

Characterization of *Bacillus mojavensis* KJS-3 for the Promotion of Plant Growth

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Scientists have recently shown an interest in the characteristics of *Bacillus mojavensis* strains because of their increasing use in plants as a defense against diseases and mycotoxins. We have shown here that *B. mojavensis* KJS-3 possesses the typical characteristics of *B. mojavensis* strains including a strong resistance to high temperatures ($\leq 50^{\circ}\text{C}$), tolerance to high salt concentrations (7% NaCl), ethanol tolerance (40% ethanol), and pH range for growth (pH 5-9). *B. mojavensis* KJS-3 has been used for the production of cyclic lipopeptides including important antifungal substances such as surfactin, iturin, and fengycin. Polymerase chain reaction analysis in this study showed that *B. mojavensis* KJS-3 can be used for the production of fengycin and the findings of LC-MS/MS analyses suggest that *B. mojavensis* KJS-3 can be used to produce iturin and surfactin. Antifungal activity analysis is confirmed that *B. mojavensis* KJS-3 has antifungal effects on *Botrytis cinerea*, *Rhizoctonia solani* AG-4, *Sclerotinia sclerotiorum*, and *Colletotricum goeosporioides*. A microscopy assessment of the roots of wild ginseng plants planted together with *B. mojavensis* KJS-3 revealed that the roots contained *B. mojavensis* KJS-3, confirming the bacteria to be a plant growth promoting endophyte (PGPE) which acts against plant diseases and mycotoxins. Our findings lead us to conclude that *B. mojavensis* KJS-3 can be produced at an industrial level as a microbial pesticide or microbial fertilizer.

Key words : Antifungal activity, *Bacillus mojavensis* KJS-3, plant pathogenic fungi, plant growth promoting endophyte

Introduction

B. mojavensis strains discovered in the Mojave Desert and found to have antibacterial and antifungal activities have been broadly utilized to protect plants against diseases and mycotoxins [1, 2]. *B. mojavensis* strains were confirmed to be a novel species distinct from *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus atrophaeus* [1, 18]. However, based on Bergey's Manual of Systematic Bacteriology which serves as an aid in the identification of those bacteria that have been described and cultured, these *Bacillus* species are more closely related to each other, and exhibit similar biochemical characteristics.

B. mojavensis KJS-3, a new *B. mojavensis* strain, was initially found in food waste and has been registered in the Korean Culture Center of Microorganisms (KCCM) under the ac-

cession number KCCM10961P [5] with specific patterns of isoquinones. Previous studies have shown that *B. mojavensis* KJS-3 exhibits almost all typical characteristics of *B. mojavensis* strains: the KJS-3 strain bacteria are rod-shaped, Gram-positive, and endospore-forming aerobic bacteria. *Bacillus* species are used in many medical, pharmaceutical, agricultural, and industrial processes that take advantage of the bacteria's wide range of physiological characteristics and their ability to produce a host of enzymes, antibiotics, and other metabolites [5, 12, 13, 16]. The rhizosphere, a narrow region of soil that is directly influenced by root secretions, is associated with microbial activity [3]. Based on the different positions in plant micro-ecosystems, plant growth promoting microorganisms can be categorized as either plant growth promoting rhizobacteria (PGPR) or PGPE, where PGPRs grow on or around the roots and PGPEs grow inside the roots, specifically in the intercellular space of roots. These microorganisms affect plant growth in three different ways: (1) by synthesizing and providing particular compounds to the plants [7], (2) by facilitating the uptake of certain nutrients from the environment [4], and (3) by protecting plants from certain diseases [11]. The potential of PGPEs to improve plant health has led to a lot of research

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into the applied use of these bacteria as microbial pesticides, primarily in agricultural crops [8]. The potential for microbial pesticides to reduce the need for chemicals such as chemical pesticides makes them important in the development of sustainable agricultural practices.

The overall objectives of this study were (1) to examine the biochemical characteristics of *B. mojavensis* KJS-3, predominantly resistance characteristics including survival at high temperatures, pH tolerance, salt tolerance, and ethanol tolerance; (2) to identify antifungal substances extracted from *B. mojavensis* KJS-3 and assess the potential antimicrobial activity of these substances; (3) to demonstrate that *B. mojavensis* KJS-3 functions as a PGPE to protect plants against diseases.

Materials and Methods

Bacterial strains and media

The bacterial strain *B. mojavensis* KJS-3 (KCCM 10961P) was obtained from Dr. Jae Seon Kang (Department of Pharmacy, Kyungshung University, Korea). Bacteria were grown in tryptic soy broth (TSB) for routine use. For colony selection for bacterial culture, bacterial strains were grown on tryptic soy agar (TSA), from which single colonies were transferred to tubes containing 5 ml TSB and grown aerobically in a shaking incubator (160 rpm) overnight at 37°C. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and all microorganisms were obtained from the KCCM and the Korean Agricultural Culture Collection (KACC).

High temperature tolerance

B. mojavensis KJS-3 was cultured in 5 ml TSB medium overnight, after which 100 µl preculture was diluted with 900 µl sterile distilled water. The preculture was continually diluted in sterile distilled water in this way to a final bacterial concentration of 10³ CFU/ml.

The final diluted solution (500 µl) was plated onto TSA plates which were incubated at various temperatures (20-50°C) and bacterial growth on the plates was assessed the following day.

Acid and alkali tolerance

To determine the optimum pH range for *B. mojavensis* KJS-3 growth, the bacteria was cultured in 8 ml TSB medium overnight, and six tubes each containing 100 ml TSB medium

were adjusted to different pHs (pH 2, 4, 6, 7, 8, and 9) using 6 M HCl and 3 M NaOH. An aliquot (1 ml) of the overnight TSB culture of *B. mojavensis* KJS-3 was added to each of the six tubes which were then incubated (160 rpm) for 8 hr. After 8 hr the optical densities (600 nm) of the six cultures were measured using a UV-Vis spectrophotometer (UV-Mini1240; Shimadzu, Japan), allowing for the growth status of *B. mojavensis* KJS-3 at each pH to be determined.

Salt tolerance

B. mojavensis KJS-3 was cultured in 10 ml TSB medium overnight. A different amount of NaCl (1, 3, 5, 7, and 9 g) was added to each of five tubes containing 100 ml TSB medium each and another tube of 100 ml TSB medium lacking NaCl was included as a negative control. After high pressure sterilization of the six tubes of medium, 1 ml of the overnight TSB culture of *B. mojavensis* KJS-3 was added into each of the six tubes which were then incubated (160 rpm) for 8 hr. After 8 hr, the optical densities (600 nm) of the six cultures were measured using a UV-Vis spectrophotometer.

Ethanol tolerance

B. mojavensis KJS-3 endospores were produced using the method described by Choi *et al.* [5]. Endospore powder (0.01 g) was added to 1 ml sterile distilled water to produce a bacterial suspension. Preparations of 20, 40, 80, and 95% ethanol were sterilized by autoclaving, after which 100 µl bacterial suspensions were added into each ethanol solution (20, 40, 80, and 95%). The bacteria-containing ethanol solutions were continually diluted in sterile distilled water to a final concentration of 10⁶ CFU/ml, after which 500 µl of each final dilution was plated on to a TSA plate and cultured at 37°C overnight. The following day the bacterial growth on the plates was assessed.

Extraction and identification of antifungal substances

Most *Bacillus* strains produce antibiotics such as iturin, fengycin, and surfactin. For the assessment of potential antibiotic production by *B. mojavensis* KJS-3, several colonies of the bacteria were transferred into tubes containing 5 ml TSB medium and grown aerobically in a shaking incubator (180 rpm) overnight at 37°C. The 5 ml of preculture was transferred to a 1 l triangular flask containing TSB medium and the *B. mojavensis* KJS-3 culture was then incubated, shaking, for 24 hr at 25°C. After 24 hr the culture was subjected to centrifugation at 8,000 rpm for 15 min and 500 ml of the

resulting supernatant was adjusted to pH 2 using 6 M HCl. Methanol was used to dissolve 150 mg of the resulting precipitate and the methanol was subsequently concentrated using a rotary evaporator. Evaporator bottoms were extracted using methanol and then reconcentrated by using a vacuum evaporator. 10 mg of residue was antimicrobial substances of *B. mojavensis* KJS-3.

After extraction and purification from the *B. mojavensis* KJS-3 culture, 10 mg of residue for the potential detection of iturin and surfactin was dissolved in methanol and the sample was further diluted to a final concentration of 100 µg/ml for analysis by LC-MS/MS (Agilent 6410 Triple Quadrupole LC/MS; Agilent Technologies, Santa Clara, CA, USA). For LC, the chromatographic conditions were as follows: Gemini NX-C185 µm stationary phase (150×4.6 mm i.d.; Phenomenex, Torrance, CA, USA); mobile phase: A=water with 0.1 % TFA and B =acetonitrile with 0.1 % TFA; elution mode: gradient 0-3 min (B=50%), 3-25 min (B =100%). For MS, the interface conditions used were as follows: the MS was set to electrospray ionization (ESI) mode with a mass range of 100-1,500 ms; SIM polarity in positive mode, gas temperature 350°C, gas flow 10 l/min, nebulizer pressure 40 psi, capillary voltage 4.8 kV, and iturin A (molecular weight 1044.6 [M+H]⁺) and surfactin (molecular weight 1022.3 and 1036.3 [M+H]⁺) as internal standards.

Polymerase chain reaction (PCR) amplifications for the identification of fengycin were performed using a PCR Thermal Cycler (M-2325; Takara Bio, Otsu, Japan). PCR amplifications were performed in 20 µl ready mix PCR reaction mixtures containing 1 tube TOP simple DryMIX-Tenuto (Enzynomics, Daejeon, Korea), 1 µl genomic DNA template, 1 µl forward primer (FENTD1F, 5' -ttggcagcaggagaagttt-3'), 1 µl reverse primer (FEND1R, 5' -gctgtccgttctgcttttc-3'), and distilled water to make up the 20 µl reaction volume [17]. The mixture was gently mixed by vortexing and then subjected to centrifugation for the entire mixture to be collected at the bottom of the tube. The PCR program used was with denaturation, annealing and extension cycle. The amplified DNA was then evaluated by agarose gel electrophoresis and subsequent ethidium bromide staining.

Anti-fungal activity

Four phytopathogenic fungi (*Botrytis cinerea* KACC 40573, *Rhizoctonia solani* AG-4 KACC 40142, *Sclerotinia sclerotiorum* KACC 41065, and *Colletotrichum gloeosporioides* KCCM 11220) were included in the antifungal activity analysis carried out

in this study. The phytopathogenic fungi were cultured on potato dextrose agar (PDA) medium at 37°C, while *B. mojavensis* KJS-3 was cultured aerobically in 5 ml TSB medium in a shaking incubator (180 rpm) overnight at 37°C. For each of the phytopathogenic fungi, a small piece of agar was cut out after 48 hr of culture and transferred onto a new PDA plate. On each of these new plates, a line of *B. mojavensis* KJS-3 culture was drawn and all plates were cultured at 37°C for 48 hr.

PGPE of *B. mojavensis* KJS-3

Wild white ginseng was harvested from the jiri mountains in Hamyang-gun, Korea and cultivated wild ginseng seeds were then combined with *B. mojavensis* KJS-3 as follows: 10 kg deshelled ginseng seeds were mixed with 1 kg corn starch, 10 g *B. mojavensis* KJS-3 (1×10⁹ CFU/g), and 1 l distilled water. The ginseng seeds combined with the bacteria were then planted on land surface of farmland, cultivated for 3 years, and harvested. The roots of the plants were washed with sterile water, after which they were divided with a sterile razor on a clean bench and observed under a microscope (100×, MTV-33K9HN; Mintron, New Taipei City, Taiwan).

Results and Discussion

Stability of *B. mojavensis* KJS-3

The tolerance of *B. mojavensis* KJS-3 to different pH, temperature, and salt conditions is shown in Fig. 1. The results of the temperature tolerance analysis revealed the bacterial strain to have a growth temperature range of 20-50°C and an optimum growth temperature of 45°C (Fig. 1A). *B. mojavensis* KJS-3 was found to survive in neutral or slightly acidic pHs, with the optimal pH range for growth being pH 6-8 (Fig. 1B). No significant decreases in cell viability were observed at neutral (pH 6.0, 7.0, and 8.0) pHs. As shown in the results (Fig.1C), *B. mojavensis* KJS-3 cell viability is not affected by NaCl concentrations ≤7%, indicative of the bacteria being highly tolerant to salt. Microbial growth is greatly affected by external environmental conditions and salt is a naturally occurring element in soils and water [10]. Salt tolerance is therefore essential for the survival of microorganisms in soil and water [10]. With increasing human development, soil salinization is one of the most important problems resulting from land degradation and basic environmental problems in arid and semi-arid regions [10]. The

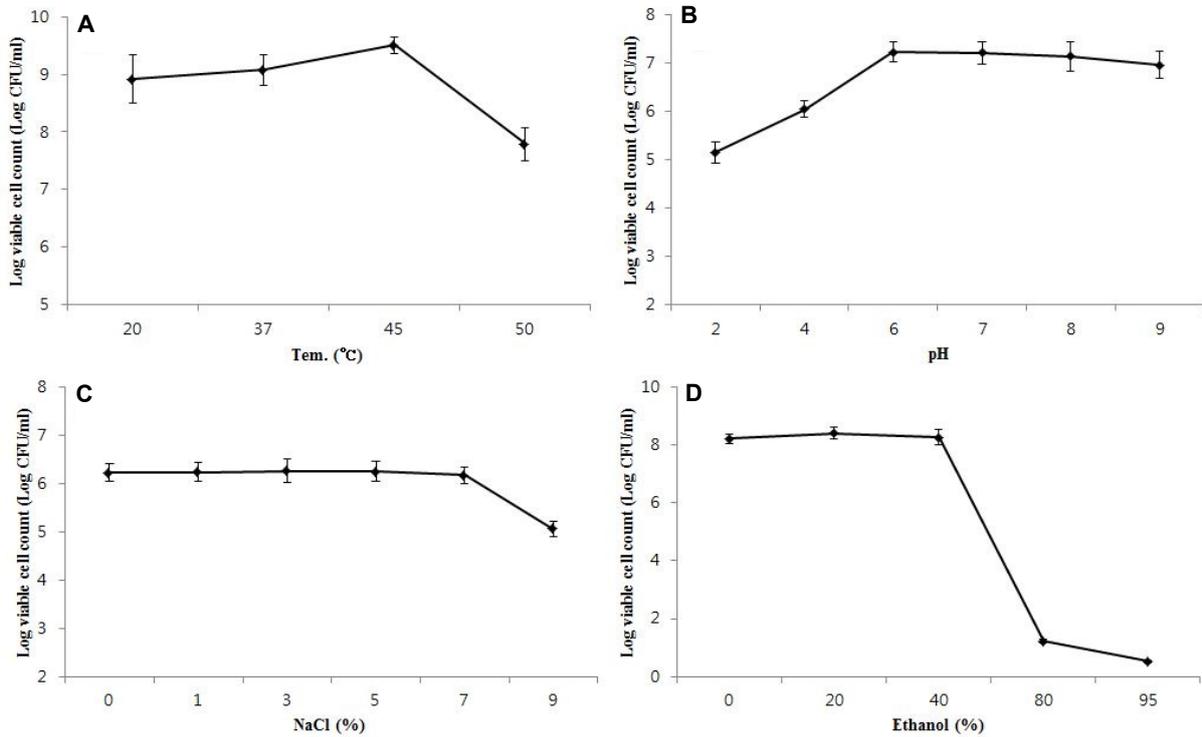


Fig. 1. Viability of *B. mojavensis* KJS-3 cultivated with various (A) Temperature (°C), (B) pHs, (C) NaCl (%), (D) ethanol (%) on TSB medium.

results presented here are consistent with those previously reported regarding the viability of *B. mojavensis* KJS-3 endospores under conditions of varied pH, temperature, and NaCl concentration [5]. Ethanol is commonly used to kill bacteria including *B. mojavensis* KJS-3. The endospores of *B. mojavensis* KJS-3, however, were found to have a strong tolerance to ethanol, surviving in solutions containing ≤40% ethanol (Fig. 1D). These findings have implications for industrial fermentation which is widely applied in terms of fermentation by microorganisms such as probiotics to produce microbial products useful to humans.

Identification of fengycin, iturin, and surfactin

B. subtilis KCCM 11316 was used as a positive control for fengycin detection by PCR analysis in this study. In the agarose gel visualizing the resulting PCR products, a prominent band representing the fengycin synthetase gene of *B. subtilis* was observed at 1.2 kbp and a corresponding band, also at 1.2 kbp, was observed for *B. mojavensis* KJS-3 (Fig. 2). In the LC-MS/MS analyses carried out, the fractions containing standards (surfactin and iturin A) were found to contain quasi-molecular ions at $m/z = 1022.3$ and 1036.3 ($[M+H]^+$) for surfactin and at $m/z = 1044.6$ ($[M+H]^+$) for iturin A. Based

on the peaks of the standard samples and the molecular weights detected for the test substances (surfactin: $1036.3 m/z$ $[M+H]^+$; iturin A: $1044.7m/z$ $[M+H]^+$; Fig. 3) it can be concluded that the surfactin and iturin A produced by *B. moja-*

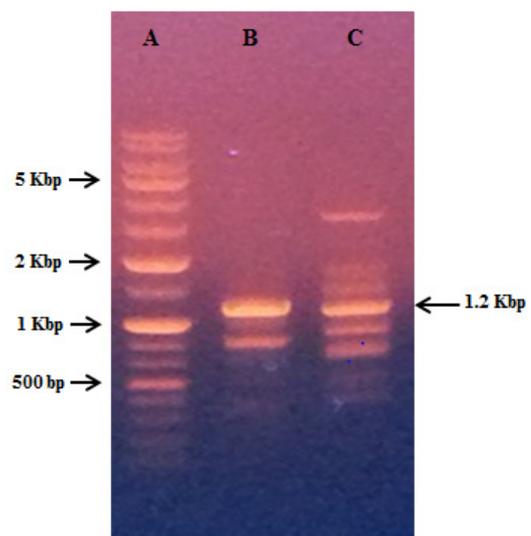


Fig. 2. Identification of Fengycin produced from *B. mojavensis* KJS-3 on TSB medium by PCR analysis. (A) Marker, (B) *B. mojavensis* KJS-3, (C) *Bacillus subtilis* KCCM 11316 (positive control).

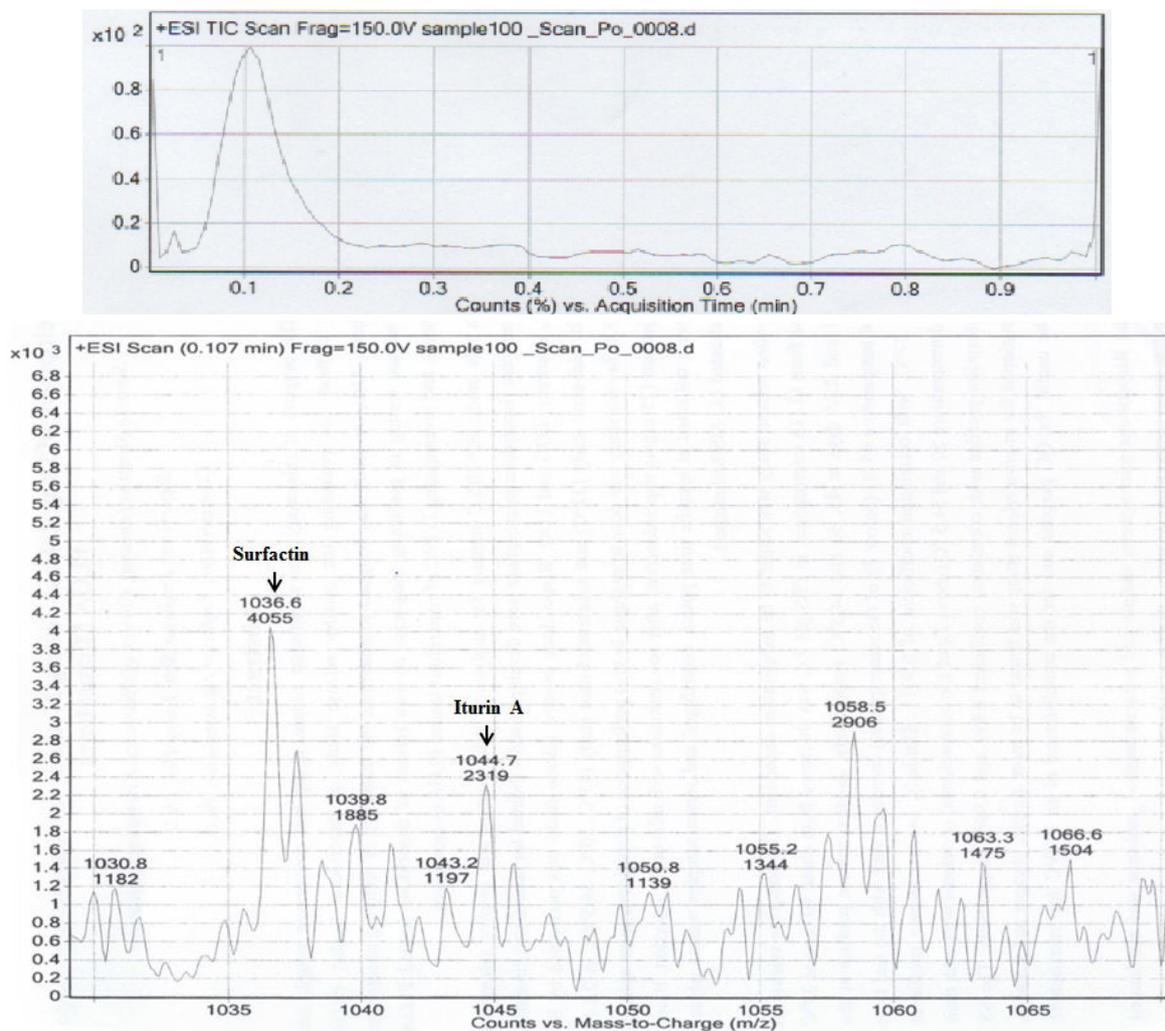


Fig. 3. Identification of Surfactin and Iturin A produced from *B. mojavensis* KJS-3 on TSB medium by LC-MS/MS. LC (upper) and MS/MS (below) data.

vensis KJS-3 are similar to commercially available surfactin and iturin A standards, respectively. These findings therefore indicate that fengycin, surfactin, and iturin A can be produced by *B. mojavensis* KJS-3. Cyclic lipopeptides including surfactin, iturin, and fengycin are the major classes of biosurfactants which are known to be produced by *Bacillus* species [15]. In the LC-MS/MS spectrum, well-resolved groups with peaks between 1,000 and 1060 m/z were observed, and these peaks can be attributed to the isoform ensembles of surfactins, iturins, and fengycins, which are included in the well-known surfactin products produced by *Bacillus* strains [21]. The cyclic lipopeptides have furthermore been shown to have higher antimicrobial and antifungal activity against Gram-positive cocci than against Gram-negative bacilli [6, 20].

Antifungal activity of *B. mojavensis* KJS-3

Cultured *B. mojavensis* KJS-3 were found to exert antifungal activity against *Botrytis cinerea* KACC 40573, *Rhizoctonia solani* AG-4 KACC 40142, *Sclerotinia sclerotiorum* KACC 41065, and *Colletotrichum gloeosporioides* KCCM 11220. *B. mojavensis* KJS-3 was found to inhibit the growth of all four fungal species (Fig. 4): inhibitive belts were clearly formed and *B. mojavensis* KJS-3 was shown to inhibit the *in vitro* growth of the mycelia of these phytopathogenic fungi. Several strains of *B. mojavensis* have been shown to inhibit the *in vitro* growth of *Fusarium moniliforme*; however, all strains of *B. mojavensis* produce different antifungal substances [1]. The antifungal compounds produced by *Bacillus* spp., such as fengycin, iturin, and surfactins, have been extensively studied for potential biocontrol activity [14].

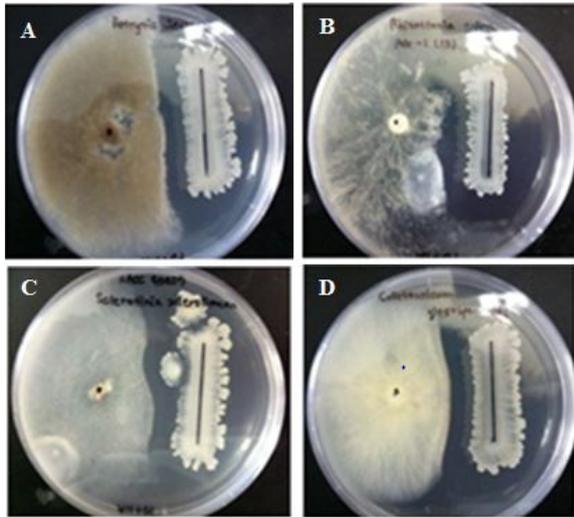


Fig. 4. Antifungal activity of the cocultivated *B. mojavensis* KJS-3 (Vertical line) on mycelia growth of (A) *Botrytis cinerea*, (B) *Rhizoctonia solani* AG-4, (C) *Sclerotinia sclerotiorum*, (D) *Colletotrichum gloeosporioides*. All strains are cultivated on PDA medium.

***B. mojavensis* KJS-3 as a PGPE**

An unknown bacterial strain suspected to be *B. mojavensis* KJS-3 was observed inside the roots of cultivated wild white ginseng plants by microscopy (Fig. 5). To confirm the identity of the selected bacterial strains, the enzymes produced by the unknown strains were shown to be the same as those produced by *B. mojavensis* KJS-3. *B. mojavensis* KJS-3 as a plant endophyte is able to enter plant roots and survive in the intercellular space, and furthermore, as a PGPE, exerts a protective effect on plants. Many studies have demonstrated PGPEs to be effective biocontrol agents for plant protection *in vitro* and *in vivo* and field application of suspensions of *Bacillus* spp. in the growth season of plants more over resulted in significantly reduced disease incidence [1,

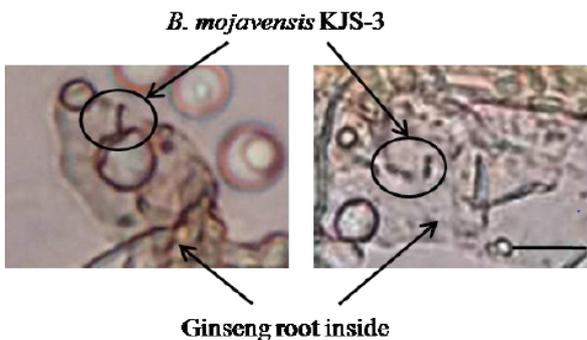


Fig. 5. Identification of *B. mojavensis* KJS-3 cocultivated with wild white ginseng seeds on land surface of farmland for 3 years throughout microscopy. Bar = 10 µm.

9, 14, 19]. PGPEs are rhizosphere bacteria that can enhance plant growth in a wide variety of applications such as microbial pesticides, microbial fertilizers, and animal feed additives. This study confirms that *B. mojavensis* KJS-3 is a promising probiotic that can be produced at an industrial level as a microbial product. The potential use of *B. mojavensis* KJS-3 as a biological control agent for field applications is supported by the presently reported results of laboratory bioassay analyses, which serve as relative predictors of resistance to pathogenic fungi. The findings of this study indicate that the industrial production of *B. mojavensis* KJS-3 may have wide applications in various areas, especially in agriculture.

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초록 : 식물 성장 촉진에 사용에 있어 *Bacillus mojavensis* KJS-3의 특징

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최근 식물 성장에 있어 곰팡이 독소에 관련된 질병에 효과가 있는 *Bacillus mojavensis* 균주 사용의 보고가 있다. 우리는 *B. mojavensis* KJS-3균주의 다양한 온도, 염도, 에탄올, pH에서 성장하는 특징을 확인 하였다. *B. mojavensis* KJS-3균주는 Polymerase chain reaction 분석에 의해 fengycin을 LC-MS/MS 분석을 통해서서는 iturin 및 surfactin 와 같은 cyclic lipopeptides를 생산함을 확인 하였다. *B. mojavensis* KJS-3균주는 식물 유해 곰팡이 균주인 *Botrytis cinerea*, *Rhizoctonia solani* AG-4, *Sclerotinia sclerotiorum*, *Colletotrichum goeosporioides*에 항곰팡이 효과가 있음을 확인 하였고 이 결과를 바탕으로 인삼재배에 있어 *B. mojavensis* KJS-3를 사용하여 성장을 관찰한 결과 뿌리 내에서 성장하여 plant growth promoting endophyte가 있음을 알 수 있었다. 이러한 특징들에 의해 미생물 농약 및 비료로 사용할 수 있을 것이다.