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ARTICLE

Isolation of *Burkholderia cepacia* JBK9 with plant growth-promoting activity while producing pyrrolnitrin antagonistic to plant fungal diseases

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Abstract *Burkholderia* species are widely distributed across wide ecological niches. Many genera of *Burkholderia* are known to be associated with plants and are involved in processes such as suppression of soil-borne pathogens, acceleration of plant growth and endophytic colonization. In the present study, a strain belonging to the *Burkholderia cepacia* complex, which was termed JBK9, was isolated. The strain JBK9 showed broad-spectrum antifungal activities against *Phytophthora capsici, Fusarium oxysporum*, and *Rhizoctonia solani*, which are representative phytopathogenic fungi, inhibiting their growth by 59.56, 51.92, and 34.22%, respectively. The strain produced an antifungal compound that was confirmed to be pyrrolnitrin by TLC, HPLC, and NMR analyses. Using an in vitro assay for plant root colonization, we observed that the population densities of *B. cepacia* JBK9 on the upper 1 cm of host plant roots were significantly different between *Burkholderia* species. The high motility of these strains is likely to have contributed to their efficient root colonization. The isolated strain was evaluated in vivo for its ability to control *Phytophthora* blight via a pot test. Compared with *Burkholderia* strains KCTC 2973 and ATCC 25416, *B. cepacia* JBK9 demonstrated a stronger antifungal activity against *P. capsici*. The strain *B. cepacia* JBK9 could be further developed as a biological control agent for pepper plants.

Keywords Antifungal activity · Biological control · *Burkholderia cepacia* · Pyrrolnitrin

Introduction

Various phytopathogenic fungi such as *Phytophthora capsici* and *Fusarium oxysporum* are existed in soil [1]. Among them, *P. capsici* causes rotting in the roots and fruits of commercially important crops [2]. In general, farmers have been in trouble to control plant diseases caused by *Phytophthora* species because of their resistance to metalaxyl, a systemic fungicidal compound that is effective against most oomycetes. Generally, wide range of chemical fungicides are needed to manage *P. capsici* which has a broad host range [3]. But, such chemicals can also eliminate soil insects and microorganisms that are beneficial for the rhizosphere. In addition, the excessive use of chemicals to control *P. capsici* has affected human health concerns, as well as the development of tolerance to target microbial populations [4].

Biological control is an alternative method to replace chemical fungicides. Many bacterial agents have been discovered via in vitro or in vivo screening of large numbers of microorganisms that inhibit plant pathogens [5]. Potential bacterial antagonists that have been discovered using this strategy include *B. cepacia* [6], *Pseudomonas* spp. [7], and *Bacillus* spp. [8]. Nevertheless, potential biological control agents discovered using this method

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must be subjected to further screening by repeating analyses, which is laborious and requires facilities with controlled environmental conditions. Therefore, an effective screening strategy should begin with a fast and simple in vitro or in vivo analysis of antagonistic action [5].

The genus *Burkholderia* is an organism with remarkable production of various secondary metabolites [9], including many compounds with antibacterial, antifungal, insecticidal, or herbicidal properties [10]. *Burkholderia cepacia* complex (BCC) is known to produce pyrrolnitrin and cepacin, natural substances with antimicrobial activity.

Pyrrolnitrin is a potent antimicrobial material produced by *Burkholderia*, *Pseudomonas*, *Myxococcus*, *Serratia*, and *Enterobacter* genera. BCCs protect themselves by this substance against phytopathogenic fungi such as *Rhizoctonia solani*, *P. capsici*, and *F. oxysporum* [7, 11]. Several *Burkholderia* species have been suggested as effective biocontrol agents, and some of their metabolites are developed to commercial products. In this study, we focused on isolating a bacterial strain for the effective biocontrol of plant pathogens and investigated its practical application in pepper plants via a pot experiment.

Materials and methods

Screening of antagonistic bacteria from soil

A total of 25 soil samples were collected from 25 different locations in Gyeongsangbuk-do area, Republic of Korea for bacterial screening. The upper soil layer samples (approximately 100 g) were placed into clean dry and sterile polythene bags. The sample was suspended in 0.85% of NaCl and spread on Luria–Bertani (LB) agar. After incubation for 2 days at 30 °C, bacterial colonies with different morphological characteristics were selected and re-streaked on fresh LB agar until the homogeneous colonies were appeared. Isolated strains were subcultured in LB broth on a rotary shaker. For long-term storage of the strain, 50% (v/v) glycerol solution was added to culture and freezed at − 80 °C until use.

Fungal strains used in the present study include, *Rhizoctonia solani* KACC 40101, *Fusarium oxysporum* KACC 40032, *Phytophthora capsici* KACC 40476, and *Corynespora cassiicola* KACC 40964 were obtained from the Korean Agricultural Culture Collection. The antifungal activity of each isolated strain was determined by the grow inhibition of pathogenic fungi. Test was performed with three replicates. The growth rate of pathogenic fungi was assessed using the modified method by Reyes-Chilpa et al. [12]. Antagonistic activity was defined according to the size of the inhibitory zone after 1 week as follows: +++++ (very strong), > 20 mm; ++++ (strong), > 15–20 mm; +++ (moderate), > 10–15 mm; + (very weak), 0–5 mm; ++ (weak), > 5–10 mm; − no inhibition zone.

Identification of isolated strain and phylogenetic analysis

Each isolate was identified by 16S rRNA gene sequencing and comparison according to the methods of Mejri et al. [13]. PCR amplification was performed with *KOD* DNA polymerase using the universal primer set 27F (5′-GAGAGTTTGATCCTGGCTCAG) and 1492R (5′-CTACGGCTACCTTGTTACGA). The 16S rRNA gene fragments were purified using PCR purification kit (Enzynomics, Daejeon, Korea). DNA sequencing was conducted SolGent (Daejeon, Korea). The sequences were analyzed using the EzTaxon-e database for species identification [14]. The phylogenetic analysis was done using the neighbor-joining algorithm and the *p*-distance model in MEGA version 6.0 [15].

Scanning electron microscopy

To obtain SEM image, a 1-cm^2 transverse section of bacterial strain on LB agar plate was obtained and fixed in 2.5% glutaraldehyde buffer for 3 h at 4 °C. The cell was washed and then fixed for 1 h at 25 °C in 1% osmium tetroxide. After washing, the cell was dehydrated with 50–100% of graded series of ethanol, dried critical point, and platinum-coated using an ion-sputter coater. The specimen was observed at an operating voltage of 2 kV using a field-emission scanning electron microscopy/energy-dispersive X-ray spectrometry microscope (model S-4100, Hitachi, Tokyo, Japan).

Motility (swarming and swimming) evaluation

To measure motility of the cell, 10-μL overnight culture broth of bacteria was spotted onto swarming agar plate (0.4% glucose in LB broth and 0.45% agar) and/or swimming agar plate (1% tryptone, 0.5% NaCl, and 0.3% agar) and incubated for 24 h [16]. Swarming and swimming agar plates were dried for overnight at 25 °C and preheated to 30 °C before use.

Rhizosphere colonization using a double-layered filter paper method

This experiment followed the protocol of Bae et al. [17]. Surface of red pepper seeds were disinfected by treatment of 1% sodium hypochlorite for 5 min. After treatment, the seeds were carefully washed with sterile water, and dried. One gram of red pepper seeds were treated with 10^9

Colony Forming Unit (CFU) of bacterial solution (10^9 CFU / mL) and air-dried for 1 h. Then, two sheets of Whatman No. 1 filter paper (Whatman, UK) were placed on the 11-cm Petri dish and soaked in 5 mL of sterile water. After the filter paper had absorbed the water uniformly, 10 seeds coated with bacteria (*B. cepacia* JBK9, *B. pyrrocinia* KCTC 2973, and *B. cepacia* ATCC 25416) were placed vertically, seed side up, between the filter paper sheets on the far side of a Petri dish. This allowed the root to grow downwards across the Petri dish. The lid was covered and sealed with paraffin film and the Petri dish was incubated at 30 °C without light for 7 days. After incubation, the roots were taken from each Petri dish and cut into 1-cm segments. The quantity of each bacterial strain (CFU / mL) on the rhizoplane of each root segment was determined by plating the root segments on LB medium.

Biocontrol of *P. capsici* by *B. cepacia* JBK9

For the pot trial, V8 juice agar medium (10% V8 juice, 0.1% $CaCO_3$, and 2% agar) was used for to prepare zoospores of *P. capsici*. The fungus inoculated agar plate was incubated for 5 days at 30 °C under the fluorescent light to produce sporangia. Chilling at 4 °C for 0.5 h was done to release zoospores. Sterilized water suspension of the zoospore was carefully filtered by cheesecloth. The final concentration of the zoospore was 10^5 zoospores mL^{-1} [18]. Pepper seeds (*Capsicum annuum* L., Chungok) were planted on the soft seeding soil. Sprouted pepper were implanted to artificial sterile soil (soil/sand/vermiculite, 2:1:1, v:v:v) and then cultivated at 25 °C with 16-h period 10,000 l× of light. After 6 weeks of transplanting, 5 mL of a zoospore suspension of *P. capsici* (10^5 zoospores / mL) was poured into the pot for the infected samples. For the bacterial treated samples, 50 mL of each culture broth (10^9 CFU / mL) was applied during the day. The same volume of sterile medium was used as a control. Pepper plants and rhizospheric soils were collected after 20 days of treatment.

Extraction of antifungal compound from *B. cepacia* JBK9

In order to extract the antifungal compound, the culture broth of strain JBK9 was centrifuged at 10,000×*g* for 20 min. The supernatant was carefully corrected and filtered to remove any bacterial cells using a 0.2-μm cellulose acetate filter. The filtered culture was mixed 1:1 (v/v) with *n*-hexane. The organic phase was evaporated under vacuum at 40 °C using a rotary evaporator (Sunil Eyela, Seongnam, Korea). The dry residues were resuspended in 1 mL of 100% methanol. Extracts were stored in microtubes at − 20 °C until use.

Thin-layer chromatography (TLC) assay

TLC was performed to fractionate compounds from secondary metabolic extracts of *B. cepacia* JBK9. A TLC silica gel 60 F254 plate (Millipore, Billerica, MA) with dimensions of 20 × 20 cm was used. Secondary metabolite extracts were spotted on a 2 cm TLC plate from the base at least 2 cm away across the horizontal baseline. The plates were developed vertically in a glass chromatography tank containing 100 mL of solvent (3:1 [v/v] *n*-hexane/ethyl acetate) until the solvent tip reached 1 cm from the top edge of the TLC plate. Samples were visualized using ultraviolet radiation at two wavelengths (256 and 354 nm). Pure pyrrolnitrin (3-chloro-4-[3-chloro-2-nitrophenyl]-pyrrole; Sigma-Aldrich, St. Louis, MO) was used as a compound of positive reference for the newly isolated active compounds.

Reverse-phase HPLC and NMR analyses

The bioactive fraction was further purified by HPLC. A C_{18} column (Phenomenex, Torrance, CA) was used with following HPLC instrumental conditions: (1) CBM-10 system controller coupled with an ultraviolet-visible detector set at 225 nm and an LC-10AD pump; (2) Solvent A, 45% H_2O, 30% acetonitrile, 25% methanol; (3) 1 min/mL flow rate for 30 min. The pyrrolnitrin (Sigma-Aldrich) was used as a standard. The NMR spectrum of pyrrolnitrin was obtained using a Bruker's NMR machine (Advance Digital, Bruker, Billerica, MA) operating at 500 MHz (^1H, ^{13}C).

Results

Isolation of strain JBK9

We collected 25 soil samples (100 g, each) from the rhizosphere of cultivated crops in Gyeongsangbuk-do, Republic of Korea. Bacteria were obtained both from rhizospheric portion of various plants. A total of 115 microorganism strains were isolated from rhizospheres of several agricultural crop fields. The antagonistic activities of those microorganisms against fungal pathogens were assessed by the dual-culture method. A total of 150 isolates that appeared as separate colonies were isolated from soil rhizosphere where sampled from Gyeongsanbuk-do area. All isolated bacteria were tested for antifungal activity as follows: a 1 cm^2 fungal plug was inoculated in the center of a plate with PDA; each isolated bacterium was sown with a sterile stick at a distance of 2.5 cm from the fungi. The isolate named JBK9 showed antagonistic activity against *R. solani, P. capsici,* and *F. oxysporum.* When strain JBK9 was inoculated with each of the three fungal

pathogens and cultivated for 7 days, it inhibited the growth of *P. capsici, F. oxysporum*, and *R. solani* by 59.56, 51.92, and 34.22%, respectively (Table 1).

Morphology and identification of the strain JBK9

Strain JBK9 was a gram-negative and rod-shaped bacterium (Fig. 1A). The 16S rRNA gene region (1421 bp) was amplified by the universal primers 27F and 1492R. After sequencing, the sequence was aligned against other sequences used by the EzTaxon program. The strain showed the highest similarity to *B. cepacia* ATCC 25416^T (Fig. 1B). The results of the homology analysis confirmed that *B. cepacia* strain JBK9 exhibited 98.9% maximal similarity with *B. cepacia* strain ATCC 25416^T and 98.4% similarity with *B. pyrrocinia* strain LMG 14191^T; this finding confirmed the isolated strain as a *Burkholderia* species by comparison against the EzTaxon database.

Antifungal activity of the *n*-hexane-extracted fraction of *B. cepacia* strain JBK9

Antifungal activity assay was performed to identify the fraction containing the antifungal compound. The fraction that was soluble in *n*-hexane fraction demonstrated antifungal activity against several tested fungal pathogens. The hexane fraction showed significant activity against *R. solani, P. capsici,* and *F. oxysporum* with corresponding growth-inhibitory effects of 58.11 ± 1.96%, 31.27 ± 1.52%, and 59.43 ± 1.64% against *R. solani, P. capsici*, and *F. oxysporum*, respectively (Table 2). Thus, the above results confirmed the antagonistic activity of *B. cepacia* JBK9 against these fungal pathogens.

Identification of the active antifungal compound

The antifungal compound isolated from *B. cepacia* strain JBK9 exhibited a characteristic spot on the TLC plate using pyrrolnitrin as a standard (data not shown). Following HPLC analysis of the active fractions yielded a peak with a retention time of 10.5 min (Fig. 2B), which was in the same as the pyrrolnitrin standard (Fig. 2A). Its antifungal activity against the fungal pathogens was confirmed in a plate assay. This compound had a strong inhibitory effect against *P. capsici*, *F. oxysporum*, and *R. solani*. The ^{1}H-NMR spectrum showed three aromatic protons, two pyrrole protons and an additional broad NH signal (Fig. 2D). And the ^{13}C-NMR spectrum revealed the presence of pyrrole CH, aromatic CH, and carbon signals (Fig. 2C). Thus, all the spectroscopic data showed that isolated compound from the strain was pyrrolnitrin.

Motility and root colonization of *B. cepacia* JBK9

Several behaviors of *Burkholderia* species, including swarming and swimming, were examined on solid media containing different concentrations of agar. *B. cepacia* JBK9 displayed two types of motility, namely swimming (spreading through the semi-soft medium) and swarming (spreading across the surface). *B. cepacia* JBK9 demonstrated higher swarming and swimming abilities than other *Burkholderia* strains (Table 3). In contrast, *B. cepacia* ATCC 25416 and *B. pyrrocinia* KCTC 2973 were apparently unable to swarm and seemed to lack flagella. We assessed the root colonization capacity of strain JBK9 in red pepper plants after seed treatment in vitro using the double-layer filter method. The results show that the red pepper samples treated with *B. cepacia* JBK9 had a significant root colonization effect compared to the samples treated with other *Burkholderia* species. As shown in Fig. 3A, the JBK9 treatments had an increase of 26 and 94% root colonization compared with *B. pyrrocinia* KCTC 2973 and *B. cepacia* ATCC 25415 treatments, respectively.

Biocontrol ability of *P. capsici* by *B. cepacia* JBK9

In all cases, initial bacterial treatment was applied when the red pepper seedlings had been grown for 50 days. The fungal pathogen, *P. capsici*, was applied 3 days later. The bacterial cell suspensions used to treat the plant roots were

Table 1 Antifungal activities of strain JBK9 against various pathogens

Fungi	Inhibition (%)	Antifungal activity
Phytophthora capsici KACC 40476	59.56 ± 1.52^a	++++
Fusarium oxysporum KACC 40032	51.92 ± 1.64^b	++++
Rhizoctonia solani KACC 40132	34.22 ± 1.96^c	++

Antifungal activity (inhibition zone): − no inhibition zone; + (very weak), 0–5 mm; ++ (weak), > 5–10 mm; +++ (moderate), > 10–15 mm; ++++ (strong), > 15–20 mm; +++++ (very strong), > 20 mm as the distance between the fungal pathogen and the area of antagonist growth after 7 days. The mean values were calculated from 5 replicates

Values with different alphabetic superscripts in the same column are significantly different ($p \leq 0.05$) according to Duncan's multiple-range test

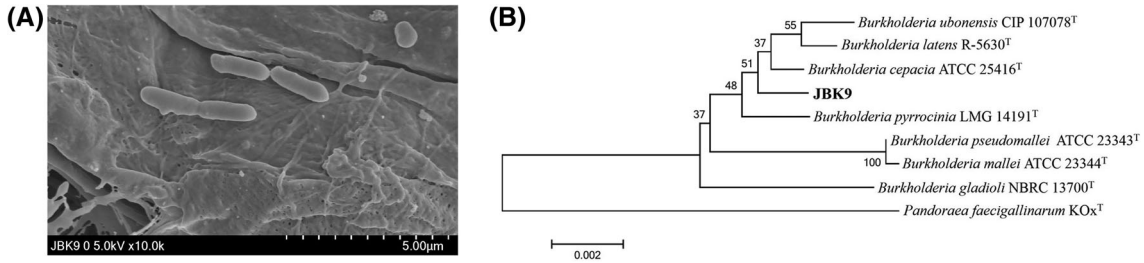

Fig. 1 Features of *B. cepacia* strain JBK9. (**A**) Morphology of *B. cepacia* strain JBK9 using scanning electron microscopy. The magnification was ×10,000. (**B**) A phylogenetic tree showing the genetic relationship between *Burkholderia cepacia* strain JBK9 and reference strains based on 16S rRNA sequence analysis. The branching pattern was generated by the neighbor-joining method. The number on each branch indicates the bootstrap values based on 1000 replicates

Table 2 Antifungal activities of *n*-hexane-extracted fraction of *B. cepacia* JBK9 against various pathogens

Fungi	Inhibition (%)	Antifungal activity
Phytophthora capsici KACC 40476	31.27 ± 1.52^{b}	++
Fusarium oxysporum KACC 40032	59.43 ± 1.64^{a}	++++
Rhizoctonia solani KACC 40132	58.11 ± 1.96^{a}	++++

The result of antifungal activity was expressed as the distance between the fungal pathogen and the area of antagonist growth after 7 days. − no inhibition zone; + (very weak), 0–5 mm; ++ (weak), > 5–10 mm; +++ (moderate), > 10–15 mm; ++++ (strong), > 15–20 mm; +++++ (very strong), > 20 mm The mean values were calculated from 5 replicates

Values with different alphabetic superscripts in the same column are significantly different ($p \leq 0.05$) according to Duncan's multiple-range test

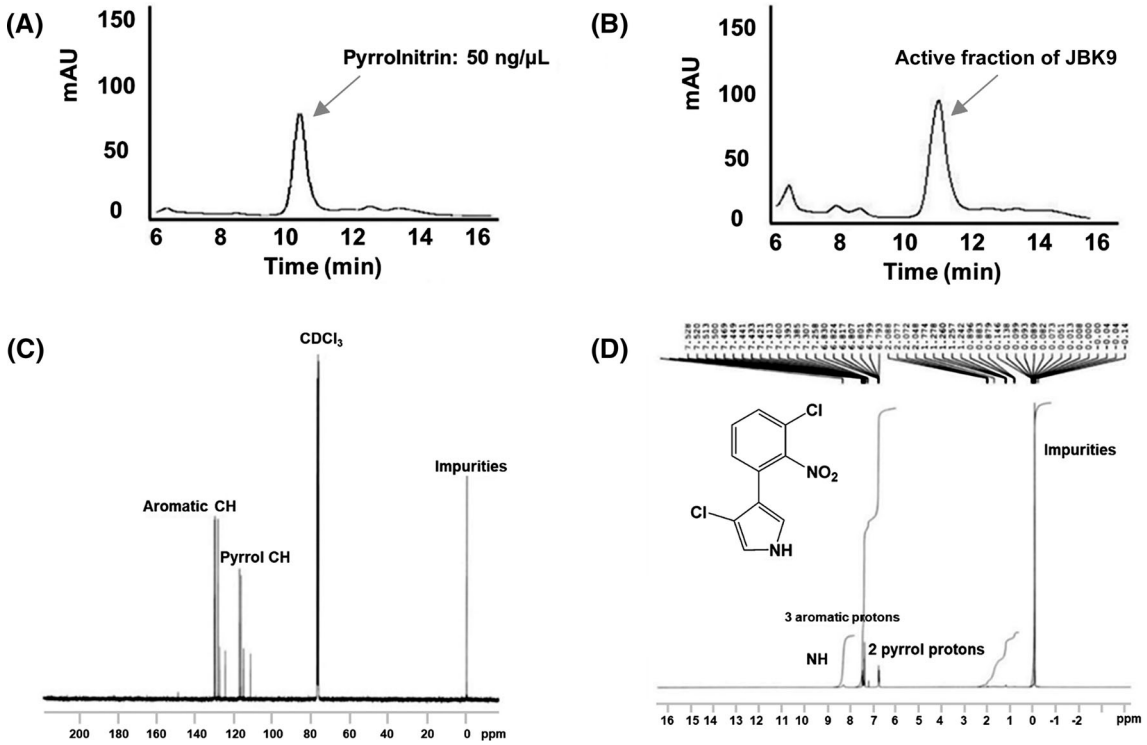

Fig. 2 Identification of pyrrolnitrin from *B. cepacia* strain JBK9. High-performance liquid chromatography analysis of (**A**) pyrrolnitrin standard and (**B**) isolated active fraction from *B. cepacia* strain JBK9. Active fraction's (**C**) ^{13}C-NMR spectrum and (**D**) ^{1}H-NMR spectrum

Table 3 Motility test of *Burkholderia* species

Strains	Swarming test	Swimming test
B. cepacia JBK9	+++++	+++++
B. pyrrocinia KCTC 2973	–	–
B. cepacia ATCC 25416	–	–

The results of swarming and swimming test were expressed as the distance cell moved out from the center of plate. – not detected; +, > 1.5 cm, < 2 cm; ++, > 2 cm, < 2.5 cm; +++, > 2.5 cm, < 3 cm; ++++, > 3 cm, < 3.5 cm; +++++, > 4 cm

diluted to a final concentration of 10^7 CFU/mL before inoculation. The spore concentration of *P. capsici* was determined by hemocytometer counting and adjusted to 10^5 conidia/mL of water. The plant growth was examined after treatment with water only, LB broth only, or *P. capsici* plus the bacterial strains *B. cepacia* JBK9, *B. pyrrocinia* KCTC 2973, and *B. cepacia* ATCC 25416 or *P. capsici* only (Fig. 3C). The disease control efficacies measured for the above treatment conditions were 100, 100, 93.2, 92.1, 30.7, and 9.5%, respectively (Fig. 3B). Therefore, compared with two other *Burkholderia* species

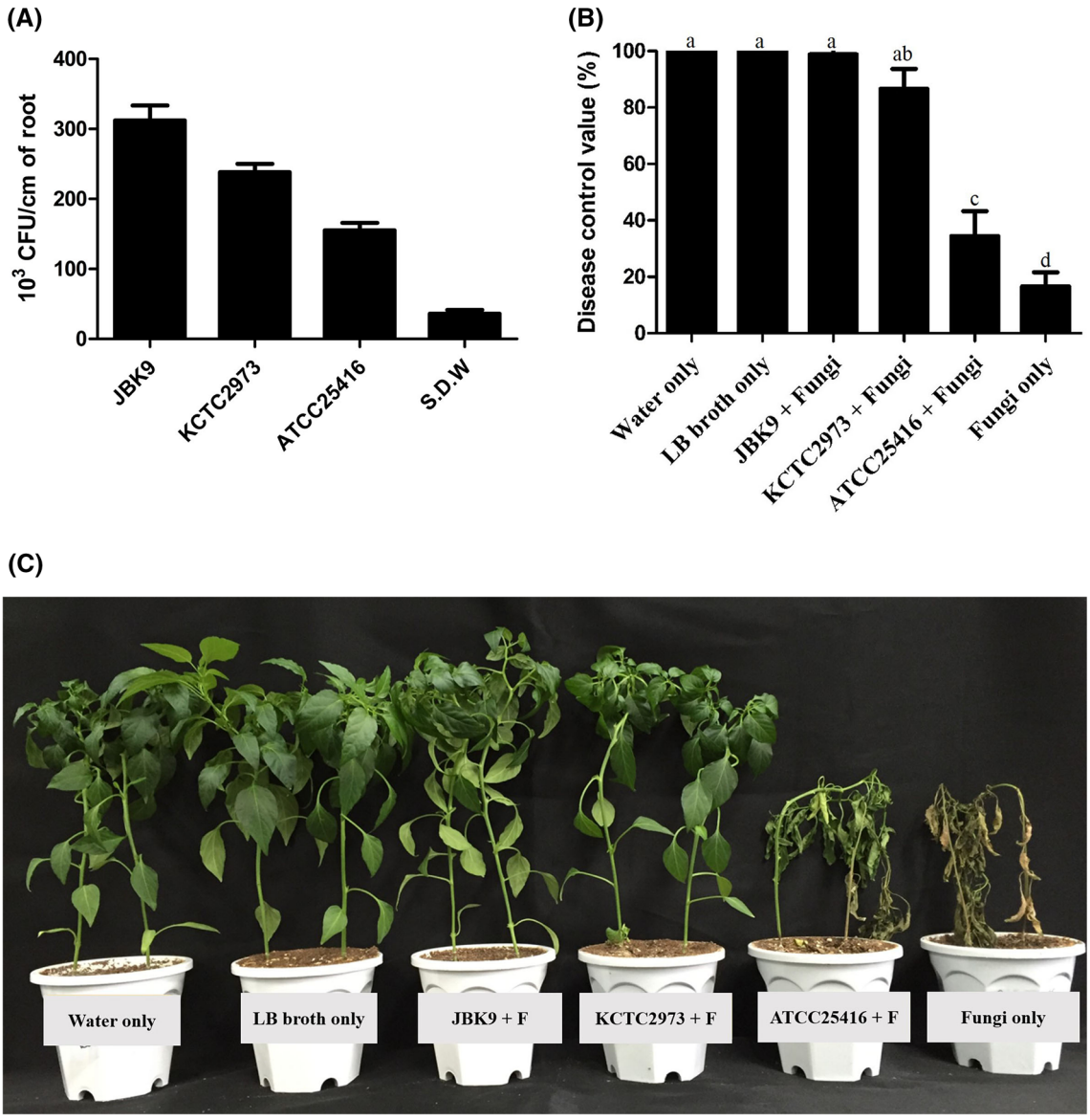

Fig. 3 The plant growth-promoting activity of the *B. cepacia* strain JBK9. (**A**) The density of root colonization by *Burkholderia* species. S.D.W, sterile distilled water. (**B**) Disease control efficiencies of *Burkholderia* species. The data represent means from four independent experiments with ten plants per treatment (± SE, n = 40). Different letters in rows show significantly different one-way ANOVA and Duncan's post hoc tests ($p < 0.05$). (**C**) Biocontrol by *Burkholderia* species against *Phytophthora* blight of red pepper plants

(*B. pyrrocinia* KCTC 2973 and *B. cepacia* ATCC 25416), *B. cepacia* JBK9 showed a stronger antifungal activity against *P. capsici*. Prior to the in vivo pot experiment, we confirmed the antifungal activities of these *Burkholderia* species using an in vitro dual-culture method. This demonstrated that the antifungal activity of *B. cepacia* JBK9 was higher than that of other *Burkholderia* species.

Discussion

In this study, we collected 25 soil samples and isolated a bacterial strain that exhibited antifungal activity using the dual-culture method. The isolated strain showed antifungal activity against *P. capsici, F. oxysporum*, and *R. solani*. The isolated strain was named JBK9 and was identified as *B. cepacia* based on comparison of 16S rRNA gene sequence. In recent years, the genus *Burkholderia* has been phylogenetically well characterized and has been revealed to comprise species that are remarkably functionally diverse [19]. *Burkholderia* strains are isolated from the rhizosphere of healthy plants and are generally involved in promotion of plant growth and inhibition of plant disease [20]. *B. cepacia* JBK9 has extensive antifungal activities against plant pathogens. The key mechanism for the antagonistic effects of *B. cepacia* against plant pathogens is the production of secondary metabolites such as pyrrolnitrin [11], cepacin [21], volatile ammonia, and siderophores [22].

The results from HPLC and NMR analyses showed that *B. cepacia* JBK9 produces the antibiotic pyrrolnitrin, which was first reported from *Pseudomonas pyrrocinia* [23]. pyrrolnitrin production was also reported by other strains like *Pseudomonas aeruginosa*, *P. cepacia*, and *P. fluorescens*. And the following studies confirmed the biosynthesis of pyrrolnitrin from tryptophan. Not only the *Pseudomonas* species, but also the other strains like *Myxococcus fulvus* and *Enterobacter agglomerans* produce pyrrolnitrin [24, 25]. Pyrrolnitrin exhibits broad-spectrum of antimicrobial activities against phytopathogenic fungi as well as pathogenic bacteria.

Bacterial motility is a key factor in their capacity for root colonization. A high motility can confer a high rhizoplane-colonizing ability in terms of movement both from soil to roots and along the roots. The importance of motility for pathogens and useful microorganisms for competitive advantage in rhizosphere soil has been established [26]. An association between the motility and virulence of plant pathogenic bacteria such as *Agrobacterium tumefaciens* and *Pseudomonas syringae* pv. *tabaci* was demonstrated [27]. Beneficial or pathogenic bacteria with a high motility have a high competitiveness and root colonization ability in the rhizosphere. Therefore, flagella-mediated motility has been thought to assist bacteria in finding favorable environments or escaping harmful ones and successfully competing with other organisms. In the present study, the motility of strain JBK9 was found to be better than that of other *Burkholderia* species.

The potential for biological control of strain JBK9 was evaluated in a pot trial. As shown in Fig. 3C, the disease control efficiency of JBK9 in *P. capsici*-infected red pepper plants was higher than that in the controls and other *Burkholderia* strains. This result demonstrated that the excellent motility and root colonization of strain JBK9 prevented the zoospore-mediated infection of red pepper plants by the fungal pathogen *P. capsici*. The production of pyrrolnitrin by strain JBK9 may play a key role in inhibiting the mycelial growth of *P. capsici* [28]. This antimicrobial compound might also inhibit or destroy the *P. capsici* zoospores. In conclusion, red pepper plants treated with *B. cepacia* strain JBK9 were significantly protected from plant disease caused by the fungal pathogen *P. capsici*. This bacterial strain can be applied efficiently in the field as a biocontrol agent through agricultural facilities. In our next study, the detailed mechanisms of pyrrolnitrin production and root colonization by strain JBK9 will be studied by a genetic analysis using the next-generation sequencing technique.

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