth and metal accumulation in maize (Zea maysL.). Iranian J Toxi 2010;4:325–34.
[37] Sayyed RZ, Gangurde NS, Patel PR, Josh SA, Chincholkar SB. Siderophore production by *Alcaligenes faecalis* and its application for growth promotion in *Arachis hypogaea*. Indian J Microbiol 2010;9:302–7.
[38] Saha M, Sarkar S, Sarkar B, Sharma BK, Bhattacharjee S, Tribedi P. Microbial siderophores and their potential applications: a review. Environ Sci Pollut Res 2016;23:3984–99.
[39] Farh MEA, Kim YJ, Sukweenadhi J, Singh P, Yang DC. Aluminium resistant, plant growth promoting bacteria induce overexpression of Aluminium stress related genes in *Arabidopsis thaliana* and increase the ginseng tolerance against Aluminium stress. Microbiol Res 2017;200:45–52.
[40] Sukweenadhi J, Balusamy SR, Kim YJ, Lee CH, Kim YJ, Koh SC, Yang DC. A growth promoting bacteria, *Paenibacillus yonginensis* DCY84^T enhanced salt stress tolerance by activating defense-related systems in *Panax ginseng*. Front Plant Sci 2018;9:813.
[41] Nadeem SM, Ahmad M, Zahir ZA, Javaid A, Ashraf M. The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. Biotechnol Adv 2014;32:429–48.
[42] Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 2010;48:909–30.
[43] You J, Chan Z. ROS regulation during abiotic stress responses in crop plants. Front Plant Sci 2015;6:1092.
[44] Hu T, Chen K, Hu L, Amombo E, Fu J. H_2O_2 and Ca^{2+}-based signaling and associated ion accumulation, antioxidant systems and secondary metabolism orchestrate the response to NaCl stress in perennial ryegrass. Sci Rep 2016;6:36396.
[45] de Oliveira JG, Cambraia J, Ribeiro C, de Oliveira JA, de Paula SO, Olivan MA. Impact of iron toxicity on oxidative metabolism in young *Eugenia uniflora* L. plants. Acta Physiol Plant 2013;35:1645–57.
[46] GU Chibuike, Obiora SC. Heavy metal polluted soils: effect on plants and bioremediation methods. Appl Environ Soil Sci 2014;2014:1–12.
[47] Vardharajula S, Zulfikar Ali S, Grover M, Reddy G, Bandi V. Drought-tolerant plant growth promoting *Bacillus* spp.: effect on growth, osmolytes, and antioxidant status of maize under drought stress. J Plant Interact 2011;6:1–14.
[48] Etesami H. Bacterial mediated alleviation of heavy metal stress and decreased accumulation of metals in plant tissues: mechanisms and future prospects. Ecotox Environ Safe 2018;147:175–91.
[49] Dimkpa C, Weinand T, Asch F. Plant–rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ 2009;32:1682–94.
[50] Sessitsch A, Kuffner M, Kidd P, Vangronsveld J, Wenzel WW, Fallmann K, Puschenreiter M. The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. Soil Biol Biochem 2013;60:182–94.