Antagonistic Evaluation of Chromobacterium sp. JH7 for Biological Control of Ginseng Root Rot Caused by Cylindrocarpon destructans

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Abstract  Cylindrocarpon destructans is an ascomycete soil-borne pathogen that causes ginseng root rot. To identify effective biocontrol agents, we isolated several bacteria from ginseng cultivation soil and evaluated their antifungal activity. Among the isolated bacteria, one isolate (named JH7) was selected for its high antibiotic activity and was further examined for antagonism against fungal pathogens. Strain JH7 was identified as a Chromobacterium sp. using phylogenetic analysis based on 16S rRNA gene sequences. This strain was shown to produce antimicrobial molecules, including chitinases and proteases, but not cellulases. Additionally, the ability of JH7 to produce siderophore and solubilize insoluble phosphate supports its antagonistic and beneficial traits for plant growth. The JH7 strain suppressed the conidiation, conidial germination, and chlamydospore formation of C. destructans. Furthermore, the JH7 strain inhibited other plant pathogenic fungi. Thus, it provides a basis for developing a biocontrol agent for ginseng cultivation.

Keywords  Chromobacterium, Cylindrocarpon destructans, Ginseng root rot

Ginseng (Panax ginseng) is commonly used for conventional medicine and for the treatment of a variety of diseases including cancer and diabetes [1-4]. Ginseng is a perennial medicinal plant that grows in the shade and requires 4–6 years to produce mature roots, which can be used as raw material to produce red or white ginseng [5, 6]. Ginseng is vulnerable to attack by soil microbes including fungi, bacteria, and nematodes because of its long-term cultivation and shady environment [7-9]. Among these pathogens, fungal pathogens induce serious damage during ginseng cultivation. These include Colletotrichum gloeosporioides causing anthracnose [10], Alternaria panax causing alternaria blight [11], Ascochyta sp. and Macrophoma sp. causing leaf spot, Botrytis cinerea causing gray mold [12], Cladosporium spp. causing dry rot [13], Phylllosticta panacicca causing snake-eye spot [13], Phytophthora cactorum causing phytophthora blight [14], Pythium ultimum and Rhizoctonia solani causing damping-off [12], and Sclerotinia panacicca causing sclerotinia rot [12]. Among the fungal pathogens affecting ginseng, Cylindrocarpon destructans (teleomorph Nectria radicicola) is the most important [15-17].

C. destructans, a soil-borne pathogenic fungus, can cause primary root rot disease in ginseng, thereby decreasing ginseng production by 40–60% and causing significant economic losses [18-20]. In addition, C. destructans causes replant failure because of its capability to survive in the field for more than 10 years after harvesting ginseng. C. destructans, originally named Ramuraria destructans in American ginseng (Panax quinquefolius) [21], is also known to cause serious root rots in other plants such as strawberries and peonies [22, 23].

The use of fungicides to prevent ginseng root rot has improved the productivity of ginseng; however, there are side effects such as residual toxicity and environmental pollution caused by ecosystem destruction [24]. Thus, it is important to develop environmentally friendly control technologies. As an alternative solution, efforts are underway to utilize secondary metabolites from microorganisms as pesticides that are readily degraded and have pathogen selectivity [25]. Biocontrol mechanisms that control plant diseases via microorganisms have been grouped into five...
categories: (1) lytic action that degrades the cell walls of phytopathogenic fungi; (2) antibiotics produced by the Bacillus [26, 27], Penicillium [28], Pseudomonas [29], and Streptomyces [30, 31] genera that directly inhibit the growth of plant pathogenic bacteria; (3) competitive antagonistic action where bacteria inhibit fungal growth and proliferation [32]; (4) inhibition of fungal pathogenicity in the plant by depletion of the factors required for growth, such as nutrients in the rhizosphere [33-36]; and (5) inducible resistance where microorganisms are used to produce molecules, such as exopolysaccharide, lipopolysaccharide, salicylic acid, hydrogen cyanide, and 2,3-butanediol, which activate plant immune functions [37-39].

Natural antibiotics derived from microorganisms provide important materials for the development of new pesticides, and their active ingredients are valuable in the field of antimicrobial research [40]. Studies on the biological control of ginseng root rot have been performed mainly on rhizobacteria, but studies have also been performed on Bacillus spp. and Streptomyces spp. [41, 42]. In this study, microorganisms with an antagonistic effect against C. destructans were identified in ginseng rhizosphere soil. To explore the microbial activity against ginseng root rot, we investigated antimicrobial molecules, including cellulases, proteases, and chitinases, as well as siderophore production and phosphate solubilization. Furthermore, the specific antimicrobial effects of the bacteria were evaluated in the presence of the fungal pathogen, C. destructans, by measuring the suppression of hyphal growth, conidial production, conidial germination, and chlamydospore formation.

MATERIALS AND METHODS

Pathogen preparation for in vitro screening. C. destructans (KACC 41077) was cultured on potato dextrose agar (PDA) at 21°C for 10 days. These fungal cultures were used directly in a dual culture assay or were cultured again for conidial production. To obtain C. destructans conidia, C. destructans hyphae were cultured in V8 liquid medium at 21°C for 7 days with shaking at 150 rpm.

Dual culture assay of bacteria with antifungal activity against C. destructans. To explore the biological control of C. destructans, bacterial discs (4 mm in diameter) from a 10-day-old culture on PDA plates were placed in the center of 9-cm PDA plates. Bacterial isolates were grown in Luria-Bertani (LB) broth at 28°C for 16 hr with shaking at 200 rpm, and 20 μL of the bacterial suspensions were added to PDA plates with pores (4 mm in diameter) at a distance of 3 cm from the mycelial discs. Sterile distilled water was used as an untreated control. After incubation for 15 days, the antifungal activity of each bacterial isolate was measured according to the inhibition of mycelial growth of C. destructans. All other phytopathogens were analyzed using the same method, and treatments were performed in triplicate.

Identification of antifungal bacteria. Among the bacteria evaluated, the isolate with the highest antifungal activity against C. destructans was selected for further biocontrol studies. Genomic DNA was extracted from bacterial cells using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). To confirm the identities of the bacteria, 16S rRNA gene sequences of 1.4 kb were amplified using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and the Thermal Cycler Thermal Controller 2720 (Applied Biosystems, Foster City, CA, USA). The 16S rRNA sequences were used as a query for a BLAST search in NCBI and EZbiocloud (https://www.ezbiocloud.net/). Each DNA sequence was aligned using ClustalW of MEGA 7, and the phylogenetic relationship was determined using the neighbor-joining method with 1,000 bootstrap replicates.

Conidiation, germination, and chlamydospore formation assays. To suppress conidial production by bacterial treatment, C. destructans was cultured in V8 liquid medium with 1%, 0.1%, or 0.01% bacterial cultures at an optical density (OD) of 1.0 with shaking at 150 rpm, at 21°C, for 9 days. After inoculation for 3, 6, or 9 days, the number of spores was calculated using a hemocytometer. For the conidial germination assay, each bacterial culture was diluted serially (OD 1.0, 0.1, and 0.01), co-mixed with the conidium suspension (1 x 10^7 conidia/mL), and placed on a hydrophobic slide (Knittel Glaser, Braunschweig, Germany), and incubated in a humidity box at 25°C. After incubation for 0.5, 1, 1.5, or 2 hr, the number of germinating spores was counted among 100 spores per treatment. To examine the inhibition of chlamydospore formation, we used Czapek-Dox broth treated with bacteria at an OD of 0.001. A mycelial agar plug (5 mm diameter) was inoculated into each medium, and the medium was incubated at 21°C with shaking at 150 rpm. Chlamydospore production was observed after 5 days under a Carl Zeiss Axio Imager A2 microscope (Carl Zeiss, Oberkochen, Germany). Each experiment was replicated three times, and three separate experiments were conducted.

Analysis of antimicrobial and antagonistic molecules. Screening for cellulase producers was performed on carboxymethyl cellulose (CMC) agar medium as described previously [43]. Proteolytic activity was evaluated on LB agar plates containing 3% skim milk powder. After 3 days at 28°C, the presence of a clear zone surrounding the bacteria was suggestive of protease production. The chitin-degrading ability was assessed in colloidal chitin agar medium as described previously [43]. After incubation for 3 days at 25°C, a clear zone stained using the above method was suggestive of chitinase activity. Production of siderophore was measured using the Chrome azurol S (CAS) agar assay. The CAS agar plate was prepared as described previously.
The CAS agar plate was punched to make pores (4 mm diameter) using a cork-borer, which were then filled with 20 μL bacterial suspension and LB broth as a control. Siderophore production was examined after incubation at 25°C for 5 days. Phosphate solubilization screening was performed on Pikovskaya’s medium. Pikovskaya’s media plate was prepared as described previously [43]. After incubation for 10 days at 25°C, the development of a clear zone was suggestive of phosphate solubilization.

Analysis of biochemical and physiological characteristics. The API 20NE (BioMérieux, Marcy l’Etoile, France) system was used to analyze the biochemical and physiological characteristics. A substrate utilization test was performed using API 20NE system test panels. The API assay was conducted according to the manufacturer’s instructions. A stock culture of the bacteria was streaked onto LB agar plates to obtain a single colony. The colony was diluted in a 0.85% NaCl solution and the turbidity was adjusted to 1 McFarland. Two hundred milliliters of this solution were transferred into each well of the panels. To prevent contamination by other microorganisms in the air, each well was filled with mineral oil and cultured at 25°C and read 24 hr after inoculation.

Table 1. Antifungal activity of bacterial treatments on the mycelial growth of Cylindrocarpon destructans in a dual culture assay

| Strain | Mycelial growth (mm) | Inhibition rate (%)<sup>a</sup> |
|--------|----------------------|-------------------------------|
| GC12   | 18.3 ± 1.0 e         | 29.8                          |
| SH10   | 20.6 ± 0.5 f         | 21.3                          |
| AD-17  | 15.9 ± 0.8 c         | 39.1                          |
| JH7    | 11.8 ± 0.7 a         | 54.9                          |
| FT-8   | 17.4 ± 0.7 d         | 33.2                          |
| JH15   | 14.2 ± 0.7 b         | 45.5                          |
| Control| 26.1 ± 0.8 g         |                               |

<sup>a</sup>Inhibition rate = [(mycelia growth (control) – mycelial growth (treatment))/mycelia growth (control)] × 100.

<sup>b</sup>Values are means ± standard errors calculated from three independent observations. Different letters indicate significant differences based on Duncan's multiple-range test (p = 0.05).

Antifungal activity and identification of bacterial isolates. A total of 132 bacteria isolated from ginseng field soils were evaluated for antifungal activity against C. destructans. Among these isolates, six significantly decreased...
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mycelial growth of the pathogen as compared with the untreated control (Table 1). This was especially true of the bacterial isolate JH7 that showed relatively higher antifungal activity (greater than 50%) against C. destructans (Fig. 1A). JH7 formed circular, creamy-yellow-colored, mucus-like, raised colonies with non-pigmented and smooth margins (Fig. 1B). To determine the identity of this bacterial isolate, 16S rRNA sequences were analyzed. JH7 showed the highest homology (E-value of 0.0) to Chromobacterium species, including Chromobacterium sp. KJ619639 (99%), C. violaceum (HM449690, 99%), Chromobacterium sp. AB426118 (99%), C. haemolyticum (KJ845679, 99%), and C. aquaticum (JQ582944, 99%) (Fig. 2).

Biochemical and physiological characterization of JH7. JH7 was characterized based on its biochemical and physiological properties (Table 2). The API 20NE tests revealed that JH7 utilizes D-glucose, L-arabinose (weak), D-mannitol, N-acetylglucosamine, potassium gluconate, capric acid, malic acid, trisodium citrate, and phenylacetic acid, but not D-mannose, maltose, or adipic acid. The test was positive for potassium gluconate, D-glucose fermentation (weak), arginine dihydrolase, urease, and gelatin hydrolysis (weak), but negative for tryptophan deaminase, esculin hydrolysis, and p-nitrophenyl-β-D-galactopyranoside. This result showed five differences in substrate utilization (urease, gelatin, L-arabinose, D-mannitol, and phenylacetic acid) from that by Chromobacterium violaceum (HM449690), which exhibited the closest relationship to JH7 based on 16S rRNA analysis (Fig. 2). Therefore, we identified strain JH7 as a Chromobacterium sp.

Table 2. Biochemical and physiological characterization of the JH7 strain

| Substrate                        | JH7 | Cylindrocarpon violaceum |
|----------------------------------|-----|--------------------------|
| Potassium nitrate                | +   | +                        |
| Tryptophan                       | −   | −                        |
| Glucose                          | w   | +                        |
| Arginine                         | +   | +                        |
| Urea                             | +   | −                        |
| Esculin                          | −   | −                        |
| Gelatin                          | w   | −                        |
| p-Nitrophenyl-β-D-galactopyranoside | −   | −                        |
| Glucose                          | +   | +                        |
| Arabinose                        | w   | −                        |
| Mannose                          | −   | −                        |
| Mannitol                         | +   | −                        |
| N-acetyl-glucosamine             | +   | +                        |
| Maltose                          | −   | −                        |
| Gluconate                        | +   | +                        |
| Caprate                          | +   | +                        |
| Adipate                          | −   | −                        |
| Malate                           | +   | +                        |
| Citrate                          | +   | +                        |
| Phenyl-acetate                   | +   | −                        |
| API 20 NE No.                    | 5355557 | 5140555              |

+, fully metabolized; −, not metabolized; w, weakly metabolized.

JH7 production of antimicrobial molecules and siderophores. The bacterial isolate JH7 was examined for production of antibacterial molecules (Fig. 3) by assessing chitinases, proteases, and cellulases on LB plates containing colloidal chitin, skim milk, and CMC, respectively. JH7 decomposed chitin but not cellulose. In addition, strong proteolytic activity was observed in JH7. Next, we examined siderophore production and solubilization of insoluble phosphate by JH7. JH7 formed halo zones on CAS agar media, indicative of siderophore production. In addition, JH7 showed solubilization of insoluble phosphate as compared

Fig. 3. Characterization of the antagonistic molecules produced by Chromobacterium sp. JH7. Specifically, the cellulase, protease, and chitinase activity, as well as siderophore production and phosphate solubilization capacity of Chromobacterium sp. JH7 was analyzed. Briefly, tests for cellulase, protease, and chitinase were performed using 1% carboxymethyl cellulose, 3% skim milk, and 0.05% colloidal chitin, respectively. Siderophore production and phosphate solubility were tested on Chrome azurol S and Pikovskaya’s agar plates, respectively.
Effect of bacterial treatment on the suppression of conidial production, conidial germination, and chlamydospore formation. Conidiation is an important process that results in a massive production of conidia in most phytopathogenic fungi; thus, we assessed the effect of bacterial isolate JH7 on conidiation (Fig. 4A). When strain JH7 was added at a concentration of 1%, we observed low spore concentrations of $1.7, 3.7,$ and $2.7 \times 10^4$/mL at 3, 6, and 9 days, respectively. The inhibition rates were $97.4\%$, $97.6\%$, and $98.7\%$ at 3, 6, and 9 days, respectively, as compared with the untreated control. After treatment with 0.1% and 0.01% JH7, the inhibition rates on spore formation were 76.4% and 71.2% at 3 days, respectively. At 6 and 9 days, the inhibition rates were 82.9% and 87.8%, respectively. Based on these results, conidial formation was almost completely suppressed by the presence of 1% JH7, and spore formation was inhibited by 80% or more, even when the JH7 culture concentration was less than 0.1%.

Given that conidium germination is an early step in disease development, the ability of the JH7 strain to suppress conidial germination of *C. destructans* was investigated (Fig. 4B). In the control, 94% of *C. destructans* spores germinated at 2 hr. However, treatment with JH7 at an OD of 1 resulted in germination of only 1% of spores over 2 hr, thus showing 98.9% inhibition of spore germination. At an OD of 0.1 or 0.01, 80.9% or 41.5% inhibition was observed, respectively. As a result, spore germination was suppressed at a high concentration of JH7 at an OD of 1.0, with delayed or inhibited spore germination at an OD of 0.1 or less.

Chlamydospores play a role in primary inoculum, and it is important to assess the effects of JH7 against chlamydospore formation of *C. destructans*. JH7 inhibited conidial formation at a concentration of 1%; therefore, 1% JH7 was used to examine the formation of chlamydospores. Our results showed that chlamydospores formed in the mycelium in the non-treated group, but not in the JH7-treated group. Therefore, JH7 inhibited the formation of *C. destructans* chlamydospores.

**The spectrum of antimicrobial activity by JH7.** To identify additional applications of JH7, the antimicrobial activity against pathogenic fungi other than ginseng root

![Fig. 4.](image)

**Table 3.** Antifungal activity of *Chromobacterium* sp. JH7 against other fungal plant pathogens

| Class            | Taxonomy                     | JH7 |
|------------------|------------------------------|-----|
| **Dothideomycetes** | *Cladosporium cucumerinum*   | ++++* |
|                  | *Exserohilum turcicum*      | +++  |
|                  | *Cochliobolus heterostrophus* | +++  |
| **Agaricomycetes** | *Rhizoctonia solani*        | +++  |
| **Leotiomycetes** | *Botrytis cinerea*          | +++  |
| **Sordariomycetes** | *Sclerotinia sclerotiorum*   | ++   |
|                  | *Magnaporthe oryzae*        | +++  |
|                  | *Colletotrichum acutatum*   | ++   |
|                  | *Fusarium oxysporum*        | ++   |
|                  | *Fusarium solani*           | +    |

*Determined by measuring the average diameter of the clear zone of inhibition: +, < 5 mm; ++, 6–10 mm; ++++, 11–15 mm; ++++, > 15 mm.*
rot was explored (Table 2). Based on our results, the JH7 strain showed antimicrobial activity against 10 pathogenic fungi. Among them, Cladosporium cucumerinum, Exserohilum turcicum, and Cochliobolus heterostrophus, belonging to Dothideomycetes, were most strongly inhibited, and Rhizoctonia solani, B. cinerea, and Magnaporthe oryzae were moderately inhibited. However, Colletotrichum acutatum, Fusarium oxysporum, and Fusarium solani, belonging to Sordariomycetes (excluding Magnaporthe oryzae), were only weakly inhibited.

**DISCUSSION**

Fungal disease is a major problem in the cultivation of ginseng from seed stratification and soil preparation prior to planting and drying of the roots. Recent studies have shown the co-existence of many soil-borne pathogens and antagonistic microorganisms in the rhizosphere [44]. In this study, we reported the antagonistic effect of *Chromobacterium* sp. JH7 on *C. destructans*, which could be used in the development of ecologically friendly methods to control ginseng root rot, one of the most harmful diseases to this crop. JH7 isolated from ginseng-cultivating soil was identified as *Chromobacterium* sp., which showed the strongest antimicrobial activity against the ginseng root rot fungus *C. destructans*. The genus *Chromobacterium* belongs to the family Neisseriaceae (Betaproteobacteria) and was first described a century ago [21]. Members of the genus are Gram-negative, facultative anaerobic, slender, slightly curved, rod-shaped bacteria found in normal waters and soils of tropical and subtropical regions worldwide [45]. Until now, nine species have been identified as belonging to the genus *Chromobacterium* based on 16S rRNA sequencing, colony morphology, fatty acid composition, and metabolic diversity: *C. violaceum* (the type species), *C. substisuga* [46], *C. aquaticum* [47], *C. haemolyticum* [48], *C. piscinae*, *C. pseudoviolaceum* [49], *C. vaccinia* [50], *C. amazonense* [51], and *C. rhizoryzae* [52]. The JH7 strain is most likely to belong to *C. violaceum* based on 16S rRNA sequencing analysis (Fig. 2); however, other characteristics of JH7 based on physiological and biochemical metabolic analyses indicated that JH7 is not similar to *C. violaceum* (Table 2). Therefore, identification of the JH7 strain at the species level cannot be completed until additional resources for bacterial identification are available.

Previously, a *Chromobacterium* sp. was shown to have an antagonistic effect on several soil-borne plant pathogens *in vitro* and suppressed the damping-off of eggplant caused by *Rhizoctonia solani* [53]. Tn5 insertion mutants deficient in chitinolytic activity did not inhibit the mycelial growth of *Rhizoctonia solani*, and their suppression of *Rhizoctonia* damping-off was much lower than that induced by the wild-type [54]. These results suggest that chitinases of *Chromobacterium* sp. play an important role in the suppression of phytopathogenic fungi. This is because the cell walls of many phytopathogenic fungi belonging to ascomycota and basidiomycota are composed of chitins, glucan polymers, and mannoproteins [55]. Many antagonistic microorganisms (including *Chromobacterium*) secrete lytic enzymes that can degrade fungal cell walls [56, 57]. Likewise, the inhibitory effect of JH7 against several fungal pathogens appears to be associated with the secretion of biological compounds, such as chitinases and proteases (Fig. 3).

Siderophores are iron-chelating compounds secreted by microorganisms to absorb iron from their surroundings [58]. Antagonistic microorganisms that produce siderophores inhibit the growth of plant pathogens and increase plant growth by recruiting iron to the root area [59]. Because siderophores produced by antagonistic microorganisms inhibit the germination of soil pathogenic fungi [60], inhibition of *C. destructans* germination appears to be associated with siderophore production by JH7 (Fig. 3 and 4B). Therefore, siderophore production by antagonistic microorganisms is an important criterion for the development of a biological agent, especially for ginseng, which requires longer cultivation periods. JH7 was also able to dissolve insoluble phosphate compounds (Fig. 3). Phosphate uptake in plants is limited by the low solubility of phosphate compounds in soil [61]. Thus, phosphate solubilization by soil microorganisms is an important factor for plants as they provide a major nutrient required for plant growth. Many bacteria play an important role in phosphate solubilization and bioavailability by producing organic acids [62].

Extracellular antibiotics secreted by bacteria can affect the cellular functions of fungal pathogens. For example, fungal respiration, cell wall synthesis, cell membrane integrity, and transport systems are inhibited by fusaric acid [63], hydrogen cyanide [64], penicillin, butyric acid [65], cyclic lipopeptides [66], polymyxin B [67], and β-phenylethanol [68]. *Chromobacterium* produce various antibiotics. Violacin, a typical antibiotic produced by *Chromobacterium*, has numerous biological activities including anti-leishmania [69], anti-virus [70], anti-tumor [71, 72], and anti-tuberculosis activities [73]. In addition, other antibiotics produced by *Chromobacterium* include cyaniode, aerocyanidin, and acrocaravin [74, 75]. Given that *Chromobacterium* produce these antibiotics, JH7-induced inhibition of hyphal growth, conidiation, conidial germination, and chlamydospore formation of *C. destructans* may be due in part to antibiotics secreted by JH7. Our results suggest that *Chromobacterium* sp. JH7 is a promising agent for the biological control of *C. destructans* on ginseng roots. However, further studies are required to analyze the secondary metabolites, such as antibiotics, secreted by JH7 and to examine the applicability of JH7 in the field.

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