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ORIGINAL ARTICLE

# Identification and characterization of the endophytic bacterium *Bacillus atrophaeus* XW2, antagonistic towards *Colletotrichum gloeosporioides*

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**Abstract** *Bacillus atrophaeus* XW2 was isolated from healthy poplar leaves and exhibited a strong inhibitory effect against *Colletotrichum gloeosporioides*, a predominant fungus causing poplar anthracnose. The culture filtrate and bacterial lysate of XW2 were antagonistic against hyphal growth and spore germination. The average diameter of the inhibition zones of hyphal growth were 22.3 mm for the culture filtrate and 12.1 mm for the bacterial lysate. The average inhibition rates of spore germination after 24 h were 94.6 % for the culture filtrate and 88.8 % for the bacterial lysate. In the presence of both culture extracts, hyphae developed vacuoles and swelling, and abnormalities were observed in 100 % of germinated spores. Lipopeptides isolated from culture filtrate and bacterial lysate had high antifungal activities against *C. gloeosporioides*. The average diameter of the inhibition zones were 23.2 mm for culture filtrate and 11.8 mm for bacterial lysate. Crude proteins isolated from culture filtrate and bacterial lysate also showed good inhibition qualities. The average diameters for the zones of inhibition were 22.3 mm for culture filtrate and 13.4 mm for bacterial lysate. Volatiles produced by XW2 inhibited hyphal growth of *C. gloeosporioides* by 60.2 % and were antagonistic against the germination of *C. gloeosporioides* spores after 3 days. Greenhouse studies revealed that XW2 had a 49.1 % efficacy in controlling poplar anthracnose 12 days after exposure to the pathogen. We consider that *Bacillus atrophaeus* XW2 is a promising natural biocontrol agent for use against *C. gloeosporioides*.

**Keywords** *Bacillus atrophaeus* · Antagonistic activity · *Colletotrichum gloeosporioides* · Sterile bacterial lysates · Volatiles

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## Introduction

Poplar is one of the most important trees in China where it has an important role in farmland shelterbelts, soil conservation, and environmental protection. However, it is threatened by poplar anthracnose, a serious leaf disease found in cultivated areas in the north and south of the country (Li et al. 2012). *Colletotrichum gloeosporioides* is the main pathogen causing poplar anthracnose and can infect a wide range of cultivars, often leading to premature defoliation and dieback, and eventually the death of the infected tree (He et al. 1991). Chemical fungicides are currently the most widespread method used to control poplar anthracnose (He et al. 1993). The long-term use of chemical fungicides that are resistant to degradation is environmentally unfavorable and can lead to the evolution of resistance in pathogenic fungi (Stockwell et al. 1997; McMullen and Bergstrom 1999). The use of chemical fungicides to control poplar anthracnose is a limited long-term strategy, and alternative approaches that are potent and environmentally friendly need to be developed.

The use of biocontrol agents to manage plant diseases has recently attracted more attention because they offer an effective and safe way to circumvent the drawbacks of chemical fungicides. Biocontrol can prevent or delay the development of resistance to chemical fungicides and reduce environmental risks associated with their pollution (Steel 1996; Sun et al. 2010; Naing et al. 2014). Endophytic bacteria can be important biocontrol agents because they colonize the internal tissues of their host plants and produce natural products able to control diseases (Beck et al. 2003; Strobel et al. 2004; Guan et al. 2005; Ryan et al. 2008; Ju et al. 2014).

Many endophytic bacteria have been assessed for their potential as control agents against *C. gloeosporioides*. Rahman et al. (2007) reported that four bacteria isolated from the papayan fructosphere had high antagonistic activities against *C. gloeosporioides* infection of papaya. Furuya et al. (2011) reported that *Bacillus subtilis* KS1 isolated from grape skin suppressed the growth of *C. gloeosporioides* (the causal agent of grape ripe rot). However, there are no reports about endophytic bacteria from poplar used to control *C. gloeosporioides*, the main causal agent of anthracnose in these trees.

In this study we isolated an endophytic bacterial strain (XW2) from healthy poplar leaves that showed high antagonistic activity against *C. gloeosporioides*. Using traditional methods and 16S rRNA gene sequencing, strain XW2 was identified as *Bacillus atrophaeus*. The antagonistic qualities of different extracts of XW2 were determined, and its potential to act as a biocontrol agent against *C. gloeosporioides* was evaluated.

## Materials and methods

### Plant pathogen

The plant pathogenic fungus *Colletotrichum gloeosporioides* was isolated from poplar and maintained on potato dextrose agar (PDA) at 4 °C in our laboratory.

### Isolation and screening of endophytic bacteria with properties against *C. gloeosporioides*

Endophytic bacterial isolates were obtained from the leaves of healthy poplars located in a farmland shelter forest in Xiaotangshan, Beijing, China. Leaves were surface-sterilized with 75 % ethanol for 3 min, 3 % sodium hypochlorite for 3 min, and then rinsed three times in sterile distilled water. The isolates were cultured on Luria-Bertani (LB) agar plates in the dark at 28 °C and then stored at–80 °C in LB broth with 30 % (v/v) glycerol (Yang et al. 2011).

The antagonistic activities of the endophytic bacterial isolates against *C. gloeosporioides* were determined using a dual culture assay described by Ferreira et al. (1991), with some modifications. Hyphal plugs (6 mm in diameter) were cut from an actively growing culture of *C. gloeosporioides* on PDA. Each plug was placed at the center of a PDA plate and incubated at 28 °C for 1 day. Bacteria were cultured on LB agar at 28 °C for 24 h, and then a single streak was inoculated onto both sides of the plug at a distance of 2 cm apart. The plug was further incubated in the dark at 28 °C for 7 days. Plates were checked for the presence of inhibiting zones, which were measured. Plates without inoculated bacteria were used as controls, and all tests were repeated three times. Strain XW2 showed the highest antifungal activity against *C. gloeosporioides* and was selected for further study.

### Identification and characterization of strain XW2

#### *Morphological and cultural characterization*

Morphological characteristics of strain XW2 were observed using light and electron microscopy (Sneath 1986; Dong et al. 2001). Cell dimensions, Gram staining, and the presence of flagella, spores, and capsules were determined.

Cultural features of strain XW2 were assessed using single colonies, and growth characteristics were determined using a range of nutrient media (Sneath 1986; Nakamura 1989; Dong et al. 2001) as follows: solid nutrient media: PDA, LB agar, nutrient agar (NA), tryptone glucose yeast extract agar (TGY agar), and tryptic soy agar (TSA); and liquid nutrient media: LB, potato dextrose broth (PDB), potato sucrose broth (PSB), nutrient broth (NB), and tryptic soy broth (TSB).

### Physiology and biochemistry

The physiological and biochemical features of XW2 were determined using standard methods (Sneath 1986; Dong et al. 2001). Utilization of different carbon sources and assessment of saccharolytic activity were determined by standard methods (Dong et al. 2001). The sources of carbon used were pentoses (L-arabinose, D-xylose, and D-fructose), hexose (glucose), disaccharides (sucrose, lactose, and maltose), polysaccharide (starch), monoalcohol (ethanol), polyalcohols (glycerol, mannitol, and D-sorbitol), and salts of organic acids (sodium citrate, sodium acetate, and sodium malonate).

Utilization of different nitrogen compounds were determined according to standard methods (Sneath 1986; Dong et al. 2001) using the following organic sources: urea, peptone, yeast extract, casein, L-glutamic acid, and L-aspartic acid; and inorganic sources: $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, $NH_4Cl$, $NH_4NO_3$, $Ca(NO_3)_2H_2O$, $KNO_3$, and $NaNO_2$.

### 16S rRNA gene

To verify identification of the isolate, genomic DNA was extracted and purified using a TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit (Takara, Japan). The 16S region of the ribosomal RNA genes was amplified using primers 63f, 5′-CAGGCCTAACACATGCAAGTC-3′, and 1387r, 5′-GGGCGGWGTGTACAAGGC-3′ (Marchesi et al. 1998). Polymerase chain reaction (PCR) conditions were as follows: 94 °C for 4 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min; and a final extension at 72 °C for 10 min. PCR products were sequenced by Invitrogen Corporation (Beijing, China). Sequences were compared with the GenBank database using BLAST search and aligned with ClustalW (ver.1.82). Phylogenetic analysis was performed using MEGA (Version 5.0), and a neighbor-joining

phylogenetic tree was constructed by bootstrap analysis with 1,000 replicates.

## Preparation of bacterial cell and fungal spore suspensions

Well-developed colonies of *C. gloeosporioides* growing on PDA were suspended in sterile distilled water and adjusted to a concentration of $1.0\times10^5$ spores $mL^{-1}$ (Todorova and Kozhuharova 2010).

Strain XW2 was cultured in LB broth at 28 °C on a rotary shaker at 200 rpm for 24 h. The cell suspension was adjusted to approximately $1.0\times10^9$ colony forming units $mL^{-1}$ (CFU) $mL^{-1}$ by used sterile distilled water.

## Preparation of sterile cultural filtrate (SCF) and sterile bacterial lysate (SBL)

Strain XW2 was incubated in 500 mL Erlenmeyer flasks containing 100 mL modified medium no. 3 containing 10.0 g $L^{-1}$ glucose, 5.0 g $L^{-1}$ peptone, 5.0 g $L^{-1}$ soybean meal, 1.0 g $L^{-1}$ $KH_2PO_4$, 0.5 g $L^{-1}$ $MgSO_4\cdot7H_2O$, 3.0 g $L^{-1}$ $NH_4Cl$, 1.0 g $L^{-1}$ $Na_2HPO_4$, and 0.5 g $L^{-1}$ yeast extract (pH 7.0–7.2). A 1 % (v/v) bacterial suspension was used to inoculate the medium and cells were incubated at 28 °C on a rotary shaker at 200 rpm for 4 days.

Two flasks of culture suspensions (200 mL) were pooled and centrifuged at 10,000×*g* at 4 °C for 20 min. The supernatant was passed through a sterile membrane filter (0.45 μm; Pall, Ann Arbor, USA) to recover the sterile culture filtrate (SCF). The pellet was collected and rinsed three times with 0.2 M $Na_2HPO_4$-$NaH_2PO_4$ buffer (pH 7.5) and finally suspended in 50 mL of the same buffer. Cell suspension (40 mL, $OD_{600}$ value is 1.4 after 10-fold dilution) was placed in a 50 mL centrifuge tube and homogenized by ultrasound (Scientz-II D, Scientz, Ningbo, China) at 20 kHz for 15 min using an alternating 5 s working and 5 s resting time. The processing tip (6 mm diameter) of the ultrasonic horn was positioned 1 cm below the surface of the bacterial suspension and 3 cm above the bottom of the centrifuge tubes. To keep the temperature of the cell suspension below 30 °C, the cell suspension was placed in an ice bath (Naveena et al. 2012; Gao et al. 2014). The cell suspension was centrifuged at 10,000×*g* and 4 °C for 15 min. The supernatant was passed through a sterile membrane filter (0.45 μm; Pall, Ann Arbor, USA) to obtain the sterile bacterial lysate (SBL).

## Extraction of lipopeptides

SCF and SBL were collected using the procedure described above. The pH of each was adjusted to 2.0 using 6 M hydrochloric acid (HCl) and refrigerated at 4 °C overnight. Suspensions were then centrifuged at 10,000×*g* and 4 °C for 30 min. The precipitates were collected and extracted three times with methanol. The methanol layers were combined (Yao et al. 2012; Zhao et al. 2014). The volume of the methanol layers are one third of SCF or SBL. Lipopeptides in methanol were sterilized by passing through a sterile membrane filter (0.45 μm; Pall, Ann Arbor, USA).

## Extraction of crude proteins

SCF and SBL were prepared as described earlier. Solid $(NH_4)_2SO_4$ was slowly added to the supernatants to achieve a 60 % saturated solution for protein precipitation. The mixtures were refrigerated overnight at 4 °C and then centrifuged at 10,000×*g* and 4 °C for 30 min. The precipitates were collected and dissolved in sterile distilled water (Zhang et al. 2013). Crude proteins liquids from the culture filtrate and bacterial lysate were obtained after dialysis. The volume of the crude proteins liquids are one half of SCF or SBL. Crude proteins liquids were sterilized by passing through a sterile membrane filter (0.45 μm; Pall, Ann Arbor, USA).

## Antifungal activities assay

The antagonistic activities of SCF, SBL, lipopeptides, and crude proteins were determined qualitatively by agar-well diffusion (Tagg and McGiven 1971), with some modifications. A 1 mL sample of a *C. gloeosporioides* spore suspension was mixed with 20 mL PDA at 45 °C~50 °C in a Petri dish (9 cm diameter) and allowed to solidify. Wells (6 mm diameter) were cut into the agar using a sterile steel borer and filled with 50 μL test solution. The zones of inhibition were measured after 2 days of incubation at 28 °C. The experiment was repeated three times.

## Effects of SCF and SBL on hyphal morphology

To determine the effects of SCF and SBL on the morphology of fungal hyphae, the hyphae of *C. gloeosporioides* at the edge of the inhibition zone were observed under an optical microscope.

## Effects of SCF and SBL on spore germination

The inhibitory effects of SCF and SBL on the germination of fungal spores were investigated by microscopy and a concave slide. SCF (20 μL) or SBL (20 μL) was placed into the recess of a concave microscope slide and mixed with 10 μL of a *C. gloeosporioides* spore suspension and incubated at 25 °C for 24 h. For the control treatment, sterile water was used instead of SCF or SBL. For each microscope concave slide examination, 100 spores were assessed for germination and morphology. Experiments were repeated three times.

### Effects of bacterial volatile compounds

The effects of volatile compounds on fungal growth were determined using sealed dishes as previously described (Arrebola et al. 2010), with some modifications. Briefly, 20 mL LB agar at 45 °C~50 °C was mixed with 1 mL of strain XW2 suspension and poured into a sterile Petri dish. A hyphal plug of *C. gloeosporioides* (6 mm diameter) was taken from the margin of the colony and placed in the center of another Petri dish containing 20 mL PDA. The fungal dish was immediately inverted over the bacterial dish (to expose the cultures to each other), and the pair of dishes were instantly sealed together using Parafilm and incubated in the dark at 28 °C for 6 days. Non-inoculated LB medium was used as a control in experiments determining the effects of bacterial volatiles. The diameters (mm) of the fungal colonies were measured, and the hyphae at the edge of the mycelium of *C. gloeosporioides* colonies were observed using light microscopy. The experiment was repeated three times. The degree of inhibition was determined using the following formula (Yang et al. 2011):

$$P(\%) = 100 \times [(C\text{-}d) - (T\text{-}d)]/(C\text{-}d)$$

P is the inhibitory rate, C is the diameter of a control colony, T is the diameter of a treatment colony, and d is the diameter of the hyphal plug.

The effects of volatiles on the germination of fungal spores were determined using the method of Yuan et al. (2012), with some modifications. 20 mL LB agar at 45 °C~50 °C was mixed with 1 mL of strain XW2 suspension and poured into a sterile Petri dish. 20 mL PDA at 45 °C~50 °C was mixed with 1 mL of a suspension of *C. gloeosporioides* and poured into another Petri dish. Then, the fungal dish was immediately inverted over the bacterial dish, and the pair of dishes were instantly sealed together with Parafilm and incubated in the dark at 25 °C for 3 days. The inhibition of spore germination by the volatiles was determined. The experiment was repeated three times.

### Efficacy of strain XW2 as a biocontrol agent against poplar anthracnose under greenhouse conditions

The efficacy of strain XW2 to control poplar anthracnose caused by *C. gloeosporioides* was determined using a pot assay under greenhouse conditions (Fu et al. 2010) using 1-year-old poplar seedlings (*Populus*× *euramericana* cv.*'74/76'*). Poplar cultivar *74/76* is a fast-growing species that is sensitive to anthracnose. Seedlings with seven to ten expanded leaves were used in experiments. Three treatments were set up spraying on both sides of the leaves, using an artist's airbrush (YD12- F111, Yudi, Zhejiang, China), as follows: 1 - SX treatment) 2 ml of strain XW2 cell suspension and 2 ml of *C. gloeosporioides* spore suspension at the same time; 2 - CK1) 2 ml sterile distilled water; and 3 - CK2) 2 ml *C. gloeosporioides* spore suspension. Each treatment consisted of 15 plants with three replicates (five plants per replicate). The diseased leaf rate (DLR), disease severity (DS), disease index (DI), and greenhouse control efficacy (GCE) were separately investigated 12 days after *C. gloeosporioides* inoculation.

The DS was expressed as a percentage of lesion area over the total surface area per leaf and divided into six ratings: 0, no lesion; 1, lesion area ≤5 %; 2, lesion area 5–25 %; 3, lesion area 25–45 %; 4, lesion area 45–65 %; and 5, lesion area >65 %. The DLR, DI, and GCE were calculated using the following formulae:

$$DLR(\%) = 100 \times (\text{No. of affected leaves}/\text{No. of total leaves})$$

$$DI = [100 \times \sum(\text{No. of affected leaves} \times \text{corresponding DS})]/(\text{No. of total leaves} \times 5)$$

$$GCE\ (\%) = 100 \times [(\text{DI of CK2—DI of CK1}) - (\text{DI of SX treatment—DI of CK1})]/(\text{DI of CK2—DI of CK1})$$

### Statistical analysis

The means and standard deviations were calculated and statistically analyzed by analysis of variance (ANOVA) and Duncan's multiple range tests ($P \leq 0.05$) using SPSS version 20.0 (SPSS Inc., Chicago, Illinois).

## Results

### Isolation and selection of endophytic bacteria with properties against *C. gloeosporioides*

A total of 35 endophytic bacteria were isolated from 11 healthy poplar leaves. Among the eight isolates that showed antifungal

activity, strain XW2 was the most antagonistic against *C. gloeosporioides* in vitro. After 7 days, the mean diameter of *C. gloeosporioides* colonies challenged with strain XW2 was 21 mm and 61 mm for the control (Fig. 1). Dual culture plates were incubated for a further 10 days, and *C. gloeosporioides* did not overwhelm the bacterial colony, indicating a persistent inhibition. Strain XW2 was deposited in the China General Microbiological Culture Collection Center (no. 7698).

## Identification and characteristics of strain XW2

### *Morphological and cultural characterization*

Cells of strain XW2 were straight rods with rounded ends and arranged in chains or pairs. They were motile, aerobic, Gram-positive, and without capsules, with dimensions of 1.5–2.5 μm long and 0.5–0.8 μm wide. Spores were elliptical and at the center of the cell (Table 1). Single colonies growing on NA, LB agar, PDA, TSA, and TGY agar were predominantly round to irregular in form, from 1 to 9 mm in diameter, had complete ends, and were creamy faintly off-white, slightly convex, and opaque, with a smooth matt surface. A dark brown or black pigment diffused into TGY. No other pigments were observed. Growth in NB produced a pellicle on the walls of the vessel. The pellicle was dense, matt, and had a slight off-white color.

### Physiology and biochemical characteristics

Strain XW2 was positive for oxidase, catalase, lecithinase, methyl red, and Voges-Proskauer reactions. It could liquefy gelatin, reduce nitrate to nitrite, produce hydrogen sulfide, and hydrolyze casein and starch. Litmus milk turned red. It was negative for the production of indole, anaerobic growth in glucose agar, degradation of tyrosine, production of gas from glucose, and phenylalanine deaminase activity. Growth ranges for temperature, pH, and NaCl concentration are presented in Table 1.

Strain XW2 utilized all carbon sources except lactose, D-xylose, ethanol, sodium acetate, and sodium malonate. The best used carbon sources were glucose and sucrose. Acid was formed without gas production for the degradation of most carbon sources. Strain XW2 actively assimilated all nitrogen sources except $NaNO_2$. Peptone and yeast extract were the best nitrogen sources of strain XW2 (Table 2).

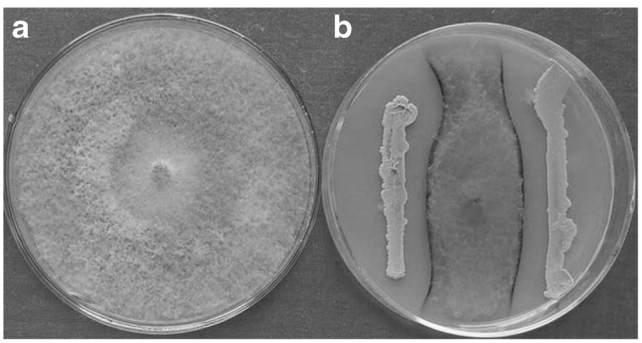


**Fig 1** Dual culture plates. (**a**) Control and (**b**) inhibition of *Colletotrichum gloeosporioides* by strain XW2

Statistical analysis of morphological, physiology, and biochemical data was used with interpreting software to identify strain XW2. Dendrograms from the hierarchical cluster analysis illustrate the step-by-step unification of strain XW2 with *Bacillus subtilis* (Fig. 2). Strain XW2 differed from *B. subtilis* in two characteristics, namely, the former did not degrade xylose and it produced dark brown or black pigments that diffused into TGY agar.

## 16S rRNA gene

The 16S rRNA sequence (GenBank accession number KF976392) was comprised of 1369 nucleotides. Sequence comparison using BLAST suggested a close relationship with *Bacillus atrophaeus*. Phylogenetic analysis (Fig. 3) indicated that strain XW2 was similar to *B. subtilis* KC990823, *B. subtilis* EU081512, and *B. velezensis* FJ713021 and that it was most closely related to *B. atrophaeus* GU994860 (99 % similarity).

## Antifungal activities of the culture filtrate, bacterial lysate, lipopeptides, and crude proteins

SCF and SBL (Fig. 4a) of strain XW2 had antagonistic qualities against *C. gloeosporioides*. The average diameters of the inhibition zones were 22.3 mm for SCF and 12.1 mm for SBL. At the edge of the inhibition zones, hyphal growth was thin and there were piles of orange conidia.

The antagonistic qualities of different components isolated from SCF and SBL were assessed. Lipopeptides (Fig. 4b) had high antifungal activities against *C. gloeosporioides*. The average diameter of the inhibition zones were 23.2 mm for SCF and 11.8 mm for SBL. Extracts of crude proteins also showed good inhibition qualities. The average diameters for the zones of inhibition were 22.3 mm for SCF and 13.4 mm for SBL (Fig. 4c).

## Effects of SCF and SBL on hyphal morphology

Treatment of *C. gloeosporioides* with SCF and SBL of XW2 resulted in morphological abnormalities to hyphae, such as vacuoles and swelling (Fig. 5b, c). Hyphae were normally smooth without swelling and vacuoles (Fig. 5a).

**Table 1** Morphological, physiological and biochemical characteristics of strain XW2

| Indicator | Result | Indicators | Results |
|---|---|---|---|
| Cell sizes, μm | 1.5-2.5×0.5-0.8 | Growth with 15 % (w/v) NaCl | - |
| Mobility | + | Liquefication of gelatin | + |
| Form of spores | Elliptical | Catalase activity | + |
| Position of spores | Central | Lecithinase activity | + |
| Capsule | - | Oxidase activity | + |
| Gram staining | + | Reduction of nitrates | + |
| Growth at 5 °C | - | Litmus milk reaction | Red |
| Growth at 10 °C | + | Production of indole | - |
| Growth at 45 °C | + | Methyl red reaction | + |
| Growth at 50 °C | - | Production of hydrogen sulfide | + |
| Growth at pH 3 | - | Voges-Proskauer reaction | + |
| Growth at pH 4 | + | pH Voges-Proskauer broth | 6.77-7.32 |
| Growth at pH 5.7 | + | Degradation of tyrosine | - |
| Growth at pH 10 | + | Production of gas from glucose | - |
| Growth at pH 11 | - | Hydrolysis of casein | + |
| Growth with 0.2 % (w/v) NaCl | + | Hydrolysis of starch | + |
| Growth with 5 % (w/v) NaCl | + | Anaerobic growth in glucose agar | - |
| Growth with 10 % (w/v) NaCl | + | Phenylalanine deaminase activity | - |

+, positive; −, negative

## Effect of SCF and SBL on spore germination

Treatment of *C. gloeosporioides* with SCF and SBL of XW2 reduced the germination rates of spores. After 24 h, germination was inhibited in 94.6 % of fungal spores treated with SCF and 88.8 % of those treated with SBL. The germinated spores treated with SCF and SBL suffered morphological aberrations such as germ tube vacuoles and swelling (Fig. 5e, f). The abnormal rates of the germinated spores were both 100 %, and untreated spores were unaffected (Fig. 5d).

## Effects of bacterial volatiles

Bacterial strain XW2 produced antifungal volatile compounds that inhibited the growth of exposed fungal colonies (Fig. 6b). Volatile compounds inhibited the growth of the hyphae and impaired the growth of *C. gloeosporioides* by 60.2 %. Hyphae challenged with volatiles in the bioassay were ramified and curved at the edge of colonies (Fig. 7b) whilst no symptoms were observed in the control (Fig. 7a). Moreover, exposure to bacterial volatiles reduced the germination rate of *C.*

**Table 2** Assimilation of different carbon and nitrogen sources by strain XW2

| **Carbon source** | **Degree of growth** | **Nitrogen source** | **Degree of growth** |
|---|---|---|---|
| Glucose | ++++, A | $(NH_4)_2HPO_4$ | + |
| Sucrose | ++++, A | $(NH_4)_2SO_4$ | ++ |
| D-fructose | +++, A | $NH_4Cl$ | ++ |
| Lactose | - | $NH_4NO_3$ | ++ |
| Maltose | ++, A | $Ca(NO_3)_2H_2O$ | ++ |
| D-xylose | - | $KNO_3$ | ++ |
| L-arabinose | ++, A | $NaNO_2$ | - |
| Starch | +, A | Urea | + |
| Mannitol | ++, A | Peptone | ++++ |
| D-sorbitol | ++, A | Yeast extract | ++++ |
| Glycerol | ++, A | Casein | ++ |
| Ethanol | - | L-glutamic acid | +++ |
| Sodium acetate | - | L-aspartic acid | +++ |
| Sodium malonate | - | | |
| Sodium citrate | +++, L | | |

A acid formation, L alkalization of medium; Evaluation ++++ very good growth, +++ good growth, ++ moderate growth, + scarce growth, − no growth

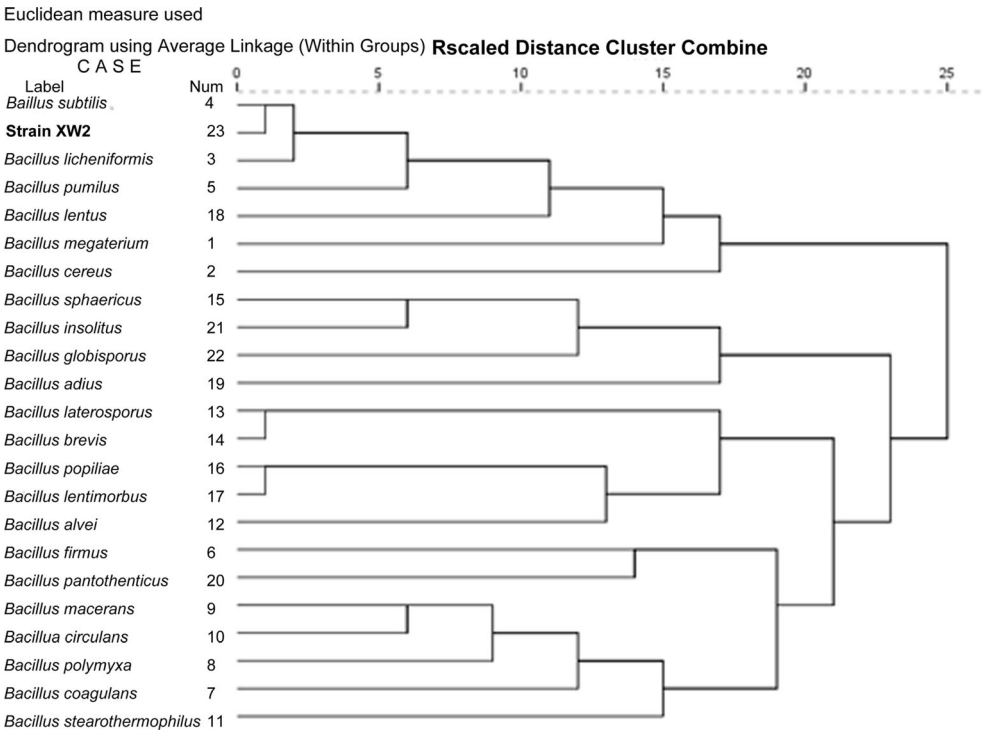


**Fig. 2** Dendrograms for the hierarchical cluster analysis of strain XW2

*gloeosporioides* spores after 3 days (Fig. 6d) compared to the untreated spores (Fig. 6c).

Efficacy of strain XW2 as a biocontrol agent against poplar anthracnose under greenhouse conditions

Strain XW2 obviously inhibited the development of poplar anthracnose in greenhouse experiments (Table 3). After 12 days, only the leaves treated with sterile distilled water had no disease symptoms. Plants treated with *C. gloeosporioides* had 50.9 % diseased leaves. The application of strain XW2 significantly reduced the proportion of diseased leaves to 37.1 %, and the DI was significantly lower than the CK2 treatment. The DI of SX treatment (strain XW2 applied on the poplar leaves) was 11.1, and the DI of CK2 treatment was 21.8. The greenhouse trial indicated that strain XW2 had a 49.1 % efficacy in controlling poplar anthracnose caused by *C. gloeosporioides*.

## Discussion

In this study we isolated an endophytic bacterial strain from poplar leaves that had strong antagonistic qualities against *C. gloeosporioides*. On the basis of morphological, cultural, physiological, and biochemical properties, strain XW2 was initially identified as *Bacillus subtilis*, however, strain XW2 could produce dark brown or black pigments that diffused in TGY agar. *Bacillus atrophaeus* can produce pigments in organic nitrogen-containing medium, and it was previously

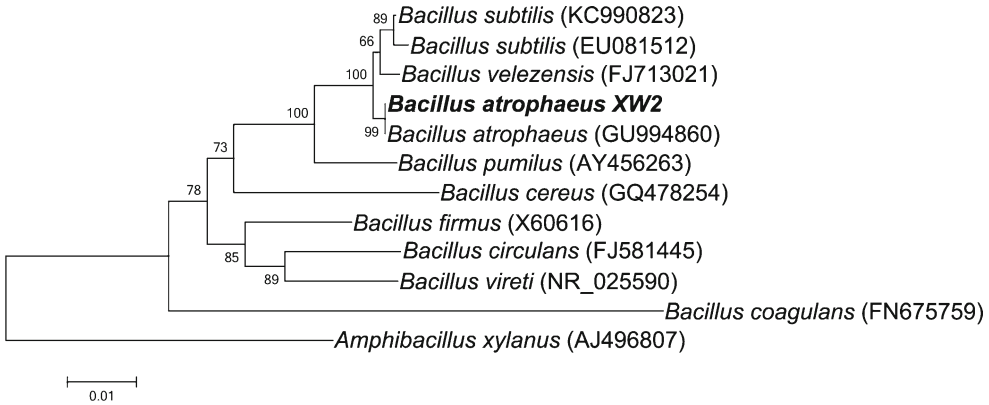


**Fig. 3** Phylogenetic tree of strain XW2 based on 16S rRNA sequence analysis, constructed using the neighbor-joining method. The level of bootstrap support (1,000 repetitions) is indicated at all nodes. *Amphibacillus xylanus* was used as an outlier to root the tree

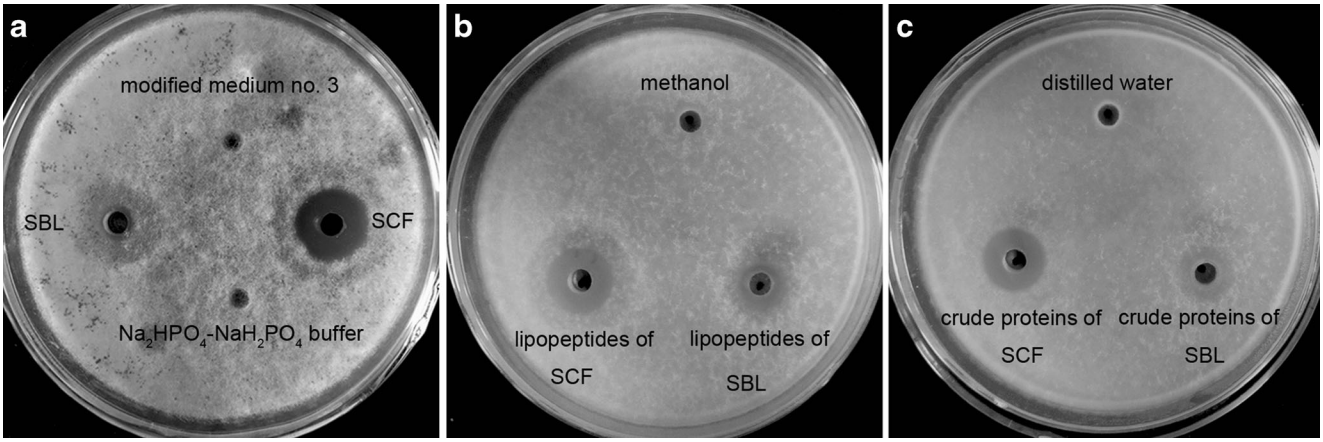


**Fig. 4** Inhibition activities of different extracts from strain XW2 against *C. gloeosporioides*. (**a**) Sterile culture filtrate (SCF) and sterile bacterial lysate (SBL), (**b**) lipopeptides from SCF and SBL, and (**c**) crude proteins from SCF and SBL

classified as *B. subtilis* var. *niger* (Nakamura 1989; Liu et al. 2009). Therefore, we identified strain XW2 as *B. atrophaeus*. Moreover, analysis of the 16S rRNA gene sequence ratified the identification. Previous studies have also reported that *B. atrophaeus* inhibits the growth of fungal plant pathogens (Xin et al. 2013; Zhang et al. 2013), however, this is the first time that *B. atrophaeus* has been used to control the growth of *C. gloeosporioides* in poplar.

Cultures of *Bacillus* sp. produce a range of extracts and products with antifungal activities including culture filtrates and volatiles (Li et al. 2010; Baysal et al. 2013; Wang et al. 2013). Antimicrobial compounds are mostly found in SCFs of *Bacillus* sp. (Li et al. 2009; Cui et al. 2012; Zhao et al. 2013), such as lipopeptide-type antibiotics and antifungal proteins (Pinchuk et al. 2002; Almenar et al. 2007; Alfonzo et al. 2012; Malfanova et al. 2012). Many of the antifungal compounds found in *Bacillus* sp. culture filtrates cause deformities in spores and hyphae (Chan et al. 2003; Rahman et al. 2007; and Furuya et al. 2011) and are important agents for cell lysis and death. In this study, the culture filtrate of *B. atrophaeus* XW2 had effective antagonistic qualities against *C. gloeosporioides*, evidenced by the development of vacuoles and swelling in the hyphae and germ tubes. Lipopeptides and crude proteins from the filtrate exhibited effective antagonism against *C. gloeosporioides*.

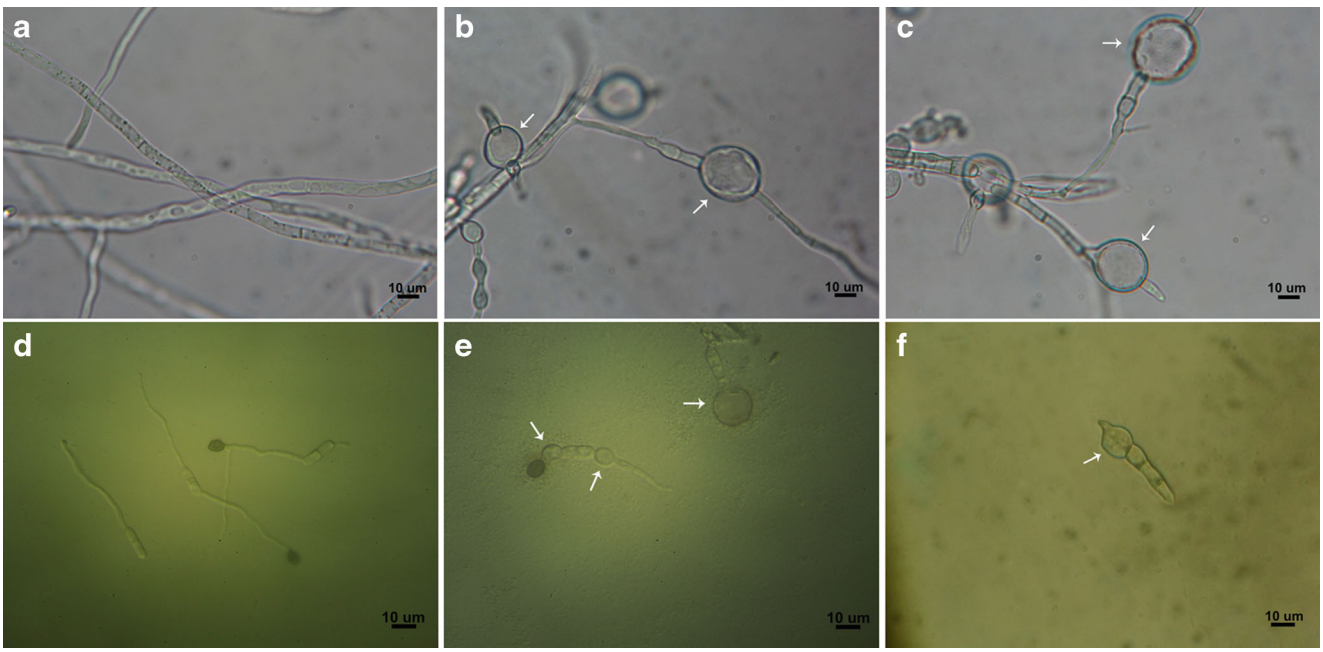


**Fig. 5** Effects of culture extracts from strain XW2 on the growth of fungal hyphae and spore germination, observed under light microscopy. Vacuoles and swelling in hyphae and the germ tube after treatment with sterile culture filtrate (**b**, **e**), sterile bacterial lysates (**c**, **f**), and control (**a**, **d**)

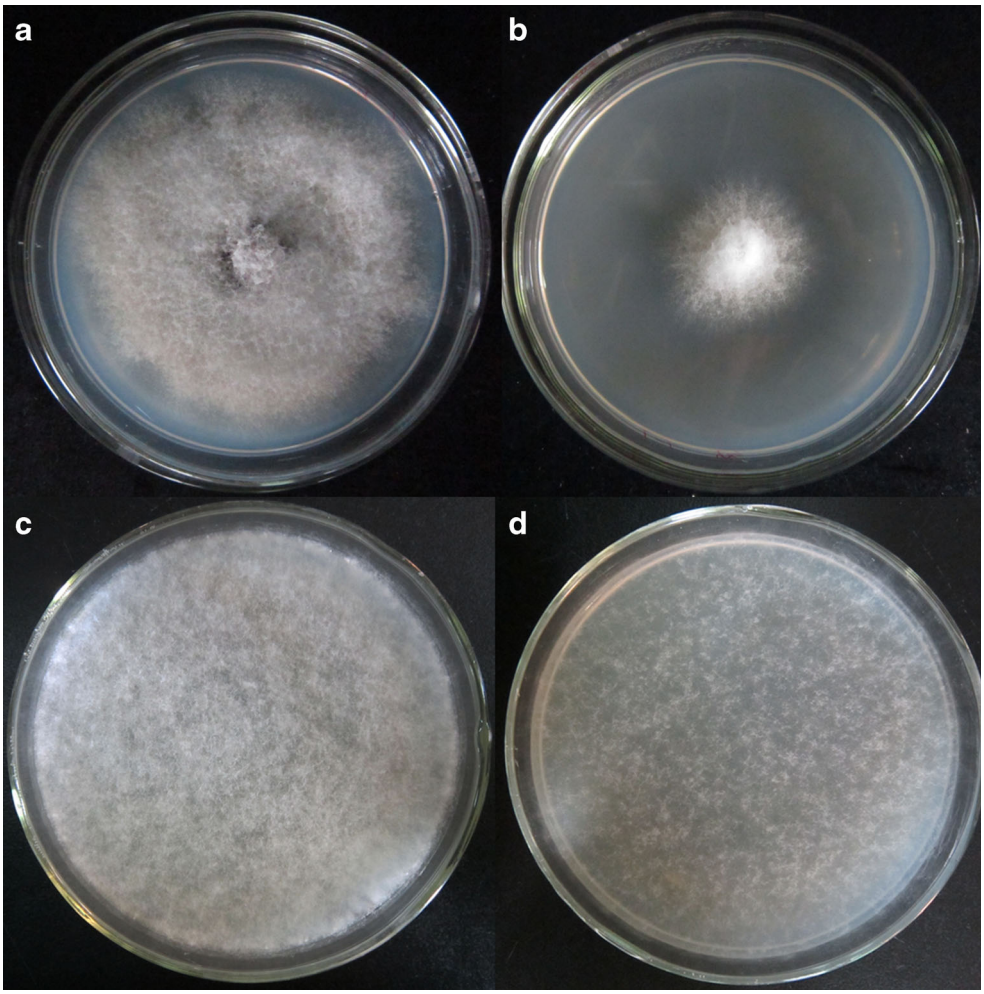


**Fig. 6** Inhibition activities of volatiles produced by strain XW2 against *C. gloeosporioides* in sealed plates. Controls (**a**) and (**c**), and inhibition of both hyphal growth (**b**), and spore germination (**d**)

Volatiles produced by *Bacillus* sp. can impair the development of hyphae and spore germination of plant pathogens (Kai et al. 2007), and several types with antagonistic qualities have been identified, such as amides, aldehydes, alcohols, ketones, and phenols (Kai et al. 2009; Yuan et al. 2012; Zheng et al. 2013). The volatiles produced by *B. atrophaeus* XW2 impaired hyphal growth and spore germination of *C. gloeosporioides*, and upon closer inspection, large amounts of ramified and curved hyphae were observed on PDA. Our findings are different from previous studies reporting

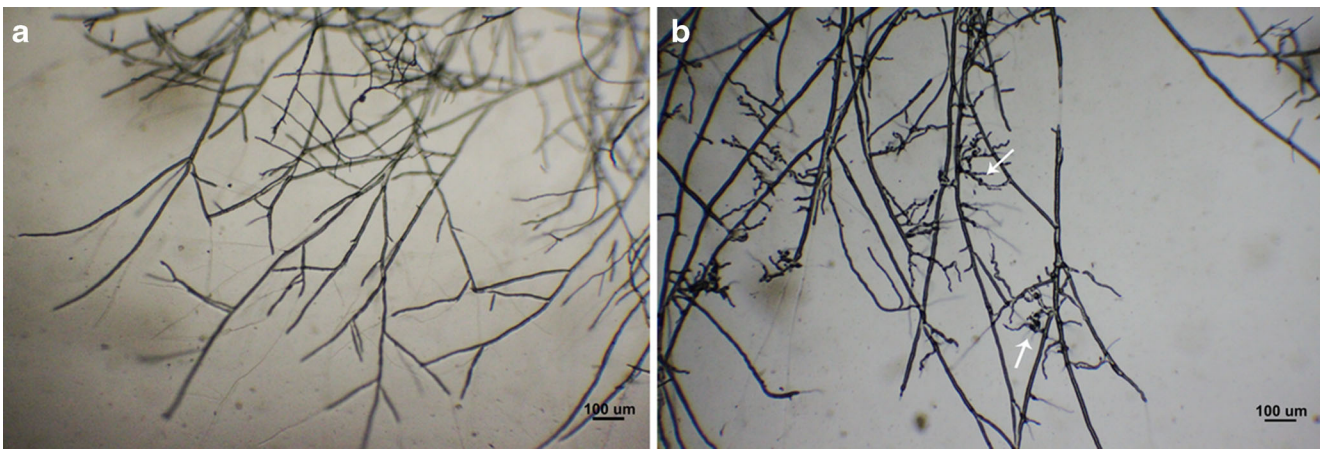


**Fig. 7** Effects of volatiles produced by strain XW2 on hyphal growth observed under light microscopy. (**a**) Control and (**b**) exposure to bacterial volatiles. Ramified and curved hyphae were observed at the edge of colonies after treatment with volatile

**Table 3** Efficacy of strain XW2 in controlling poplar anthracnose under greenhouse conditions

| Treatment | Diseased leaves rate (%) | Disease index | Greenhouse control efficacy (%) |
|---|---|---|---|
| CK1 | 0±0c | 0±0c | \ |
| CK2 | 50.9±3.0a | 21.8±3.6a | \ |
| SX | 37.1±3.4b | 11.1±1.0b | 49.1 |

CK1, only sterile distilled water applied to leaves; CK2, leaves only inoculated with *C. gloeosporioides*; SX, strain XW2 and *C. gloeosporioides* applied to leaves at the same time. The investigations were carried out 12 days after the treatments, respectively. The diseased leaves rate (DLR), disease index (DI), and greenhouse control efficacy (GCE) were calculated using the following formulae: DLR (%)= 100×(No. of affected leaves/No. of total leaves), DI=[100×∑ (No. of affected leaves×corresponding DS)]/(No. of total leaves×5), GCE (%)= 100×[(DI of CK2 - DI of CK1)-(DI of SX treatment- DI of CK1)]/(DI of CK2-DI of CK1) Values with the different letter within the same column are significantly different at $P<0.05$ according to Duncan's test. Numbers follow by the "±" are standard errors (SE). \, the data are incalculable

deformities in fungal spores and hyphae caused by volatiles from *Bacillus* sp. (Chaurasia et al. 2005; Arrebola et al. 2010; Baysal et al. 2013).

The antagonistic properties of SCFs and volatiles produced by bacteria have received much attention, however, studies focusing on the intracellular metabolites or products of potentially useful strains have received less attention (Ren and Zhao 2010; Yu et al. 2013). Our findings are similar to those of Zhang et al. (2013) who reported that the SCF and volatiles produced by *B. atrophaeus* CAB-1 were highly effective against plant pathogens. Further to the findings of Zhang et al. (2013), we provide the first report on the inhibitory qualities of sterile lysates of *B. atrophaeus* active against *C. gloeosporioides*. More specifically, our study identified morphological abnormalities of the hyphae and germinated spores of *C. gloeosporioides* upon treatment with culture extracts. Culture filtrates and lysates contained antifungal agents, which were refined through the isolation of lipopeptides and crude proteins. Both of these extracts exhibited antagonistic qualities against *C. gloeosporioide*. The results indicate that filtrates and lysates of *B. atrophaeus* XW2 cultures contain undefined antimicrobial compounds that may have intra- or extra-cellular characteristics. Some compounds with antagonistic activities may only exist in within bacterial cells because they cannot be secreted through the cell membrane, and this could have significant implications for the type of extract or biocontrol agent used, which will be studied in the future.

In conclusion, *B. atrophaeus* XW2 is an endophytic bacterium with antagonistic properties against *C. gloeosporioides*. Cultural filtrate, bacterial lysate, extracts of lipopeptides and crude proteins, and volatiles produced by *B. atrophaeus* XW2 were all effective at inhibiting the growth of *C. gloeosporioides*. Further to these findings, *B. atrophaeus* XW2 was an effective biocontrol agent against *C. gloeosporioides* in poplar trees under controlled greenhouse conditions and has much potential for use as an economical and environmentally safe way to control this disease.

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## References

Alfonzo A, Piccolo SL, Conigliaro G, Ventorino V, Burruano S, Moschetti G (2012) Antifungal peptides produced by *Bacillus amyloliquefaciens* AG1 active against grapevine fungal pathogens. Ann Microbiol 62(4):1593–1599. doi:10.1007/s13213-011-0415-2

Almenar E, Del Valle V, Catala R, Gavara R (2007) Active package for wild strawberry fruit (*Fragaria vesca L.*). J Agri Food Chem 55(6): 2240–2245. doi:10.1021/jf062809m

Arrebola E, Sivakumar D, Korsten L (2010) Effect of volatile compounds produced by *Bacillus* strains on postharvest decay in citrus. Biol Control 53(1):122–128. doi:10.1016/j.biocontrol.2009.11.010

Baysal Ö, Lai D, Xu HH, Siragusa M, Çalışkan M, Carimi F, Da Silva JAT, Tör M (2013) A proteomic approach provides new insights into the control of soil-borne plant pathogens by *Bacillus* species. PLoS One 8(1):e53182. doi:10.1371/j.pone.0053182

Beck HC, Hansen AM, Lauritsen FR (2003) Novel pyrazine metabolites found in polymyxin biosynthesis by *Paenibacillus polymyxa*. FEMS Microbiol Lett 220(1):67–73. doi:10.1016/S0378-1097(03)00054-5

Chan YK, McCormick WA, Seifert KA (2003) Characterization of an antifungal soil bacterium and its antagonistic activities against *Fusarium* species. Can J Microbiol 49(4):253–262. doi:10.1139/w03-033

Chaurasia B, Pandey A, Palni LMS, Trivedi P, Kumar B, Colvin N (2005) Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain cause structural deformations in pathogenic fungi in vitro. Microbiol Res 160(1):75–81. doi:10.1016/j.micres.2004.09.013

Cui TB, Chai HY, Jiang LX (2012) Isolation and partial characterization of an antifungal protein produced by *Bacillus licheniformis* BS-3. Molecules 17(6):7336–7347. doi:10.3390/molecules17067336

Dong XZ, Cai MY, Lu YY, Xie JY, Liu XL (2001) Identification methods of common bacteria. In: Dong XZ (ed) Cai MY(eds)Handbook of common bacteria systematic identify. Beijing Science press, Beijing, pp 349–398

Ferreira JHS, Matthee FN, Thomas AC (1991) Biological control of *Eutypa lata* on grapevine by an antagonistic strain of *Bacillus subtilis*. Phytopathology 81(3):283–287

Fu G, Huang S, Ye Y, Wu Y, Cen Z, Lin S (2010) Characterization of a bacterial biocontrol strain B106 and its efficacies on controlling banana leaf spot and post-harvest anthracnose diseases. Biol Control 55(1):1–10. doi:10.1016/j.biocontrol.2010.05.001

Furuya S, Mochizuki M, Aoki Y, Kobayashi H, Takayanagi T, Shimizu M, Suzuki S (2011) Isolation and characterization of *Bacillus subtilis* KS1 for the biocontrol of grapevine fungal diseases. Biocontrol Sci Tech 21(6):705–720. doi:10.1080/09583157.2011.574208

Gao SP, Lewis GD, Ashokkumar M, Hemar Y (2014) Inactivation of microorganisms by low-frequency high-power ultrasound: 1 Effect of growth phase and capsule properties of the bacteria. Ultrason Sonochem 21(1):446–453. doi:10.1016/j.ultsonch.2013.06.006

Guan S, Sattler I, Lin W, Guo D, Grabley S (2005) P-aminoacetophenonic acids produced by a mangrove endophyte: *Streptomyces griseus* subsp. J Nat Prod 68(8):1198–1200. doi:10.1021/np0500777

He W, Yang W, Shen RX (1991) The preliminary study on anthracnose of *Populus*×*beijingensis*. For Pest and Dis 4:7–9

He W, Shen RX, Yang W, Wang YY, Tong ZL (1993) Chemical control the anthracnose of *Populus*×*beijingensis*. For Pest and Dis 2:15–17

Ju R, Zhao Y, Li J, Jiang H, Liu P, Yang T, Bao Z, Zhou B, Zhou X, Liu X (2014) Identification and evaluation of a potential biocontrol agent, *Bacillus subtilis*, against *Fusarium* sp. in apple seedlings. Ann Microbiol 64:377–382. doi:10.1007/s13213-013-0672-3

Kai M, Effmert U, Berg G, Piechulla B (2007) Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen rhizoctonia solani. Arch Microbiol 187(5):351–360. doi:10.1007/s00203-006-0199-0

Kai M, Haustein M, Molina F, Petri A, Scholz B, Piechulla B (2009) Bacterial volatiles and their action potential. Appl Microbiol Biotechnol 81(6):1001–1012. doi:10.1007/s00253-008-1760-3

Li Z, Liang YM, Tian CM (2012) Characterization of the causal agent of poplar anthracnose occurring in the Beijing region. Mycotaxon 120(1): 277–286. doi:10.5248/120.277

Li J, Yang Q, Zhao L, Zhang SM, Wang YX, Zhao XY (2009) Purification and characterization of a novel antifungal protein from *Bacillus subtilis* strain B29. Univ Sci B 10(4):264–272. doi:10.1631/jzus.B0820341

Li BQ, Lu XY, Guo QG, Qian CE, Li SZ, Ma P (2010) Isolation and identification of lipopeptides and volatile compounds produced by *Bacillus subtilis* strain BAB-1. Sci Agric Sin 43(17):3547–3554

Liu B, Li H, Yao L, Li JX, Chen C (2009) Characteristics and applications of *Bacillus subtilis* var. *niger* ATCC9372. Chin J of Disinfection 26(2):236–237

Malfanova N, Franzil L, Lugtenberg B, Chebotar V, Ongena M (2012) Cyclic lipopeptide profile of the plant-beneficial endophytic bacterium *Bacillus subtilis* HC8. Arch Microbiol 194(11):893–899. doi:10.1007/s00203-012-0823-0

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64(2):795–799

McMullen M, Bergstrom G (1999) *C*hemical and biological control of *Fusarium* head blight 1999 projects and progress. In Proceedings of the 1999 National *Fusarium* Head Blight Forum, Sioux Falls, S.D., 5–7 December 1999, pp 61–63.

Naing KW, Anees M, Kim SJ, Nam Y, Kim YC, Kim KY (2014) Characterization of antifungal activity of *Paenibacillus ehimensis* KWN38 against soilborne phytopathogenic fungi belonging to various taxonomic groups. Ann Microbiol 64:55–63. doi:10.1007/s13213-013-0632-y

Nakamura LK (1989) Taxonomic Relationship of black-pigmented *Bacillus subtilis* strains and a proposal for *Bacillus atrophaeus* sp. nov. Int J Syst Bacterio 39(3):295–300. doi:10.1099/00207713-39-3-295

Naveena B, Sakthiselvan P, Elaiyaraju P, Partha N (2012) Ultrasound induced production of thrombinase by marine actinomycetes: Kinetic and optimization studies. Biochem Eng J 61:34–42. doi:10.1016/j.bej.2011.12.007

Pinchuk IV, Bressollier P, Sorokulova IB, Verneuil B, Urdaci MC (2002) Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Res Microbiol 153(5):269–276. doi:10.1016/S0923-2508(02)01320-7

Rahman MA, Kadir J, Mahmud TMM, Rahman RA, Begum MM (2007) Screening of antagonistic bacteria for biocontrol activities on *Colletotrichum gloeosporioides* in papaya. Asian J Plant Sci 6(1):12–20

Ren M, Zhao L (2010) Isolation and identification of a beta-cypermethrin-degrading and biocontrol endophytic bacteria in leek. Acta Agriculturae Boreali-Occidentalis Sinica 3:014

Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN (2008) Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett 278(1):1–9. doi:10.1111/j.1574-6968.2007.00918.x

Sneath PHA (1986) Endospore-forming Gram-positive rods and cocci. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 2. Williams & Wilkins, Baltimore, pp 1104–1207

Steel CC (1996) Catalase activity and sensitivity to the fungicides, iprodione and fludioxonil in *Botrytis cinerea*. Lett Appl Microbiol 22(5):335–338. doi:10.1111/j.1472-765X.1996.tb01173.x

Stockwell CA, Luz WC, Bergstrom GC (1997) Biocontrol of wheat scab with microbial antagonists. Phytopathology 87:S94

Strobel G, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic microorganisms⊥. J Nat Prod 67(2):257–268. doi:10.1021/np030397v

Sun JB, Peng M, Wang YG, Zhao PJ, Xia QY (2010) Isolation and characterization of antagonistic bacteria against Fusarium wilt and induction of defense related enzymes in banana. Afr J Microbiol Res 5:509–515. doi:10.5897/AJMR10.607

Tagg JR, McGiven AR (1971) Assay system for bacteriocins. Appli Microbiol 21(5):943

Todorova S, Kozhuharova L (2010) Characteristics and antimicrobial activity of *Bacillus subtilis* strains isolated from soil. World J Microbiol Biotechnol 26(7):1207–1216. doi:10.1007/s11274-009-0290-1

Wang BB, Yuan J, Zhang J, Shen ZZ, Zhang MX, Li R, Ruan YZ, Shen QR (2013) Effects of novel bio-organic fertilizer produced by *Bacillus amyloliquefaciens* W19 on antagonism of Fusarium wilt of banana. Biol Fertil Soils 49(4):435–446. doi:10.1007/s00374-012-0739-5

Xin HF, Meng YY, Li JH, Mao HX, Zhang X (2013) *Bacillus atrophaeus* strains colonization in wheat plant and its inhibition efficiency to *Fusarium* head blight. Chin J of Ecol 32(6):1490–1496

Yang CJ, Zhang XG, Shi GY, Zhao HY, Chen L, Tao K, Hou TP (2011) Isolation and identification of endophytic bacterium W4 against tomato *Botrytis cinerea* and antagonistic activity stability. Afr J Microbiol Res 5(2):131–136. doi:10.5897/AJMR10.81

Yao DH, Ji ZX, Wang CJ, Qi GF, Zhang LL, Ma X, Chen SW (2012) Co-producing iturin A and poly-c-glutamicacid from rapeseed meal under solid state fermentation by the newly isolated *Bacillus subtilis* strain 3–10. World J Microbiol Biotechnol 28:985–991. doi:10.1007/s11274-011-0896-y

Yu Q, Liu Z, Lin D, Zhang W, Sun Q, Zhu J, Lin M (2013) Characterization and evaluation of *Staphylococcus* sp. strain LZ16 for the biological control of rice blast caused by *Magnaporthe oryzae*. Biol Control 65(3):338–347. doi:10.1016/j.biocontrol.2013.03.016

Yuan J, Raza W, Shen QR, Huang QW (2012) Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. *cubense*. Appl Environ Microbiol 78(16):5942–5944. doi:10.1128/AEM.01357-12

Zhang XY, Li BQ, Wang YG, Guo QG, Lu XY, Li SZ, Ma P (2013) Lipopeptides, a novel protein, and volatile compounds contribute to the antifungal activity of the biocontrol agent *Bacillus atrophaeus* CAB-1. Appl Microbiol Biotechnol 97(21):9525–9534. doi:10.1007/s00253-013-5198-x

Zhao XY, Zhao XM, Wei YM, Shang QX, Liu ZP (2013) Isolation and identification of a novel antifungal protein from a rhizobacterium *Bacillus subtilis* strain F3. J Phytopathol 161(1):43–48. doi:10.1111/jph.12015

Zhao X, Han Y, Tan X, Wang J, Zhou ZJ (2014) Optimization of antifungal lipopeptide production from *Bacillus* sp. BH072 by response surface methodology. J Microbiol 52(4):324–332. doi:10.1007/s12275-014-3354-3

Zheng M, Shi JY, Shi J, Wang QG, Li YH (2013) Antimicrobial effects of volatiles produced by two antagonistic *Bacillus* strains on the anthracnose pathogen in postharvest mangos. Biol Control 65(2):200–206. doi:10.1016/j.cropro.2004.03.014