



Biocontrol of Southern Blight Caused by *Sclerotium rolfsii* in Pepper Plants Using *Bacillus subtilis* GJ6-14

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Southern blight, caused by the soil-borne fungus *Sclerotium rolfsii*, is a serious disease that affects many economically important crops. In this study, we selected *Bacillus subtilis* GJ6-14, from a total of 260 strains, to control Southern blight in pepper plants. In both seedling and plant tests, GJ6-14 significantly suppressed disease incidence and severity compared to control, furthermore, GJ6-14 demonstrated efficient colonization in the rhizosphere by maintaining the population from log 5.41 to log 3.92 in the pathogen-inoculated plants, indicating its potential as a biocontrol agent. Molecular analysis revealed up-regulation of defense-related genes, such as a 7.6-fold increase in *LOX1* and 15.5-fold increase in *PR1*, at 72 hr after inoculation of *S. rolfsii* in GJ6-14-treated plants, suggesting activation of plant defense mechanisms. Overall, our findings highlight the promising role of *B. subtilis* GJ6-14 as a potential biocontrol agent in sustainable management of Southern blight in pepper plants.

Keywords: Antagonism, Biocontrol, Southern blight

Pepper (*Capsicum annuum* L.) is a commercial vegetable cultivated worldwide (Karim et al., 2021). According to the Ministry of Agriculture, Food, and Rural Affairs, as of 2021 in Korea, the pepper-cultivated area is 33,000 ha, which is the largest cultivated area among condiment vegetables, and pepper production is approximately 60,000 tons. Pepper plants are susceptible to multiple soil-borne pathogens, including *Sclerotium rolfsii*, which is a causal agent of Southern blight; this disease leads to considerable losses in pepper production (Dwivedi and Prasad, 2016). The Southern blight of pepper caused by *S. rolfsii* was first reported in South Korea in 2004 (Kwon and Park, 2018). In 2018, an outbreak of the disease occurred in pepper plants in Wanju province, with infection rates of 25.4% in July and 46.7% in August (Kim et al., 2018b). Infected pepper plants develop water-soaked

lesions, a dense white mycelial mat of *S. rolfsii* on the crown and lower stems near or at the soil surface, and the foliage eventually wilts (Remesal et al., 2010). Sclerotia are formed at the beginning of the disease outbreak and can overwinter in the soil, making them difficult to remove (Mullen, 2001). Current methods for managing Southern blight include soil solarization and the use of compost to increase soil temperature (Bidima et al., 2022). Chemical fungicides, such as flutolanil, thifluzamide, pyraclostrobin, and fluxapyroxad, can also be used (Keinath and DuBose, 2017; You et al., 2021). However, the use of chemical agents can lead to the development of pathogen resistance to fungicides and environmental contamination due to misuse or residual presence in soil (Pirttilä et al., 2021). In addition, over-application of fungicides can inhibit mycelial growth and sclerotia formation of *S. rolfsii*, which can increase the risk of pathogen resistance to the fungicide (Dwivedi and Prasad, 2016).

Microbial biocontrol is an alternative to chemical control, and it has the potential to be more environmentally friendly

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and effective (Elnahal et al., 2022; Etesami et al., 2023). Biocontrol can be achieved through direct or indirect mechanisms. Direct mechanisms involve the use of microbes that produce antimicrobial compounds or enzymes, parasitism, or competition for nutrients or niches. Indirect mechanisms include inducing systematic resistance in plants (Kamle et al., 2020; Raymaekers et al., 2020). *Bacillus* species are the most prominent biocontrol and plant growth-promoting agents because they are capable of rapid growth and production of endospores that are tolerant to various adverse environmental stresses (Tsoetsi et al., 2022). *Bacillus* spp. can suppress plant pathogens by directly producing numerous lipopeptide compounds, such as surfactin, iturin, and fengycin, or indirectly competing with the pathogens for nutrients and niches (Dimkić et al., 2022). They also play a key role in conferring abiotic and biotic stress tolerance to plants, and improving crop production and soil health (Mahapatra et al., 2022). The objectives of this study were to select an antagonistic bacterial strain against *S. rolfsii* in pepper plants, investigate its disease-suppressive activity and related mechanisms including colonization and induction of plant defense responses, and identify the selected strain.

For preparation of plant material and pathogen, the pepper seeds (Super-manitta; NONGWOO BIO, Suwon, Korea) were sown in 50 hole-plugs and pots (diam., 10 cm) filled with a commercial potting mixture for seedling and plant assays, respectively. To isolate *S. rolfsii*, sclerotium was isolated from diseased pepper plants grown in the experimental fields of the National Institute of Agricultural Sciences, Rural Development Administration in South Korea. The isolated sclerotium was surface-sterilized and incubated in a semi-selective medium (NEON) at 28°C for 5 days. The genomic DNA of the cultured mycelia was isolated, and sequenced for the internal transcribed spacer (ITS) region (ITS4-ITS5) using the Macrogen sequencing service (Macrogen, Seoul, Korea). Comparison of the ITS sequences revealed that the sclerotium was identified as *S. rolfsii*, and it was used in this study. A total of 260 bacterial strains were isolated from the rhizosphere and bulk soil of plants in 10 different locations in South Korea during 2016–2018. The strains were cultured on tryptic soy agar (TSA; Difco, Sparks, MD, USA) as described by Jang et al. (2022). To perform the disc-diffusion assay against *S. rolfsii*, fungal mycelia on potato dextrose agar (Difco) were cut into plugs (diam., 5 mm) using a puncher and placed on the center of TSA. Sterile filter paper discs (diam., 8 mm) were

inoculated with 50 µl of the bacterial cell suspension and placed on the three corners of the plate, equidistance (3.5 cm) from the center of the plate. After incubation at 28°C for 4 days, mycelial growth inhibition was measured. The experiment was conducted twice with five replications each.

For seedling and plant assays, fourth-leaf stage and eighth-leaf stage peppers were used for the seedling and plant assays, respectively. To inoculate the pathogen, a mixture of barley (150 g) and distilled water (225 ml) was autoclaved at 121°C for 15 min. Ten mycelial plugs (diam., 5mm) of *S. rolfsii* were then inoculated into the sterile barley. After incubating *S. rolfsii* in barley at 30°C, 160 rpm for 10 days, the *S. rolfsii*-cultured barley was used as the inoculum. In the seedling assay, the inoculum was placed into two holes (2 cm away from the plants, 1 cm deep, one grain/hole). Six hr after inoculation, 10 ml of bacterial culture broth ($OD_{600}=0.2$, 10^7 colony forming units [cfus]/ml) was drenched into each seedling. Seven days after inoculation, the incidence of damping-off in the seedlings was assessed. The seedling assay was conducted twice with three replications, each consisting of 10 seedlings. In the plant assay, the inoculum of *S. rolfsii* was placed in four holes (2 cm away from the plants, 1 cm deep) of the pepper plants. Six hr later, 20 ml of bacterial culture broth ($OD_{600}=0.2$) was drenched into each pot. Fourteen days after inoculation, the blight and wilting in the plants were evaluated as disease severity (0, no symptoms to 5, dead plant) according to Latunde-Dada (1993). The 1/10-diluted tryptic soy broth (TSB) and a fungicide (fluxapyroxad) were used as the negative and positive chemical controls (Keinath and DuBose, 2017), respectively. The plant assay was performed twice with 15 replications each. To evaluate bacterial colonization in the rhizosphere after drenched treatment, rifampicin-resistant mutants were prepared following the method of Sang and Kim (2012) and used. The bacterial treatment was conducted under same conditions as the plant assay described above. Rhizosphere soil samples were collected 0, 1, 4, 7, and 10 days after drenched treatment. One g of each soil sample was suspended in 9 ml of 10 mM $MgSO_4$ solution and the 100 µl aliquot of the serially diluted suspension was then smeared on TSA (Difco) amended with rifampicin (50 g/ml). cfus were counted at 72 hr after incubation at 28°C. The colonization was performed twice with five replications each. The data were analyzed and visualized using Python (version 3.9; Python Software Foundation; <http://www.python.org>). Repeated experiments' data were pooled after confirming

homogeneity of variances using Levene's test. Following verification of normal distribution by the Shapiro-Wilk test, significant differences were performed by Tukey's test at $P < 0.05$.

As a result of the study, 24 out of 260 bacterial strains isolated from the rhizosphere and bulk soils were antagonistic to *S. rolfsii*. Seven of these strains showed over 70% inhibition

activity against *S. rolfsii*, which was used as an arbitrary threshold (Table 1). In the seedling assay, three bacterial strains, JE3-11, GJ6-14, and BC1-43, significantly reduced the disease incidence compared to the control (Fig. 1). In the plant assay, GJ6-14 significantly reduced disease severity by 2.2 ± 0.4 , however, JE3-11 and BC1-43 did not show significant disease suppression compared to the control (Fig.

Table 1. Antifungal activity of bacterial culture broth against *Sclerotium rolfsii*

Strain	Mycelial growth ^a (mm)	Strain	Mycelial growth (mm)
Control	31.64±2.77 a	DY10-04	5.73±2.59 l,m
JJ2-01	8.23±2.57 h-m	DY10-06	8.86±5.77 g-l
JE2-01	11.97±4.74 c-h	JS1-05	11.95±4.89 c-h
JE2-06	14.09±3.02 b-e	BM1-09	5.05±2.20 m
JE3-11	8.58±1.59 h-m	BM1-12	14.88±1.41 b-d
GJ6-04	9.52±1.45 f-k	BM1-13	12.54±1.93 c-g
GJ6-14	7.69±2.20 i-m	BC1-06	10.75±2.63 e-j
GJ8-03	12.82±2.72 c-f	BC1-07	10.77±1.65 e-j
BC1-01	12.53±3.23 c-g	BC1-27	17.52±1.45 b
NS4-04	11.23±1.67 d-i	BC1-43	6.51±1.89 k-m
SC3-09	15.15±5.11 b,c	BC1-71	7.43±2.67 j-m
SC3-12	13.03±3.84 c-f	BC1-72	11.14±1.87 d-j
SC3-03	13.45±2.03 c-e	BC1-73	14.71±2.69 b-d

Values are presented as mean±standard error ($n=10$). Different letters on the columns indicate significant differences ($P < 0.05$) based on Tukey's test.

^aMycelial growth was measured at 4 days after treatment.

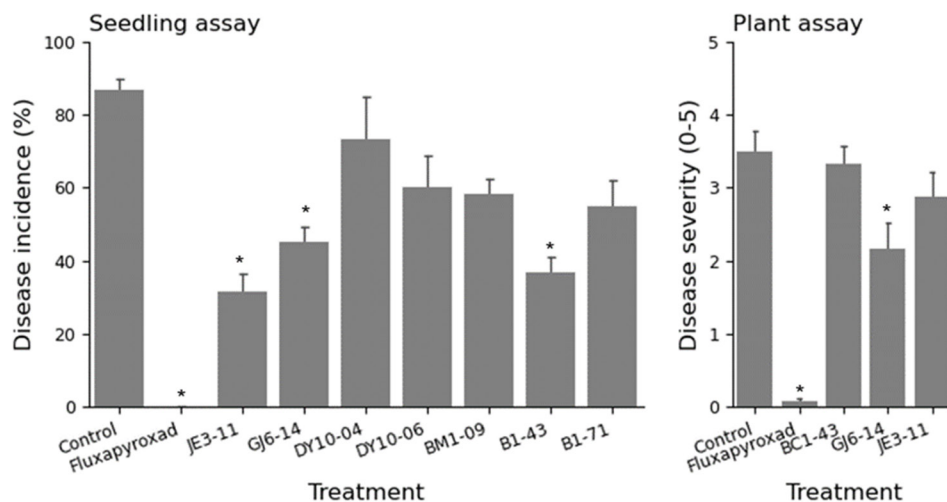


Fig. 1. Disease incidence on seedling assay (left) and disease severity on plant assay (right). Pepper seedlings and plants were treated with bacterial culture broth, control (1/10-diluted tryptic soy broth), and fungicide (fluxapyroxad, a positive chemical control). An asterisk on the bar indicated significant differences based on Tukey's HSD test ($P < 0.05$); error bars indicated standard error ($n=6$ for seedling assay, $n=30$ for plant assay).

1). As a result of disease suppression, GJ6-14 was selected as a biocontrol strain for the subsequent experiments. The colonization of GJ6-14 ranged from log 5.41 to log 3.92 (approximately 2.6×10^5 to 8.3×10^3 cfu/g) in the rhizosphere of pepper plants inoculated by *S. rolf sii* (Fig. 2).

The GJ6-14 exhibited remarkable inhibition of *S. rolf sii*'s mycelial growth through the production of antagonistic metabolites. In addition to displaying antifungal activity in the agar diffusion assay, GJ6-14 exhibited disease suppression in pepper plants. Among the seven tested antifungal bacteria, GJ6-14 significantly reduced disease development in both seedling and plant assays. While some antifungal-producing bacteria exhibit effective antifungal activity, it is important to note that not all of them necessarily lead to significant disease suppression in plants (Jang et al., 2022; Kim et al., 2008). The interactive factors of host-antagonist-pathogen-environment can have a significant impact on the ability of

bacteria to suppress disease. Consequently, after seedling and plant assays to evaluate the biocontrol activity in pepper plants, GJ6-14 was selected as a biocontrol agent for subsequent studies.

Bacterial colonization is recognized as essential for effective disease management (Etesami et al., 2023). Successful biocontrol involves robust colonization of the root surface and rhizosphere, often through the formation of biofilm. This strategy establishes a competitive niche that competes for nutrients released by the roots, in turn, limits the availability of nutrients for pathogen's utilization and protect the infection site. Hence, bacterial colonization is a pivotal mechanism behind the disease suppressive effects of various biocontrol agents, including *Bacillus* species (Etesami et al., 2023). In our result, the population of GJ6-14 maintained from log 5.41 to log 3.92 (approximately 2.6×10^5 to 8.3×10^3 cfu/g) in pathogen-inoculated plants during the disease development, with a slight decline over time. Although the population of GJ6-14 showed a significant decrease at 10 days after treatment and inoculation, it still fell within the colonization range of successful biocontrol agents. For instance, Maurer et al. (2013) reported the colonization of four bacterial biocontrol agents against *Verticillium* wilt with from log 2.9 to log 4.7 (approximately 7.9×10^2 to 5.0×10^4 cfu/g) in the rhizosphere, leading to plant growth promotion in hops. Similarly, Fan et al. (2017) demonstrated that the *B. subtilis* 9407, a biocontrol agent against bacterial fruit blotch, colonized log 4.87 (approximately 7.4×10^4 cfu/g). Their findings highlighted that the biocontrol activity of the bacterial agents was a result of a coordinated interplay between surfactin-mediated antibacterial activity and colonization. Therefore, the dual attributes of colonization capability and antifungal compound production of GJ6-14 could be important contributors to its biocontrol efficacy against *S. rolf sii* in pepper plants.

To assay defense-related gene expression, eighth leaves of pepper from the bottom were collected at 0, 24, 48, and 72 hr after inoculation with *S. rolf sii*, as described in the plant assay above. The leaves were frozen and homogenized described above. Total RNA extraction and complementary DNA synthesis were performed as described Jang et al. (2022). The quantitative real-time polymerase chain reaction using primers (Supplementary Table 1) was programmed as follows: 95°C for 10 min (initial denaturation): 40 cycles of 95°C for 15 sec (denaturation), 58°C for 30 sec (annealing), and 72°C for 30 sec (extension). The experiment was conducted

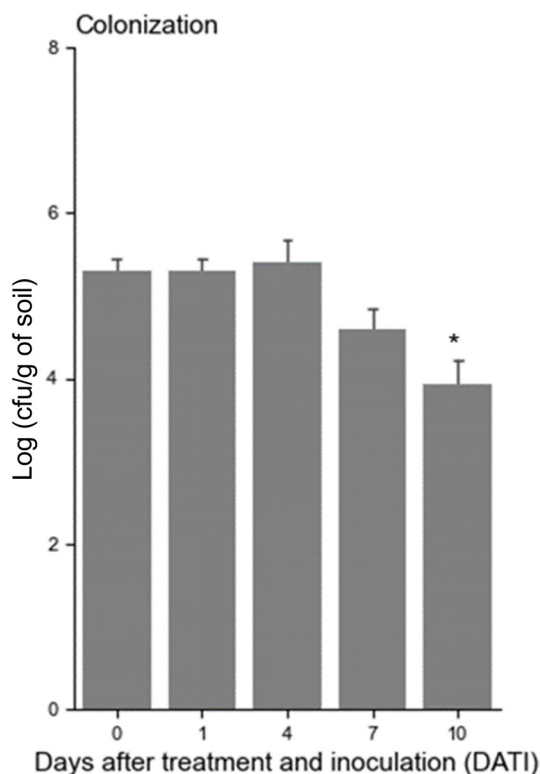


Fig. 2. Population of antagonistic bacterial strain GJ6-14 in rhizosphere soil of pepper plants un-inoculated or inoculated by *Sclerotium rolf sii*. A spontaneous rifampicin-resistant mutant of GJ6-14 was used; rhizosphere soil samples were collected at 0, 1, 4, 7, and 10 days after bacterial treatment and inoculation (DATI). Bacterial colony forming units (cfus) were determined on tryptic soy agar supplemented with rifampicin. Mean \pm standard error ($n=10$). An asterisk indicates statistical difference ($P < 0.05$) compared to control based on Tukey's test.

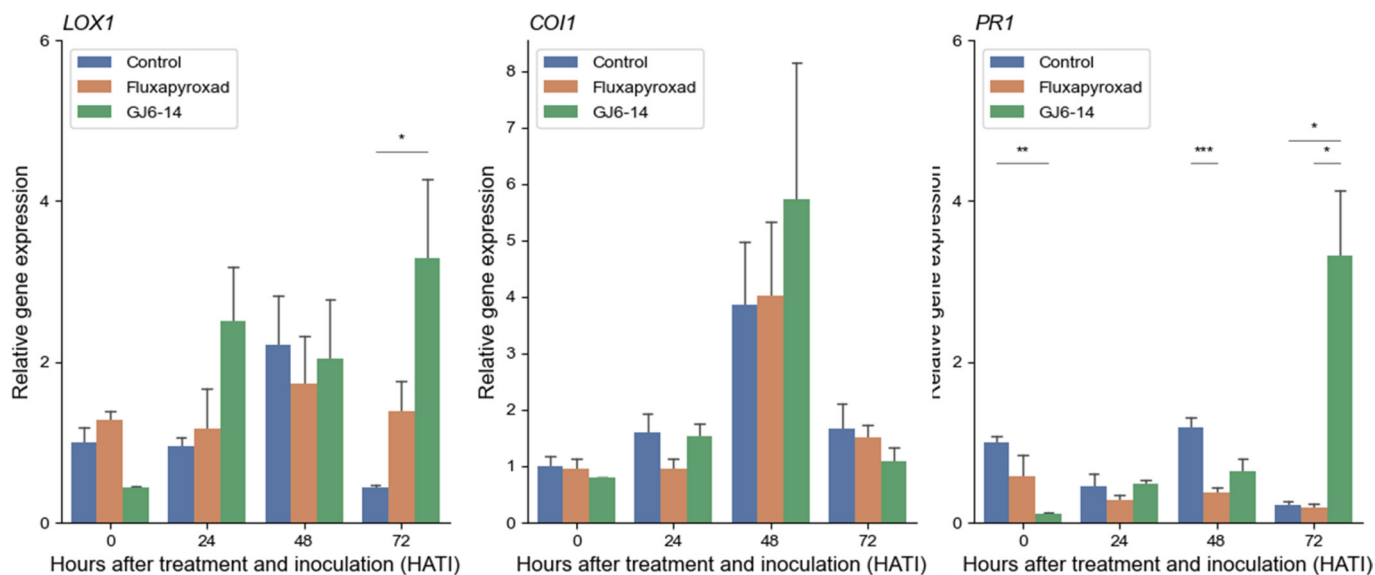


Fig. 3. Relative expression of *LOX1*, *COI1*, and *PR1* in pepper plants treated with GJ6-14, control (10^{-1} -diluted tryptic soy broth), and fungicide (fluxapyroxad, a positive chemical control) under *Sclerotium rolfsii*-inoculation. Asterisks indicated significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) based on Tukey's HSD in each time.

twice with three replications each. Data analysis and visualization were conducted described above, the significant difference between each treatment were analyzed using the *t*-test.

The relative gene expressions of *LOX1* and *PR1* at 72 hr after inoculation were significantly ($P < 0.05$) increased by 7.6-fold, 15.5-fold compared to that of the control, respectively. However, the relative expression of *COI1* was not affected by GJ6-14 treatment compared to that of the control (Fig. 3). Fluxapyroxad treatment, as a positive chemical control, did not affect the relative expression of *COI1*, *LOX1* for 72 hr after treatment and inoculation (HATI), and *PR1* except at 48 HATI. Plant defense are intricately regulated by the interplay of defense hormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene (Fernandes and Ghag, 2022; Yang et al., 2019). Our results indicated that GJ6-14-treated plants exhibit 7.6-fold up-regulation of *LOX1*, which is involved in JA biosynthesis, and 15.5-fold of *PR1* expression, which is involved in SA pathway, 72 hr after *S. rolfsii* inoculation. These results might indicate that the GJ6-14 could impact on JA and SA pathway, however, further investigation is required to discover the precise mechanism underlying the function of GJ6-14.

Strain GJ6-14 was identified by amplifying and sequencing the 16S rRNA gene using universal internal primers 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') provided by Macrogen (Macro-

gen, Inc., Suwon, Korea). The *gyrB* gene was amplified and sequenced using primers UP1 (5'-GAAGTCATCATGACCGTTCTGCA YGCNGGNGGNAARTTYGA-3') and UP2r (5'-AGCAGGGTACGGATGTGCGAGCCRTCNCACRTCNCRCNTCAT-3') described by Yamamoto and Harayama (1995). The 16S rRNA gene sequences were compared with those of type strains using the EzBioCloud database (<https://www.ezbiocloud.net/>). The *gyrB*, encoding for B subunit of the gyrase, sequences were compared using National center for biotechnology information. Phylogenetic trees were constructed using the maximum likelihood method of the Molecular Evolutionary Genetics Analysis (MEGA 6.0 program; <http://megasoftware.net>). The strain (KACC 92431P) has been deposited in the Korean Agricultural Culture Collection (KACC, Wanju, Korea). Bacterial characterizations were assayed according to methods of Lee et al. (2014) and Kim et al. (2018a). The 16S rRNA gene sequence (1,102 bp) of strain GJ6-14 showed 99.82% similarity to *B. tequilensis* (AYTO01000043), *B. cabrialesii* (MK462260), and *B. inaquosorum* (AMXN01000021), and 99.73% similarity to *B. subtilis* (ABQL01000001) (Fig. 4). The *gyrB* gene sequence (1,121 bp) exhibited 98.93% similarity to *B. subtilis* (CP034484.1) and 93.84% similarity to *B. inaquosorum* (CP029465.1), therefore, GJ6-14 was identified as *B. subtilis*, and it has activities such as siderophore production, phosphate solubilization, hydrolysis of cellulose, starch and casein (Table 2). The genera *Bacillus* is

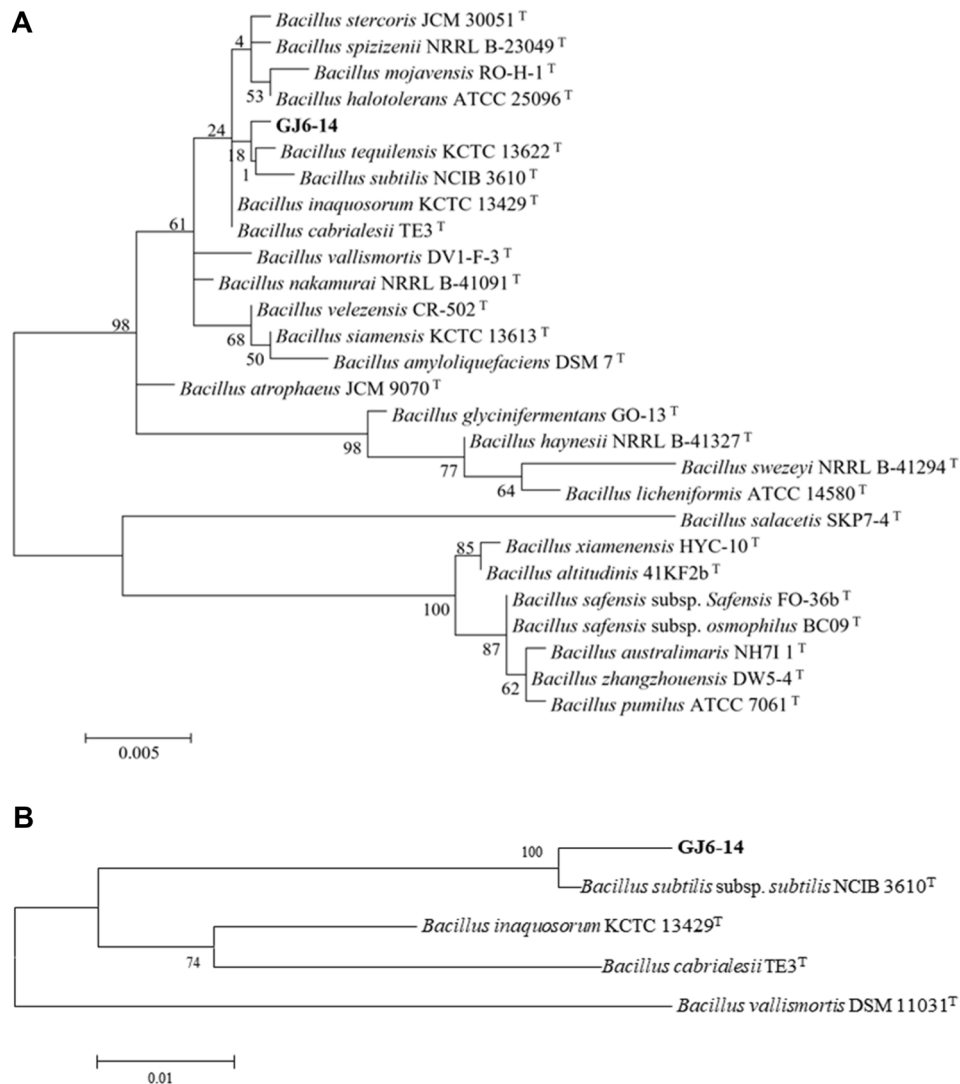


Fig. 4. The phylogenetic trees of GJ6-14 were constructed by the maximum likelihood analysis based on 16S rRNA (A) and *gyrB* (B) sequences in MEGA 6. The scale bar indicates the five-nucleotide substitution per 1,000 nucleotides of the 16S rRNA and one-nucleotide substitution per 100 nucleotides of *gyrB* gene.

Table 2. Characteristics of strains GJ6-14

Character	Reaction
Siderophore production	+
Phosphate solubilization	+
ACC deaminase production	-
HCN production	-
Cellulose hydrolysis	+
Chitin hydrolysis	-
Starch hydrolysis	++
Casein hydrolysis	+

+, week positive reaction (<20 mm); -, not detected; ++, positive reaction (>20 mm).

renowned for generating antagonistic compounds, encompassing volatiles, lipopeptides, and siderophore (Dimkić et al., 2022; Grahovac et al., 2023). Various *Bacillus* species, including *B. velezensis* LBSB1, *B. subtilis* BMB26, *B. pumilus* LX11, have been documented to possess antifungal effects against *S. rolfisii* (Chen et al., 2020; Xu et al., 2020). Similarly, *B. subtilis* GJ6-14 had exhibited comparable characteristics in terms of antifungal activity, siderophore production, and hydrolytic enzyme secretion.

Overall, antagonistic *B. subtilis* GJ6-14 was a potential biocontrol agent against *S. rolfisii*, effectively colonizing and triggering plant defense response related to SA and JA signal pathways. The application of strain GJ6-14 led to reduced severity and incidence of Southern blight, while also elevating

the expression of defense-related genes in pepper plants. Consequently, this investigation into *B. subtilis* GJ6-14 holds substantial promise for advancing the development of sustainable strategies to effectively manage Southern blight in pepper plants.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at Research in Plant Disease website (<http://www.online-rpd.org/>).

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