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Isolation and Identification of Ginseng Growth Promoting Bacteria from Korean Ginseng Rhizosphere

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Abstract: Rhizosphere bacteria interact with plant roots in various ways and significantly affect plant growth and development. Ginseng is a specialty crop with high pharmacological effects and has been cultivated in Korea for a long time. In this study, we aimed to isolate and identify rhizosphere bacteria in ginseng cultivation areas that stimulate growth for sustainable ginseng cultivation. Among 12 strains of bacteria isolated from the rhizosphere of ginseng cultivation areas, two bacteria, *Bacillus zanthoxyli* strain GGS1 and *Paenarthrobacter nicotinovorans* strain GGS3, were isolated that showed ginseng growth-promoting effects. Ginseng treated with GGS1 or GGS3 showed a root weight increase of approximately 33% or 35%, respectively. The two bacteria especially promoted the development and growth of lateral roots of ginseng. The number of lateral roots increased approximately 2-fold in both bacterial treatment groups. We predicted that this growth-promoting effect of ginseng was due to indole-3-acetic acid (IAA) produced by GGS1 and GGS3 strains. We confirmed the amount of IAA produced by GGS1 and GGS3 by Salkowski reaction and liquid chromatography-mass spectrometry. In this report, we demonstrated that these two ginseng soil bacteria are promising biofertilizers that can be used for ginseng cultivation.

Keywords: Ginseng, Indole-3-acetic acid (IAA), PGPR, Rhizobacteria

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Introduction

Soil microorganisms have a profound impact on plants, especially in the rhizosphere the area surrounding the plant's roots. Within this zone, plant growth-promoting rhizobacteria (PGPR) play a vital role in improving plant growth through their interactions with plants [1]. Excessive utilization of chemical fertilizers and synthetic substances can have detrimental effects on plant health, reducing stress tolerance in various environments and against pathogens, as well as causing harm to soil microorganisms and contributing to environmental pollution [2]. Conversely, the application of biofertilizers such as PGPR not only assists plants in their growth but also allows for environmentally-friendly cultivation practices, minimizing pol-

lution [3,4]. PGPR, which stands for Plant Growth-Promoting Rhizobacteria, are microorganisms that inhabit the rhizosphere of plants, with bacteria being the most prevalent [5]. PGPR are recognized for their diverse functions, including the promotion of root growth, regulation of plant stress, and synthesis of plant growth hormones such as IAA (Indole-3-Acetic Acid) through their interactions with plants [6,7]. The role of PGPR is paramount in soil nitrogen fixation and the facilitation of soil nutrient accessibility to plants through phosphate solubilization, thereby significantly contributing to their nutrient uptake [8]. Moreover, PGPR plays a pivotal role in bolstering plant resistance against abiotic stresses, as evidenced by a body of research [9-12]. Numerous studies have consistently provided compelling evidence of the growth-promoting prowess exhibited by PGPR. When PGPR was administered during the plant cultivation phase, there were alterations in the expression of genes associated with ROS elimination and ethylene production in potatoes [13]. Similarly, in the case of wheat, considerable improvements in length, weight, and chlorophyll content were observed under conditions involving plant growth hormone synthesis and salt stress [14]. In a study by Chu et al. (2020) [15], when inoculated PGPR on both corn and bamboo, with a notable involvement in the synthesis pathway of Indole-3-Acetic Acid (IAA) and the promotion of root hair development in plants. Additionally, Rodríguez et al. (2020) [16] reported that the application of isolated strains not only heightened plant growth and non-biological stress tolerance in tomatoes but also led to an increase in root length and weight. Various microorganisms are engaged in enhancing plant growth, with indole-3-acetic acid (IAA) being a crucial component [17]. IAA produced by microorganisms not only initiates changes in plant root structure but also promotes the development of root hairs Hadas et al. (1987) [18] and by controlling IAA levels, plants can adjust their defense mechanisms, thereby reducing their vulnerability to various stresses [19]. In a study conducted by Goswami et al. (2014) [20], peanut plants cultivated in saline soil and treated with IAA-producing bacterium 2M4 exhibited a remarkable 17% increase in total length and a 13% enhancement in fresh biomass. Ginseng, distinguished as a prominent functional food with a spectrum of health advantages, poses notable challenges during its cultivation. Typically, the cultivation of ginseng entails a time frame of approximately five years, demanding meticulous care. It exhibits a pronounced sensitivity to humid and hot environmental conditions, further compounded by its susceptibility to diseases, attributed to its semi-shade plant nature. Remarkably, ginseng manifests a notably sluggish growth rate relative to its extended cultivation period. Additionally, the plant's delicate and fragile root system complicates the application of fertilizers, including chemical variants [21,22]. Consequently, cultivating ginseng is demanding, especially when aiming for full maturation over a seven-year period. In response to the multifaceted challenges inherent to ginseng cultivation, ongoing research endeavors are directed toward harnessing the potential of PGPR to amplify ginseng growth and enhance its resilience to stressors. In a study as Bak et al. (2010) [23], this research meticulously documented both the inhibitory effect on plant pathogenic fungi and the root growth-promoting attributes exhibited by isolated microorganisms, employing mung bean seedlings in ginseng soil. An additional referenced study, identified as Um et al. (2014) [24], involved the isolation of endophytic fungi from ginseng seeds. Subsequently, a comprehensive examination was conducted to assess their biocontrol efficacy when co-inoculated with pathogenic bacteria, along with the verification of Indole-3-Acetic Acid (IAA) production. Finally, in a study by Choi et al. (2000) [25], the application of microbial agents to ginseng roots resulted in a significant enhancement in root weight, spanning from 7% to 13%, when compared to the control group. The treated ginseng exhibited pronounced improvements in root diameter and weight relative to the control group. In this way, numerous investigations are actively exploring microorganisms known for their plant growth-promoting attributes, leading to the development of corresponding microbial agents. However, there remains a notable deficiency in research dedicated to microorganisms and microbial agents specifically aimed at promoting ginseng growth. Cultivating ginseng is a demanding task, and efforts to enhance its growth and stress resistance through the utilization of PGPR.

In our study, we focused on isolating and identifying microorganisms inhabiting the rhizosphere of ginseng. Interestingly, we discovered two types of microorganisms that showed beneficial effects on ginseng growth.

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Materials and Methods

Bacterial analysis using high-throughput 16S rRNA sequencing

To identify the microorganisms within the ginseng soil, we conducted 16s rDNA gene sequencing. Subsequently, microbial DNA extraction was carried out in accordance with the previously described and established genomic DNA extraction procedure. The extracted DNA was employed for the analysis of the 16S rDNA gene [26]. To amplify the 16S rDNA gene region, PCR reactions were carried out, utilizing the DNA oligo primers 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTAC GGGNGGCWGAAG-3′ and 5′-GTCTCGTGGGCTCGGAGATGTCTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. Following amplification, the PCR products underwent purification using the QIAquick PCR Purification kit provided by Qiagen (Hilden, Germany).

Bacterial genomic DNA extraction

Genomic DNA extraction from the soil followed the experimental protocol by [27] utilizing a buffer solution. Soil samples were combined with Edwards buffer, consisting of 0.5% (w/v) SDS, 200 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 25 mM EDTA (pH 8.0), with the addition of SDS to reach a final concentration of 1% (w/v). The mixture was incubated at 65°C for 5 minutes. Subsequently, 1M potassium acetate was added to precipitate proteins, and the mixture was left on ice for 5 minutes. Genomic DNA was then precipitated by centrifuging the mixture at 13,000 rpm, followed by the addition of 300 mM NaOAc (Sodium acetate) and 50% (v/v) isopropyl alcohol. After another centrifugation at 13,000 rpm, the precipitated genomic DNA was washed with 70% (v/v) ethanol and ultimately dissolved in distilled water. To ascertain the quantity and purity of the extracted genomic DNA, a spectrophotometer (ND-1000, Nanodrop, Waltham, Massachusetts) was employed.

Bacterial isolation and identification

Ginseng soil collection was conducted at the ginseng fields in Yeonguri, Masan-myeon, Haenam-gun, Jeollanam-do, over a two-year cultivation period. Soil was collected from around the ginseng roots and storing it at 4°C. To isolate and identify microorganisms from ginseng soil, a 1:1 mixture of soil and sterilized water was prepared and allowed to settle for 24 hours at 24°C. The supernatant was collected, and soil particles were removed from the upper layer using Celite 545 (DSP-1255, Duksan, Korea). The resulting soil water was diluted to 10^{-2} or 10^{-3} and inoculated onto LB (Luria-Bertani broth), TSA (Tryptic soy agar), TSA-YE (Tryptic soy agar-yeast extract), and R2A (Reasoner's 2A agar) media. Incubation of the media was carried out at 30°C for one day. The isolation of pure strains involved the careful selection of single colonies, which were subsequently streaked onto media of identical composition and then incubated at 30°C for one whole day. DNA plasmid miniprep kits from Qiagen (Hilden, Germany) were employed to extract microbial DNA following the provided protocol. Subsequently, the 16S rRNA gene sequence, 16s rDNA sequence was amplified and sequenced using the Macrogen sequencing service on the Miseq™ platform (Macrogen, Korea). Microorganism identification was performed based on the NCBI database.

Measurement of plant growth-promoting effect

One-year-old ginseng was planted after blending cultivation soil (biosoil1, Hungnong, Korea) with horticultural soil. Approximately 250 g of soil was used per pot and relative humidity was 60%. Light intensity was 3950 lux. To examine the effect of microorganisms on ginseng growth, we inoculated with microbes. Ginseng plants, cultivated for ten days under controlled conditions of 16 hours of light and 8 hours of darkness at 24°C, were subjected to weekly applications of 1 mL of the isolated microorganisms near their root, spanning a four-week duration. Before inoculation, the cultured microorganisms were re-suspended in sterile water, with an absorbance reading of 0.6 at 600 nm as the baseline. The ginseng stem length, weight, and lateral root count measurements were conducted and subjected to comparative analysis.

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Ginseng Growth Measurement

To evaluate ginseng growth, microorganisms were applied for 4 weeks, followed by a 1-week period of water-only supply before measurement. The length and wet weight of leaves, stems, and roots were measured to compare differences among treatment groups. The number of lateral roots was determined by counting the individual lateral roots attached to the taproot. All measurements were conducted with seven replicates for each treatment group, and the data are presented as the mean ± standard deviation (± S.D.).

IAA quantifications

The Salkowski solution was used to evaluate auxin production [28]. Initially, two microorganisms were cultured in R2A medium supplemented with 1%(w/v) tryptophan at 30°C and 170 rpm for 24 hours. Subsequently, they were transferred to the same medium and maintained under identical conditions for an additional 24 hours. The resulting culture supernatant was mixed with Salkowski reagent (comprising 35% (w/w) $HClO_4$ and 0.5 M $FeCl_3$) in a 1:2 (v/v) ratio and allowed to react in the dark condition for 30 minutes. The extent of color development were quantified at 535 nm using a UV spectrophotometer (Ubi-600, MicroDigital, Korea). For the identification of auxin, chromatography-mass spectrometry analysis was adopted (LCMS-8040, Shimadzu, Japan). To prepare experimental samples for auxin production measurement, GGS1 and GGS3 strains were cultured for 24 hours. The cultures were then centrifuged at 10,000 rpm for 10 minutes to pellet the cells, and the supernatant was collected to prepare the samples. 10 μL of sample solution were injected into a packed column (Kinetex C_{18}, 150 × 2.1 mm, 1.7 μm; Phenomenex) installed for the LCMS-8040. Samples were eluted at an oven temperature of 40℃ and a flow rate of 0.3 mL/min.10 μL of sample solution were injected into a packed column (Kinetex C_{18}, 150 × 2.1 mm, 1.7 μm; Phenomenex) installed for the LCMS-8040. Samples were eluted at an oven temperature of 40°C and a flow rate of 0.3 mL/min.

Results

Isolation and identification of bacteria in the ginseng rhizosphere soil

Rhizosphere soil microorganisms were sourced from Yeonguri, Masan-myeon, Haenam-gun, and Jeollanam-do, where ginseng had been cultivated for two years. The extraction of ginseng soil water followed the method detailed in the materials and methods section. A total of 35 single colonies were isolated from the extracted soil water using four different media types: LB, TSA, TSA-YE, and R2A. Through the primary screening for isolating ginseng growth-promoting bacteria with 35 bacterial isolates, we selected 12 distinct microorganisms, including including strains GGS1 and GGS3 (Table 1). Among 35 microorganisms identified, 12 microorganisms, excluding identical strains, were selected for the initial screening. After the first screening, experiments were repeated using only the microorganisms that showed significant effects [29].

16S rRNA sequence-based phylogeny of bacterial strains

To identify the 12 ginseng growth-promoting bacteria, we extensively analyzed the 16S rRNA gene sequences. We then analyzed sequences with the NCBI database to determine their relationships with closely related strains. GGS1 was identified as a member of *Bacillus zanthoxyli* strain P9-3 (Fig. 1A), while GGS3 showed significant homology with *Paenarthrobacter nicotinovorans* (Fig. 1B).

Plant growth activity test of ginseng rhizosphere soil microbes

To evaluate the impact of 12 different microorganisms on ginseng growth, we conducted a microbial inoculation experiment using one-year-old ginseng plants as test subjects. Repeated inoculation trials consistently demonstrated enhanced growth in gin-

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Table 1. Bacteria isolated from the Korea ginseng field. Two bacterial strains, GGS1 and GGS3, exhibiting plant growth-promoting activity, have been assigned subspecies names

Species name of isolated bacteria	Subspecies name in this report
Bacillus megaterium strain XM1	
Bacillus aryabhattai strain ZJJH-1	
Bacillus sp. strain P9-3	GGS1
Bacillus megaterium strain Pap.TC-EB01	
Paenarthrobacter nicotinovorans strain P-HV2-1	GGS3
Bacillus sp. strain RMM15B1	
Bacillus aryabhattai strain OP62	
Bacillus megaterium strain AK4	
Brevibacterium] frigoritolerans strain WS2-1	
Arthrobacter sp. 32-OD9	
Bacillus megaterium strain AVMB3	
Bacillus megaterium strain Lmb023	

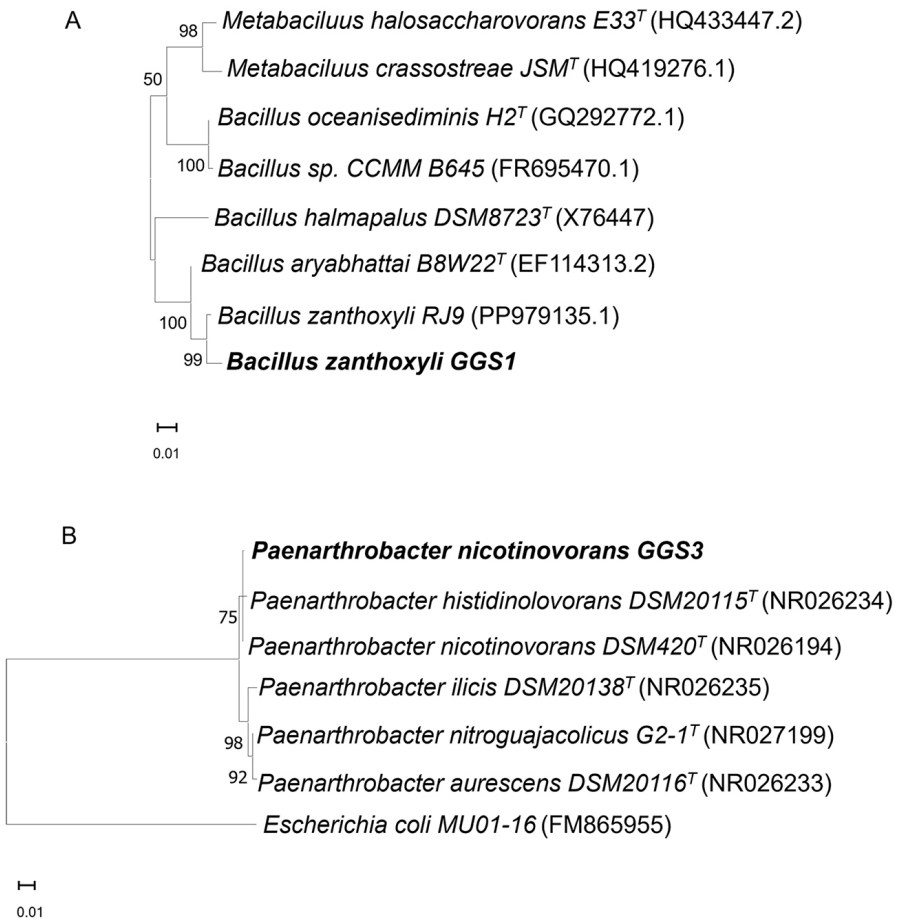

Figure 1. Phylogenetic tree based on 16S rRNA gene sequence of GGS1 (A) and GGS3 (B). The tree was inferred by a Maximum Likelihood analysis. The scale bar represents the maximum composite likelihood distance.

seng leaves and roots when treated with *B. zanthoxyli* strain. GGS1 and *P. nicotinovorans* strain. GGS3 compared to the untreated control group (Figs. 2A and 2B). GGS1 treatment resulted in approximately a 33% increase in ginseng wet weight (Fig. 2C). Similarly, GGS3 treatment led to an average weight increase of 35% compared to the control group (Fig. 2C). Only minor changes

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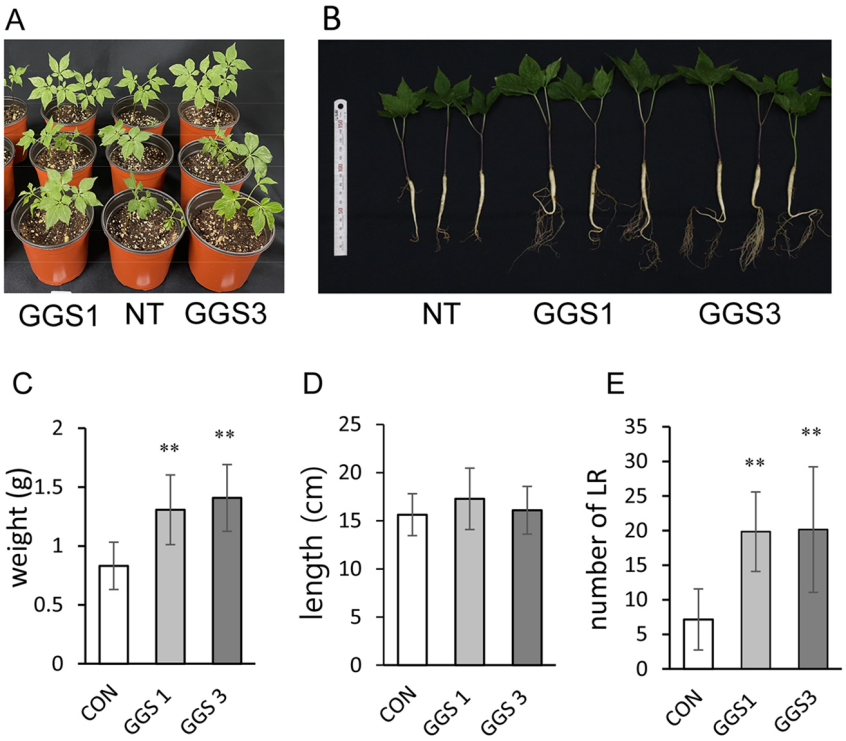

Figure 2. The ginseng growth effect of ginseng rhizosphere soil microbes.

Two types of bacteria were selected through primary irrigation for plant growth-promoting effect. They were named GGS1, GGS3 (A). Ginseng root after 4weeks of bacteria treatment (B). Subsequently, the root weight of ginseng (C), stem length (D), and number of lateral roots (LR) (E) were quantified and compared among the treatments following the 4-week bacteria treatment. All data were obtained from at least 7 plants per treatment and presented. And the average was calculated. The error bar indicates the standard deviation, and a significant difference was shown through the t-test (*, p<0.05; **, p<0.01).

were observed in the total length of the plants (Fig. 2D). Notably, the most significant effects of GGS1 and GGS3 were seen in lateral root growth. The number of lateral roots in ginseng plants inoculated with GGS1 or GGS3 approximately doubled compared to the untreated group (Fig. 2E).

Identifying the ginseng root growth factor

Auxin is a well-known hormone produced by plants and bacteria that enhances plant cell division and elongation. Among the plant growth-promoting factors induced by bacterial inoculation, auxins, particularly indole-3-acetic acid (IAA), have been widely recognized, with IAA being the natural form of auxin. Consequently, we focused on IAA, a plant growth hormone known for its role in promoting ginseng growth. The production of IAA was initially confirmed using the Salkowski reagent. A standard curve for IAA quantification with the Salkowski reagent was generated, and IAA production by GGS1 and GGS3 was visually monitored by the color change of the Salkowski reagent. The amounts of IAA produced by GGS1 and GGS3 were quantified from bacterial culture media at 6 hours and 9 hours of growth (Fig. 3). At 6 hours, GGS1 and GGS3 produced average IAA concentrations of 0.88 ppm and 0.89 ppm, respectively. After 9 hours of cultivation, IAA production increased to 1.5 ppm in GGS1 and significantly to 3.26 ppm in GGS3 (Fig. 3).

Using the more sensitive LC-MS/MS method, we successfully detected IAA production in the culture media of both GGS1 and GGS3. The culture media were directly analyzed via LC-MS/MS, revealing consistent IAA peak profiles and mass patterns for both strains (Fig. 4). The retention time of the IAA standard was 4.354 minutes, and both microorganisms exhibited identical retention times. The mass patterns matched the IAA standard perfectly in both strains. These findings conclusively demonstrate that GGS1 and GGS3 produce IAA, contributing to the enhanced growth of ginseng.

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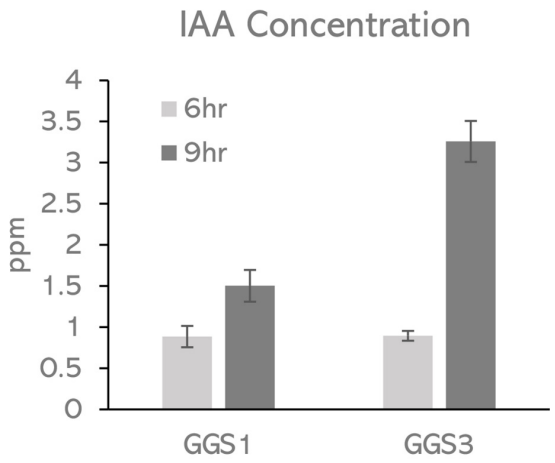

Figure 3. IAA activity of microorganisms.

To assess the ability to produce IAA, the bacteria were subjected to the Salkowski reagent test. The development of a red color, resulting from the reaction between Salkowski reagent and IAA, indicated the production of IAA by the isolated bacteria . Assessment of IAA activity at 6 and 9 hours into microbial cultivation.

Discussion

This study was conducted to isolate PGPR that can promote ginseng growth and verify the effects of these PGPR to solve the problem of ginseng quality deterioration due to abiotic stress such as heavy metals derived from chemical fertilizers and pesticides in ginseng cultivation areas [30]. The ginseng growth-promoting strains isolated from the ginseng rhizosphere soil in Haenam-gun, Jeollanam-do were *B. zanthoxyli* (GGS1) and *P. nicotinovorans* (GGS3), and these strains significantly affected the growth of ginseng. In previous studies, *B. zanthoxyli* was mainly confirmed to confer tolerance to salt stress in vegetable crops, and its plant disease suppression effect was also reported [31]. In particular, it effectively suppresses disease occurrence by inducing resistance to diseases such as cucumber anthracnose, fruit rot, and cucumber vine scab [32]. Its plant growth-promoting effect has also been confirmed [33]. *P. nicotinovorans* strains can also interact with plant roots to contribute to growth promotion, and there are cases where they have been used to improve crop productivity by producing various plant growth-promoting substances [34]. This study confirmed the growth effects of *Bacillus* sp. (GGS1) and *P. nicotinovorans* (GGS3) isolated from ginseng rhizosphere soil in Haenam-gun, Jeollanam-do, on ginseng, and analyzed their ginseng growth-promoting factors. Both strains were found to produce IAA (Indole-3-Acetic Acid) to promote plant growth. It has been previously known that microorganisms of the genus *Bacillus* produce auxin [35], and in the case of GGS3, where a high level of IAA was detected, auxin production was confirmed for the first time in this study. In the case of GGS1 and GGS3, the average total weight increased by 33% or 35%, respectively, compared to the untreated control group. In addition, the number of ginseng letheral roots in inoculated with GGS1 and GGS3 increased by about 3 times compared to the untreated group. This can be seen that the IAA produced by the microorganism expanded the plant's root structure to absorb more nutrients and helped to create long roots by promoting the development of root hair and letharal roots. [36] Among the microorganisms isolated from the ginseng rhizosphere soil, *Arthrobacter nicotinovorans* [37], *Streptomyces werraensis* [38], *Bacillus velezensis* [39], *Pseudomonas thivervalensis* [40], *Trichoderma* spp. [41], etc. are known, this study is the first to report on the promotion of ginseng letheral roots growth by the isolated *B. zanthoxyli* and *P. nicotinovorans*. In conclusion, the two microorganisms have the potential to be utilized as plant growth-promoting rhizobacteria (PGPR) that can make an important contribution to the promotion of ginseng growth. In particular, GGS1 and GGS3 showed a great effect on increasing total weight and the number of letheral roots development, and shows potential for use in ginseng cultivation in the future. It is expected that the utilization of these microorganisms can contribute to the promotion of ginseng growth and improvement of productivity. However, the limitation of this study

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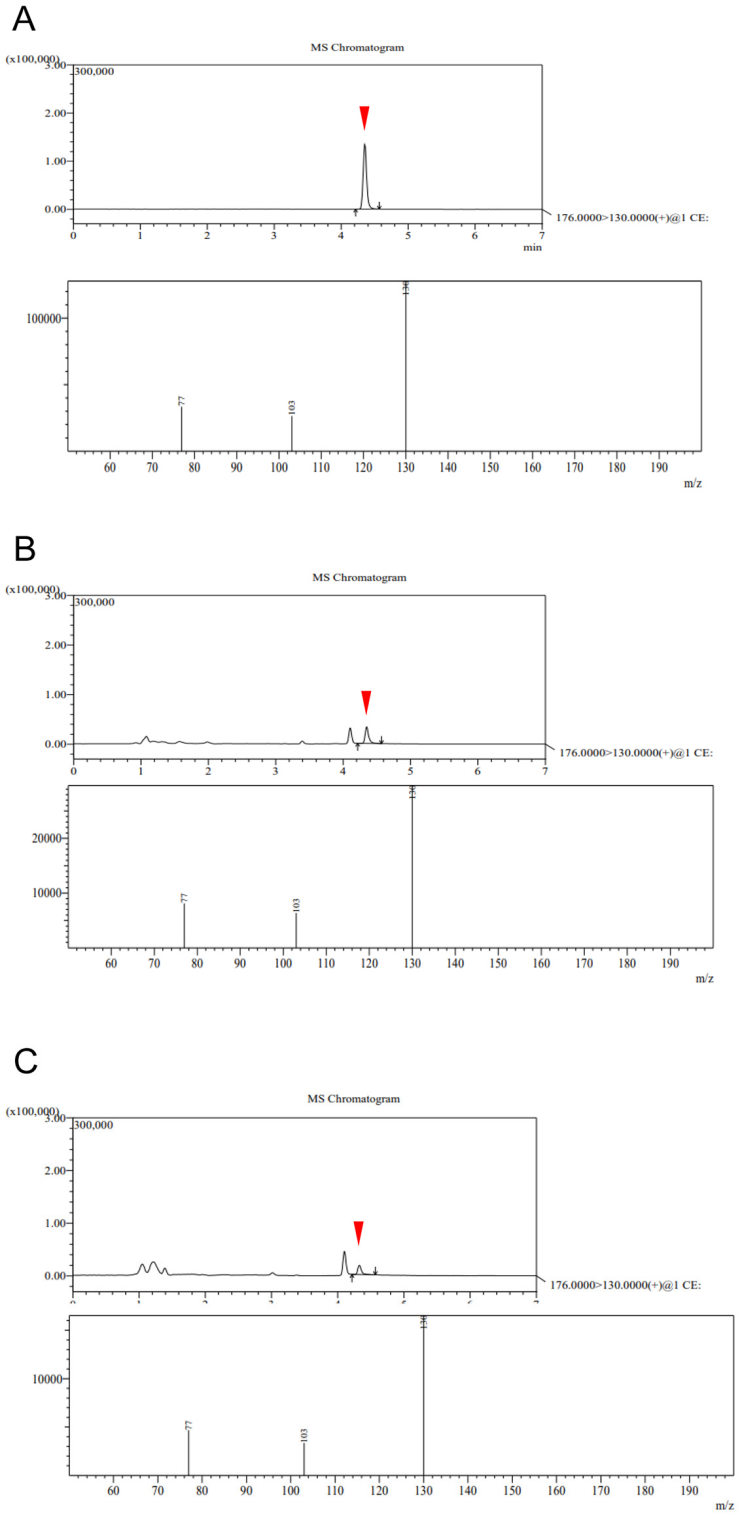

Figure 4. LC–MS/MS for detection of IAA.

Shows mean values for quantitative (50ppb) estimation of IAA standard solution (A). show LC-MS/MS plot of commercial bacterial culture supernatant GGS1 (B) and GGS3 (C) sample.

is that further research is needed on the long-term effects of the two strains on ginseng growth. Since the study was conducted over a short period of time, further research is needed to determine whether the two strains have a continuous positive effect throughout the life of ginseng and how they respond to various environmental stresses (e.g., drought, pests, etc.). Additionally,

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it is important to study how the two strains interact with other soil microbial communities in real agricultural environments.

Conclusion

Two bacteria that exhibited the capacity to enhance ginseng growth were isolated and identified from the ginseng rhizosphere soil. Both of these bacteria demonstrated the ability to produce IAA (Indole-3-Acetic Acid). Notably, *P. nicotinovorans* sp. GGS3 exhibited significant improvements in ginseng root weight, stem length, and the number of root hairs when compared to the control group. The results of this study accentuate the potential value of bacteria isolated from ginseng rhizosphere soil as Plant Growth-Promoting Rhizobacteria (PGPR), attributing to their role in enhancing both ginseng growth and the development of root hairs.

Data Availability: All data are available in the main text or in the Supplementary Information.

Author Contributions: E.K., J.B. and K.W.H. carried out experiment and collected the data; E.K. and J.B. wrote the manuscript; E.K., J.B., P.H.L., K.W.H., J.K.M., Y.K. reviewed the manuscript; P.H.L., J.K.M. and Y.K. designed the study. All authors have read and agreed to the published version of the manuscript.

Notes: The authors declare no conflict of interest.

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Additional Information:

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