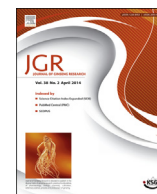


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Research article

Biological control of *Colletotrichum panacicola* on *Panax ginseng* by *Bacillus subtilis* HK-CSM-1Hojin Ryu¹, Hoon Park², Dong-Sang Suh³, Gun Ho Jung⁴, Kyungseok Park^{1,**},
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ABSTRACT

Background: Biological control of plant pathogens using benign or beneficial microorganisms as antagonistic agents is currently considered to be an important component of integrated pest management in agricultural crops. In this study, we evaluated the potential of *Bacillus subtilis* strain HK-CSM-1 as a biological control agent against *Colletotrichum panacicola*.

Methods: The potential of *B. subtilis* HK-CSM-1 as a biological control agent for ginseng anthracnose was assessed. *C. panacicola* was inoculated to ginseng plants and the incidence and severity of disease was assessed to examine the efficacy of the bacterium as a biological control against *C. panacicola*.

Results: Inoculation of *Panax ginseng* plants with *B. subtilis* significantly suppressed the number of disease lesions of *C. panacicola* and was as effective as the chemical fungicide iminoctadine tris(albesilate). The antifungal activity of *B. subtilis* against *C. panacicola* was observed on a co-culture medium. Interestingly, treatment with *B. subtilis* did not significantly affect the diameter of the lesions, suggesting that the mechanism of protection was through the reduction in the incidence of infection related to the initial events of the infection cycle, including penetration and infection via spore germination and appressorium formation rather than by the inhibition of invasive growth after infection.

Conclusion: Our results suggest that *B. subtilis* HK-CSM-1 can be used as an effective and ecologically friendly biological control agent for anthracnose in *P. ginseng*.

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1. Introduction

Korean ginseng (*Panax ginseng*) is one of the most important perennial herb plants grown and used in Asia. Its clinical value as a medicine has been recognized for over a thousand years [1,2]. The pharmacologically active compounds in ginseng are primarily located in the roots. Long cultivation periods (4–6 yr) maximize the concentrations of these root compounds. Therefore, in Korea, *P. ginseng* plants are generally cultivated for several years, usually in shady areas. However, successive cultivation in the same soil for a long period of time leads to a deterioration in the physical and

chemical properties of the soil, frequently providing favorable conditions for infection by various soil-borne pathogens; this can potentially lead to severe reductions in yield. Chemical pesticides have been applied to control disease in *P. ginseng* plantations. However, the accumulation of deleterious pesticide residues in ginseng roots and in the surrounding soil has become a serious environmental concern. As a result, the organic production of ginseng is being increasingly favored. Ginseng anthracnose, caused by the fungus *Colletotrichum panacicola*, has a severe impact on yields, necessitating the development of an organic control method.

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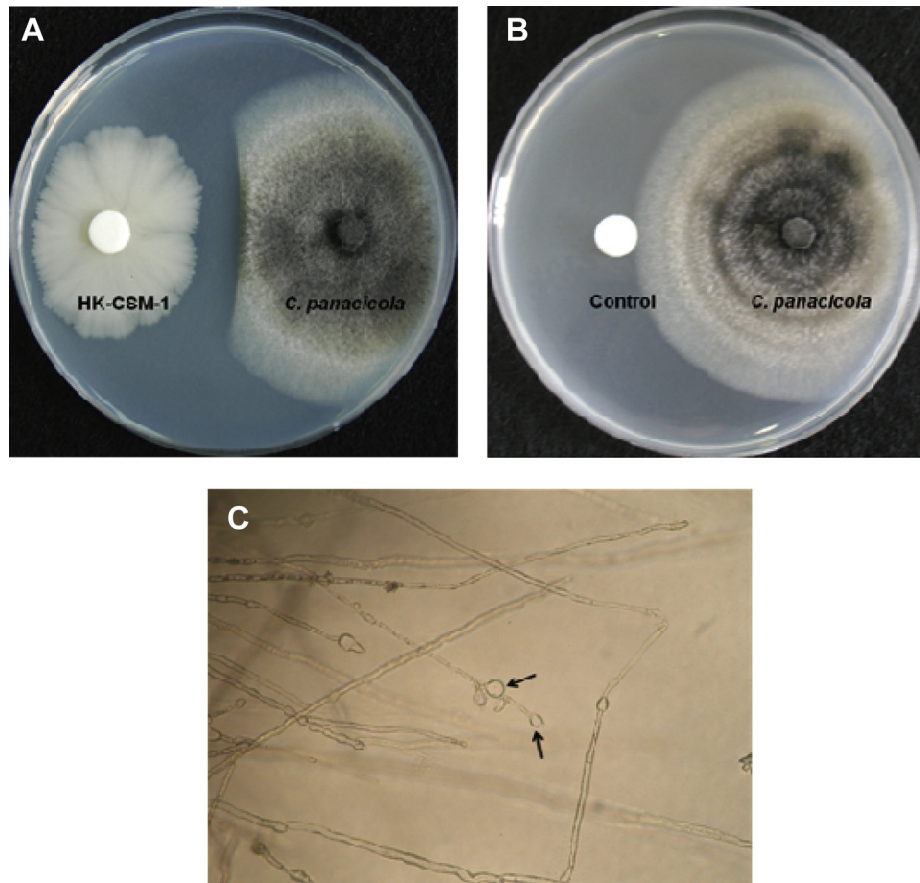


Fig. 1. Antagonistic activity of *Bacillus subtilis* HK-CSM-1 on mycelial growth of *Colletotrichum panacicola*. (A) Inhibition zone of mycelial growth achieved by cocultivation with *B. subtilis* HK-CSM-1. (B) Normal growth phenotype of *C. panacicola* on control. (C) Hyphal swelling (arrows) of *C. panacicola* induced by *B. subtilis* HK-CSM-1 after 10 d of incubation on the potato dextrose agar medium.

The biological control of plant diseases using beneficial rhizobacteria is an environmentally friendly method that exhibits good potential for use in ecologically friendly programs of disease management. Members of the genus *Bacillus* are known to suppress various plant diseases, such as anthracnose in red peppers [3], mangos and wax apples [4], as well as root rot in ginseng caused by *Fusarium cf. incarnatum* and *Cylindrocarpon destructans* [5,6]. Furthermore, *Bacillus subtilis* has been reported to be relatively benign to humans and several *B. subtilis* strains are listed by the Organic Materials Review Institute [7]. Several active compounds with potentially inhibitory effects on pathogen growth have been identified in *B. subtilis* and many of these compounds have shown antibiotic activity against anthracnose in mangos and wax apples [4].

Although the use of *B. subtilis* as a biological control agent for anthracnose in ginseng plants has been proposed, the effects of this species or other members of the genus *Bacillus* have not been evaluated for their activity against *C. panacicola*. In this study, we evaluated the antifungal activity of *B. subtilis* HK-CSM-1 against *C. panacicola*. We also verified whether its antagonism towards the growth of *C. panacicola* could be used as a criterion in the protection of ginseng plants from anthracnose disease.

2. Materials and methods

2.1. Antifungal activity in vitro

B. subtilis HK-CSM-1 was initially isolated from soils in ginseng fields [8] and stored in order to survey its potential as a biological

control agent for ginseng anthracnose. Mycelial growth inhibition activity was performed by the dual-culture method. Paper discs (0.5 cm diameter) were dipped into a suspension of *B. subtilis* HK-CSM-1 (1×10^7 cfu/mL) and placed on the edge of potato dextrose agar (PDA) plates. Inoculum discs (0.5 cm diameter) of *C. panacicola* were placed on the opposite edges of the plates, which were then incubated at 25°C for 10 d.

2.2. Inoculum

C. panacicola was isolated from infected ginseng leaf tissues and identified based on its morphological and cultural characteristics. The pathogenicity of the fungus was confirmed by its successful reinfection of ginseng seedlings. For inoculum preparation, the pathogen was cultured on PDA plates at 25°C for 10 d, mechanically blended, and then filtered through gauze, yielding a suspension of 10^7 spores/mL.

2.3. Container test

Ten ginseng seeds were sown per container, which was filled with soil (parent material, weathered granite). After the seedling leaves fully unfurled, a conidial suspension was sprayed on the seedlings. To induce anthracnose, the seedlings were grown in a growth chamber at 25°C for the first 7 d, after which they were grown at 22°C for a further 7 d. The incidence of disease was recorded.

2.4. Treatment spray

Four different treatments were assayed, namely: a bacterial suspension of *B. subtilis* HK-CSM-1 (1×10^7 cfu/mL); tryptic soy broth (TSB) as a control; the fungicide iminoctadine tris(albesilate) (ITA; a.i. 400 ppm), and water as another control. Treatments were conducted after the pathogen inoculation spray had dried on the seedlings.

Five replicates were performed for the container experiment; the containers were arranged in a randomized block design. All results were analyzed using Duncan's multiple range test.

2.5. Disease incidence and severity assessment

Disease incidence was rated as the mean number of diseased lesions per container and the total number of lesions was counted. Disease severity was rated as the mean diameter of the lesions; all the lesions on two seedlings per container were measured using a Vernier caliper. The percentage of leaf area per seedling covered with lesions was estimated visually. The protection rate of disease incidence (PI) was calculated as $PI (\%) = (N_c - N_t) / (N_c \times 100)$, where N_c = number of lesions in the control and N_t = number of lesions in the treatment sample. The inhibition rate (IR) of lesion size was defined as $IR (\%) = (D_c - D_t) / (D_c \times 100)$, where D_c = mean diameter of lesions in the control and D_t = mean diameter of lesions in the treatment. The protection rate of disease severity (PS) was defined as $PS (\%) = (A_c - A_t) / (A_c \times 100)$, where A_c = total area of lesions in the control [$N_c \times \pi \times (D_c/2)^2$] and A_t = total area of lesions in the treatment [$N_t \times \pi \times (D_t/2)^2$].

Table 1

Effect of *Bacillus subtilis* HK-CSM-1 and the Chemical Fungicide Iminoctadine Tris(albesilate) on the Control of Ginseng Anthracnose Caused by *Colletotrichum panacicola*

Treatment	Lesion number ¹⁾	Lesion diameter (mm) ²⁾	Disease severity (mm ²) ³⁾
<i>Bacillus subtilis</i> HK-CSM-1	1.80 ± 1.03 ⁴⁾ (85.8)a	2.98 ± 1.13 (0.0)a	10.08 (86.6)a ⁵⁾
ITA	0.25 ± 0.26 (98.0)a	0.95 ± 0.79 (61.7)b	0.41 (99.1)a
TSB	6.43 ± 4.07 (54.8)b	2.40 ± 1.11 (9.5)a	32.79 (69.6)a
Control	15.88 ± 6.16 (0.0)c	2.62 ± 0.78 (0.0)a	99.62 (0.0)b

ITA, iminoctadine tris(albesilate); TSB, tryptic soy broth

¹⁾ Mean number of disease lesions per container

²⁾ Mean spot size (diameter) per lesion

³⁾ Disease severity [total area of lesions per container = lesion number × (lesion radius)² × 3.14]

⁴⁾ Data presented as mean ± standard deviation of five replications

⁵⁾ The same letters in a column denote no significant difference at $p < 0.05$ by Duncan's multiple range test

3. Results and discussion

To test if *B. subtilis* HK-CSM-1 had antagonistic effects on the growth of *C. panacicola*, we first carried out a dual-culture test on a PDA medium. An inhibition zone was evident, produced by the inhibition of mycelial growth via the antifungal activity of *B. subtilis* HK-CSM-1 (Fig. 1A). However, normal growth of the fungus was observed in the control (Fig. 1B). Several previous studies have documented the antagonistic effects of beneficial microorganisms towards fungal pathogens as a result of the inhibition of conidial germination and inducement of germ tube swelling [4]. In our

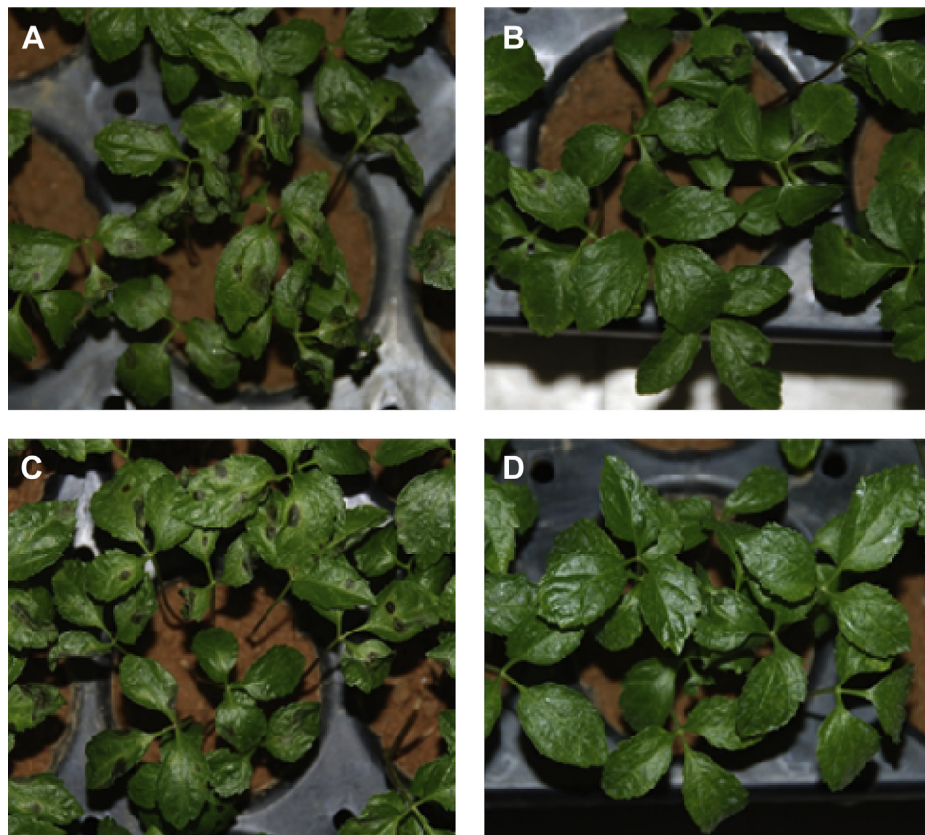


Fig. 2. Effect of *Bacillus subtilis* HK-CSM-1 on the control of ginseng anthracnose caused by *Colletotrichum panacicola* (14 d after treatment). (A) Control (pathogen only). (B) *B. subtilis* HK-CSM-1 treatment + pathogen. (C) Tryptic soy broth medium + pathogen. (D) Iminoctadine tris(albesilate) treatment + pathogen.

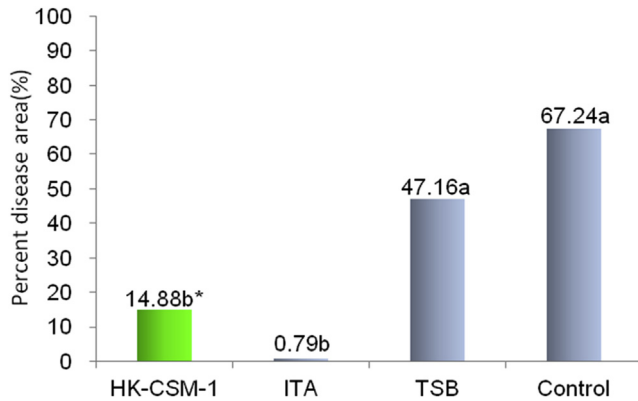


Fig. 3. Effect of *Bacillus subtilis* HK-CSM-1 on the percentage area of *Panax ginseng* leaves affected by anthracnose. * No significant difference ($p < 0.01$) between means with the same letter (assessed by Duncan's multiple range test). ITA, iminoctadine tris(albesilate); TSB, tryptic soy broth.

study, frequent and consistent hyphal swelling of *C. panacicola* mycelia was induced by cocultivation with *B. subtilis* HK-CSM-1 (Fig. 1C). Together, these results indicate that *B. subtilis* HK-CSM-1 inhibits the growth of *C. panacicola*.

We then investigated the possibility of using *B. subtilis* HK-CSM-1 as a biological control agent against *C. panacicola* *in vivo* and determined its efficacy relative to treatment with the chemical fungicide ITA. The fungicide demonstrated good control of anthracnose in ginseng leaves (Fig. 2D). Interestingly, as shown in Fig. 2B, *B. subtilis* HK-CSM-1 effectively attenuated the infection of *C. panacicola* on ginseng seedlings, whereas symptoms of an advanced infection were observed on the water and TSB controls (Figs. 2A and 2C). The number of infected lesions per container is indicated in Table 1. *B. subtilis* HK-CSM-1 was not significantly different ($p < 0.05$) from ITA (Table 1) in control efficacy 14 d after inoculation with the pathogen. In addition, the reduction in the number of lesions displayed a significantly negative linear correlation with effect variation ($r = -0.9899$, $p < 0.05$). These results are similar to those in previous studies, which reported variations in the inhibitory effects of microbial pesticides against pathogen growth [9].

The degree of protection, in terms of the percentage reduction in the number of disease lesions, is displayed in Table 1. No significant difference ($p < 0.05$) was detected between the *B. subtilis* HK-CSM-1 and ITA treatments. The TSB control also displayed a protective effect ($p < 0.05$) compared with the control, but lower than that of *B. subtilis* HK-CSM-1.

Anthraco-nose infection processes can be divided into two stages, referred to initially as biotrophs and later switching to necrotrophs. The first biotrophic stage involves spore germination and the formation of an appressorium, then penetration into plant tissues by a thin penetration peg. In the second necrotrophic stage, the invaded hypha is developed in the plant tissues, resulting in death and collapse to form a sunken area [10,11]. To verify the attenuation of disease symptoms, we also surveyed the differences in size of anthracnose lesions. Interestingly, as displayed in Table 1, treatment with *B. subtilis* HK-CSM-1 was not significantly different from the control in terms of lesion size (area). However, the disease severity was significantly reduced in plants treated with *B. subtilis* HK-CSM-1 compared with the controls. This suggests that *B. subtilis* was able to inhibit virulence at the penetration stage, but not at the tissue invasion stage. This implies that treatment during the penetration stage is critical in protecting against anthracnose.

Lastly, we investigated the area of the lesions as a percentage of the total leaf area, which is equivalent to disease severity. As shown in Fig. 3 and Table 1, there was no significant difference in the control of anthracnose between *B. subtilis* HK-CSM-1 and ITA ($p < 0.01$). Furthermore, the percentage of leaf area covered by lesions indicated significant linear correlation ($r = 0.95038$, $p < 0.05$) with the number of lesions. This again suggests that the penetration stage is critical in the effective control of anthracnose in ginseng. These observations also confirm the veracity of visual assessments.

4. Conclusion

We have reported an effective approach to achieve the ecologically friendly control of ginseng anthracnose, one of the most harmful diseases of this crop. The protective effects of *B. subtilis* HK-CSM-1 were similar to those of the commercial fungicide ITA. However, this study was conducted on containerized plants and further studies are required to investigate whether these results hold true under field conditions. To develop an effective biological control standard, it is necessary to test the protective effects of *B. subtilis* in the field, including the determination of the optimum time for the treatment. In addition, formulations prolonging the survival of the bacterium on crop plants are necessary. This study provides a foundation to enable the development of ecologically friendly agricultural biotechnology methods for protection against ginseng diseases.

It has been suggested that the genus *Bacillus* can be considered as a microbial "factory", as the species in this genus produce a wide variety of antibiotic metabolites. These compounds, including lipopeptides, have shown diverse inhibitory effects on the growth of various phytopathogens. Furthermore, approximately 4–5% of the genome of *B. subtilis* contains genes suitable for the synthesis of antibiotics; it has been proposed that over two dozen structurally diverse antimicrobial compounds are produced by this species [12]. Based on these reports, it is likely that the antifungal activity of *B. subtilis* HK-CSM-1 is due to the production of certain antibiotic compounds. Identification of these putative antibiotic compounds may be helpful in expanding our understanding of microbial functions in ecosystems, with the purpose of developing biotechnological tools to control a broad range of plant diseases.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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