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FULL RESEARCH PAPER

Compounds inhibitory to nematophagous fungi produced by *Bacillus* sp. strain H6 isolated from fungistatic soil

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Abstract Soil fungistasis can cause inconsistent control by nematophagous fungi of plant-pathogenic nematodes in field situations. Recent studies have shown that production of fungistatic compounds by bacteria was the principal explanation for soil fungistasis. The culture filtrate of *Bacillus* sp. strain H6, a strain representative of the dominant colony types isolated from fungistatic soils, showed strong inhibitory activity against nematophagous fungi by inducing unusual swelling in the conidia and the germ tubes of nematophagous fungi, thereby preventing the fungi from proliferating. This inhibitory mechanism is novel in comparison with other known mechanisms. Antifungal activity of the culture filtrate of strain H6 was maximal after culture in Luria Bertani (LB) broth (pH 7.0) at 36°C for 36 h. The inhibitory effect of the compounds produced by *Bacillus* sp. strain H6 was not significantly influenced by pH, and the inhibitory compounds in the culture filtrate were thermostable. After being partially purified by thin layer chromatography (TLC) on silica gel plates, characterization by colour reactions and positive fast atom bombardment mass spectrometry indicated that the inhibitory compounds showed similarity to iturin A group compounds.

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Keywords *Bacillus* · Fungal germination · Fungistatic inhibition · Lipopeptide · Nematophagous fungi

Abbreviations

DMSO	Dimethyl sulphoxide
EtOAc	Ethyl acetate
LB	Luria Bertani
PDA	Potato dextrose agar
RBF	The residual fraction of the *n*-butanol extract that was extracted by EtOAc
RSF	The residual fraction of supernatant that was extracted by *n*-butanol
TLC	Thin layer chromatography
TDM	4,4′-bis(dimethylamino) diphenylmethane

Introduction

Fungistatic soils have been defined as those in which the germination of most fungal propagules is inhibited (Dobbs & Hinson, 1953). There are

many factors that affect fungistatic intensity, including chemical and physical attributes of the soil, and microbial community composition (Lockwood, 1977). Although abiotic characteristics of soil can contribute to fungistasis, soil fungistasis is often a function of the activity of the resident soil microorganisms. As for the microbial cause of fungistasis, the nutrient-deprivation hypothesis (Lockwood, 1977) and germination inhibition hypothesis (Liebman & Epstein, 1992) are the two most widely accepted. More significant data (Ellis, Timms-Wilson, & Bailey, 2000; Liebman & Epstein, 1992;) supported the germination inhibition hypothesis. Moreover, the study of Wietse, Verheggen, Gunnewiek, Kowalchuk, and Veen (2003) indicated that the bacterial community composition was a determining factor in the development of fungistasis, and that the antifungal compounds produced by them were the principal explanation for fungistasis.

Rootknot, caused by the phytoparasitic nematode *Meloidogyne incognita*, is a condition commonly observed in tobacco fields in Yunnan province (one China's southwest provinces), one of the major tobacco production areas, and the pest results in serious economic losses (Lei, Li, Kong, & Yang, 1998). The potential of biological control of phytoparasitic nematodes with fungi will become increasingly important, as methyl bromide, a major nematicide for agriculture and horticulture, was phased out in 2005 (Thomas, 1996). From this point of view, several microbial nematicides were tested in field trials and showed potential control effects (Zhu, Zhang, Li, Xia, & Yang, 2001). However, under field conditions fungal biocontrol agents have often been proved to be less efficacious and to give more variable disease control than chemical pesticides. Such inconsistent biocontrol may be due to soil fungistasis (Bae & Knunsen, 2000), and the initial failure of biocontrol agents in the rhizosphere was related to unfavorable soil conditions, such as temperature, moisture content, pH and aeration, as well as competition from the indigenous microbiota (Ahmed, Ezziyyani, Sánchez, & Candela, 2003; Bae & Knunsen, 2000; Dik, Koning, & Kohl, 1999). Predicting the effects of all these factors on the biological control of nematodes in the field is a considerable challenge, but studies on the effect of soil fungistasis may help us to understand some of the environmental limitations of control efficacy and determine when they may be most effective.

In our previous research (Xu, 2004), we studied the bacterial community composition of tobacco soils fungistatic to nematophagous fungi from various regions of Yunnan and isolated nine strains of bacteria that were dominant colony types, not only because of their high population frequency, but also because of their strong inhibitory activity. To understand a relationship between the inhibitory metabolites produced by these dominant bacteria and the fungistatic nature of soils to biocontrol agents, it is necessary to identify these compounds, although there are many factors that affect the ability of microorganisms to produce antibiotics in soils (Raaijmakers, Vlami, & Souza, 2002). The objectives of this study were to identify the bacterium strain H6, a strain representative of the nine most dominant bacterial colony types isolated, and the inhibitory compounds it produced.

Materials and methods

Identification of bacterial strain

The bacterial strain H6 was examined for Gram reaction, spore formation, cellular morphology, pigmentation, motility and the production of catalase (Bergey, Buchanan, & Gibbons, 1974).

Production of antifungal compounds

A loop of H6 cells from a slant culture of fresh nutrient agar was used to inoculate a 300 ml Erlenmeyer flask containing 150 ml LB broth (pH 7.0). The flask was incubated on a rotary shaker at 180 rev min^{-1} for 24 h at 36°C. The fresh culture (3 ml each) was used to inoculate three other fresher flasks containing the same medium. The flasks were incubated under the same conditions as described above for 96 h. During incubation, samples of 2 ml were collected every 4 h from each of the three flasks, and the three samples were pooled. The combined sample was subjected

to the antifungal bioassay described below, pH determination, and cell density measurement. Cell density was measured at an optical density of 580 nm (OD_{580}) using a photometer. The experiment was conducted with three replicates and repeated (trial 2). The data of this experiment and of others presented in this article include the data from repeat(s) of experiments.

The culture filtrate of strain H6 incubated for 36 h under the same conditions as described above was harvested by centrifugation at $7{,}000 \times g$ for 15 min. The supernatant was subjected to the antifungal and conidial germination bioassays described below.

The LB broth containing 10%, 25%, 50% or 100% of the filter-sterilized culture filtrate, was bioassayed for antifungal activity described below. LB broth (pH 7.0) was used as a control. The experiment was carried out with three replicates and repeated three times.

Bioassay for measuring antifungal activity

Three nematophagous fungi, *Paecilomyces lilacinus* (CGMCC No. 0241), *Pochonia chlamydosporia* (CGMCC No. 0418) and *Clonostachys rosea* (CGMCC No. 0807), originally obtained from the China General Microbiological Culture Collection Centre (CGMCC), were grown on potato dextrose agar (PDA) for 7 days at 28°C. Spore suspensions were prepared by dislodging the conidia with sterile water containing 0.1% (v/v) Tween 80. The resulting suspensions were adjusted with sterile water to a concentration of 1.0×10^4 conidia ml^{-1}. An aliquot (0.1 ml) of spore suspension was then mixed with 20 ml molten PDA precooled to 45°C in each Petri plate (9 cm diam). Wells (8 mm diam) were produced using a flame-sterilized cork-borer precooled to room temperature. Samples (each 0.2 ml) were sterilized by filtering through a 0.2 μm pore size sterile membranes and loaded in each well. The diameter of the inhibition zone was measured from the well to the perimeter of the clear inhibition zone of fungi after the plate was incubated at 28°C for 3 days. Every sample had one well in each of three replicate plates for each fungal species, and the results were recorded as the mean of the three plates. For the culture filtrate of bacterial strain H6, the LB broth (pH 7.0) was used as a control.

Conidial germination assay

An aliquot (200 μl) of *P. lilacinus* conidial suspension of 1.0×10^4 conidia ml^{-1} was centrifuged, and the pellet containing the conidia was resuspended in the 200 μl LB broth (pH 7.0) in the presence of 10%, 25% or 50% of the filter-sterilized culture filtrate of bacterial strain H6. As a control, the pellet was resuspended in 200 μl LB broth (pH 7.0). During incubation at 28°C, approximately 100 conidia of *P. lilacinus* were taken out every 4 h and examined at 400× magnification. The percentage of germinated conidia of each suspension was calculated. A conidium was considered germinated if the germ tube was longer than one-half of conidium diameter. The experiment was done with three replicates and repeated three times.

Conidial and hyphal morphologies exposed to the culture filtrate of bacterial strain H6

A Petri plate (5 cm diam) filled with 2 ml filter-sterilized culture filtrate of strain H6 was mixed with PDA (about 10 ml) precooled to 45°C so that concentration of the culture filtrate in the test was regarded as 17% (v/v). The *P. lilacinus* conidial suspension (50 μl, about 1×10^4 conidia ml^{-1}) was spread evenly on the surface of the Petri plate with a sterilized glass scraper. The Petri plate was then incubated at 28°C. For the control plate, the conidia were spread on the PDA. The conidial and hyphal morphologies were observed microscopically at various intervals. The experiment was done three times. Effects of the culture filtrates on conidial and hyphal morphologies of *P. chlamydosporia* and *C. rosea* were also studied.

Thermal and pH stability of culture filtrate

Heat. Culture filtrate aliquots (pH 8.6) were stored at 25°C for 1 month, or treated at 100°C for 10 min, 30 min, 50 min or 70 min, or subjected to autoclaving at 121°C for 20 min. After allowing the heated samples to cool to 25°C, all

culture filtrate aliquots were subjected to the antifungal and conidial germination bioassays described above. Control samples were kept at –4°C in the refrigerator. The experiment was done with three replicates and repeated (trial 2).

pH. Culture filtrate aliquots were adjusted to pH 3, 4, 5, 6, 7, 8, 9, 10 or 11 using 1 N of NaOH or HCl, and were then incubated at 25°C for 24 h. The pH of each culture filtrate aliquot and the control was restored to pH 8.6. Concentration of each culture filtrate was re-standardized. They were then subjected to the antifungal and conidial germination bioassays described above. The experiment was carried out with three replicates and repeated (trial 2).

Purification of inhibitory compounds

A total of 3 l of cultures of strain H6 incubated for 36 h under the conditions described earlier was obtained for further purification. Cells were removed by centrifugation at 7,000 × *g*. The cell-free supernatant was reduced to 300 ml, in vacuo, at 45°C with a rotary evaporator and extracted with *n*-butanol (3 × 300 ml). The pooled *n*-butanol extract was reduced, in vacuo, at 45°C with a rotary evaporator to give a brown/grey tar. The tar of *n*-butanol extract was dissolved in 200 ml water and sequentially extracted with ethyl acetate (EtOAc) (3 × 200 ml). The pooled EtOAc extract was evaporated, in vacuo, at 45°C with a rotary evaporator to give a taupe/brown crystalline solid (2.0 g). The residual fraction of supernatant extracted by *n*-butanol (RSF) and the residual fraction of the *n*-butanol extract extracted by EtOAc (RBF) were also concentrated. Each fraction (*n*-butanol extract: 0.2 g; RSF: 0.2 g; EtOAc extract: 0.02 g; RBF: 0.05 g) was resuspended in a 1 ml LB broth (pH 7.0), and an aliquot (200 µl) of each fraction was then subjected to antifungal and conidial germination bioassays described above. The LB broth (pH 7.0) was used as a control.

An EtOAc extract aliquot (0.05 g) was dissolved in 1.0 ml methanol, fractioned by silica gel TLC plates (20 × 20 cm, 0.2 mm thick, G, Qingdao, China), developed in chloroform–methanol–pyridine (8:2:0.1). Each track (including visualized and non-visualized tracks) detected by I_2 vapour was scraped consecutively from the plates and resuspended in 3 ml methanol. For the control treatment, silica was scraped from the plate developed without the EtOAc extract aliquot. Each fraction was weighted in after methanol was evaporated at room temperature (about 23°C). Each fraction obtained was then re-dissolved in dimethyl sulphoxide (DMSO) (10 µl), resuspended in sterile distilled water (400 µl), and subjected to the antifungal and conidial germination bioassays described above. The DMSO (10 µl) in sterile distilled water (400 µl) was used as a control.

The bioactive fraction dissolved in methanol was applied to silica TLC plates and developed in chloroform–methanol–water (65:25:4) as the solvent system. Spots were detected with ninhydrin for compounds with free amino groups, or water for hydrophilic compounds, or with 4,4′-bis(dimethylamino)diphenylmethane (TDM) reagent (Sigma Chemical Co., St Louis, MO, USA) for peptide bonds (Brückner & Przybylski, 1984).

Mass spectrometry analysis

Mass spectra were obtained for the active fraction with the VG Autospec-3000 spectrometer employing fast-atom-bombardment (FAB) operating in the positive ion mode with 8 kV xenon. Samples were embedded in glycerol matrices on a direct insertion probe. An aqueous mixture of NaCl and KCl was added to the matrices to generate Na^+ and K^+-doped spectra to aid in the identification of the molecular ion peaks.

Results

Identification of strain H6

The microorganism was an aerobic, Gram-positive, endospore-forming, motile, and catalase-positive bacterium. Its cells were rod-shaped. From these characteristics, the microorganism was identified as a strain of *Bacillus* sp. H6 according to the description in (Bergey's et al., 1974) *Manual of Determinative Bacteriology*.

Production

The antifungal substances produced by *Bacillus* sp. strain H6 were detected by bioassay after 16 h incubation (log phase) (Fig. 1). At 36 h (end of the stationary phase), the antifungal activity was the highest. Then, the antifungal activity began to decrease gradually with incubation time. At 96 h, the culture filtrate still showed activity, but it was very weak. The pH of the culture broth changed little over the 96 h period. The LB broth (pH 7.0) containing 25% or more of the filter-sterilized culture filtrate of strain H6 showed antifungal activity whereas no antifungal activity was detected in the presence of 10% of the culture filtrate (Fig. 2).

Inhibition of fungal growth and conidial germination

The supernatant of the *Bacillus* sp. strain H6 inhibited growth of *P. lilacinus* and produced a clear inhibitory zone (Fig. 3). The supernatant also inhibited the growth of *P. chlamydosporia* and *C. rosea*. Analysis of the inhibition zone diameters revealed *P. lilacinus* to be the most sensitive fungus, followed by *C. rosea* and *P. chlamydosporia* (data not shown). The germination efficiency of *P. lilacinus* conidia after 16 h of incubation in the absence of the culture filtrate of strain H6 was nearly 100%. However, in the presence of 10%, 25%, or 50% culture filtrate, the percentage of germination decreased to 15%, 8%, or 0%, respectively (Fig. 4).

Conidial and hyphal morphologies affected by culture filtrate

Our observations showed the interference with the normal growth of mycelia by unusual hyphal swelling, lysis and complete degradation of the hyphal tip (data not shown) that were characteristics of the hyphal response to antifungal metabolites. Furthermore, the culture filtrate of *Bacillus* sp. strain H6 inhibited the germination of *P. lilacinus* conidia strongly and caused morphological changes in its conidia and hyphae (Fig. 5). Conidia incubated on PDA in the absence of the culture filtrate swelled gradually. After 10 h of incubation, the majority of the conidia were 3–5 μm diam, many had germinated (Fig. 5A), hyphae were growing radially (Fig. 5B) after 15 h of incubation, and hyphae became bushy (Fig. 5C) after an incubation of 24 h. *Paecilomyces lilacinus* began to produce conidia sparsely (Fig. 5F) after 3 days of incubation and produced conidia abundantly (Fig. 5J) after an incubation of 7 days. However, the conidia incubated for 24 h on PDA mixed with the culture filtrate were swollen markedly with diameters of 5–8 μm, and only few of them had germinated (Fig. 5D, E). Many of the swollen conidia continued swelling. The largest diameter the conidia reached was 13 μm (the majority of the conidia were 9–12 μm diam) after 3 days of incubation. Few of them germinated (Fig. 5G, H, I). As can be seen from Fig. 5G, the surface of the swollen conidia seemed rough. It was also observed that the germ tubes and hyphae of *P. lilacinus* cultured

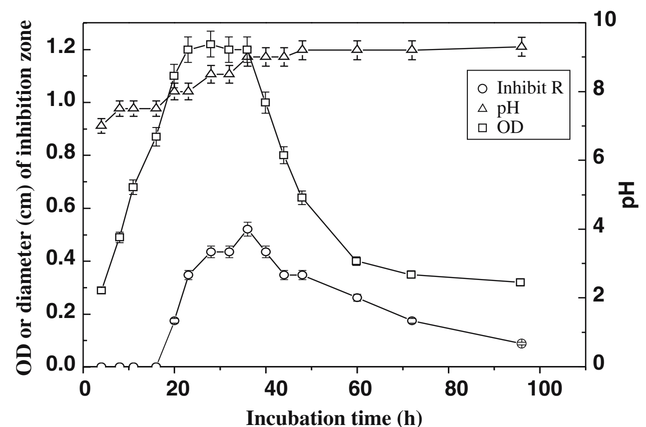

Fig. 1 The relations between the cell density of *Bacillus* sp. strain H6, the antifungal activity against *P. lilacinus*, and the pH of the culture over incubation time in Luria broth. OD is the optical density at 580 nm. Curves show mean values with standard error bars

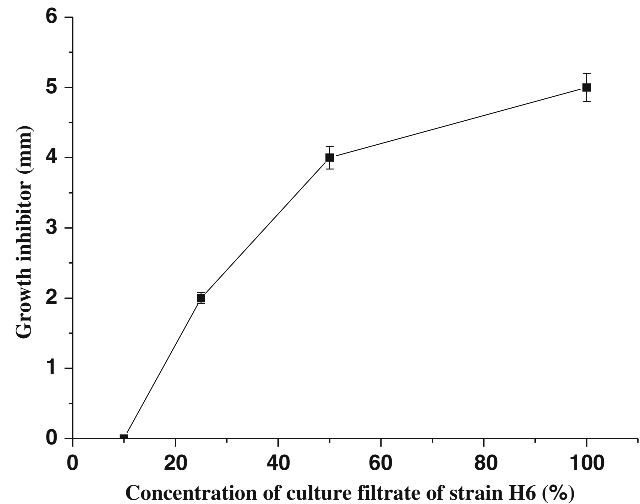

Fig. 2 Antifungal activity of the LB in the presence of 10%, 25%, 50% and 100% culture filtrate of *Bacillus* sp. strain H6 against *P. lilacinus*. Curves show mean values with standard error bars

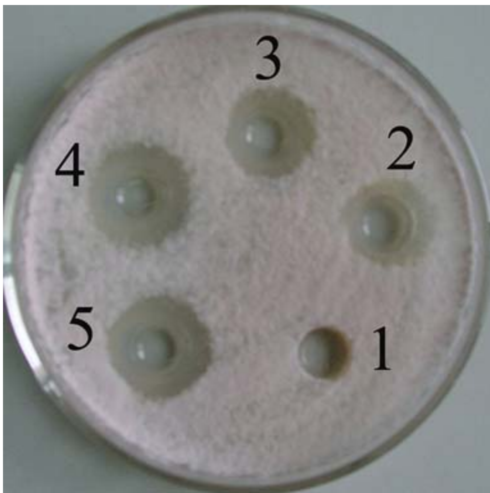

Fig. 3 Antifungal activities of the supernatant of *Bacillus* sp. strain H6: spot 1: control; spots 2 (50%), 3 (50%), 4 (100%), 5 (100%): supernatant of *Bacillus* sp. strain H6

on PDA with the culture filtrate were abnormally swollen, whereas they did not show any obvious growth aberration when cultured on PDA in the absence of the culture filtrate (Fig. 5B, C and Fig. 5D, I, L). Furthermore, the breakdown of some conidia incubated on PDA with the culture filtrate was observed after 7 days (Fig. 5K). The culture filtrate inhibited the conidial germination of *P. chlamydosporia* and *C. rosea* in the same way (low germination, conidial swelling) as it affected the conidia of *P. lilacinus* (data not shown).

Thermal and pH stability of antifungal activity in culture filtrate

The antifungal activity and inhibition of spore germination did not decrease while the culture filtrate was stored for 1 month at room temperature. The fungistatic activity was thermostable. Germination percentages and growth inhibitors did not differ significantly between the culture filtrates treated at 100°C for 10 or 30 min and the control stored at –4°C (Fig. 6). But there were significant differences between the culture filtrates treated at 100°C for 50 min or 70 min and the control stored at –4°C ($P < 0.05$). Autoclaving at 121°C for 20 min reduced activity by 50%. The antifungal activity and inhibition of spore germination in the culture filtrate were also examined after the culture filtrates were adjusted to pH values ranging from 3 to 11. Germination percentages ($F = 1.774$) and growth inhibitors ($F = 2.334$) did not differ significantly between the culture filtrates adjusted to pH values ranging from 3 to 11 and the control stored at –4°C (data not shown). So antifungal activity and inhibition of spore germination were not affected by incubation at this pH range for 24 h at room temperature.

Purification

Paecilomyces lilacinus was used as the model fungus during purification because of its

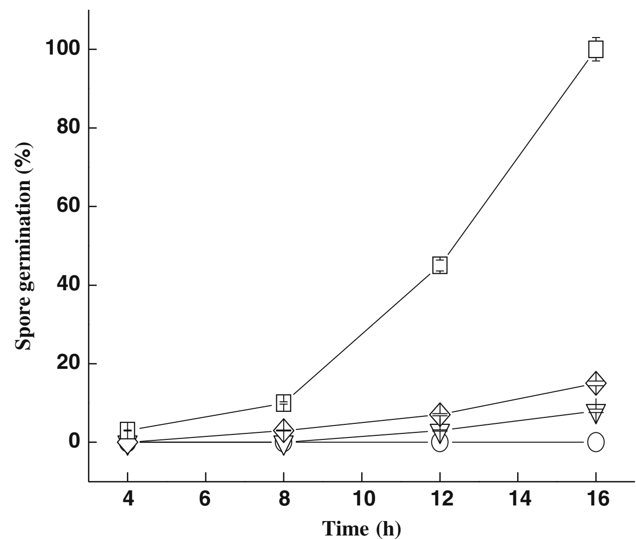

Fig. 4 Germination of *P. lilacinus* in LB (□) and LB containing 10% (◇), 25% (▽), and 50% (○) of culture filtrate of *Bacillus* sp. strain H6. Curves show mean values with standard error bars

sensitivity to the inhibitory compounds. The *n*-butanol extract showed antifungal activity and inhibited germination of *P. lilacinus* conidia, but the concentrated RSF had no activity (Fig. 7A). Although both the EtOAc extract and the RBF had antifungal activity (Fig. 7B), only the EtOAc extract strongly inhibited germination of *P. lilacinus* conidia. Thirteen fractions (including the origin) were detected by I_2 vapour in EtOAc extract that was developed in chloroform–methanol–pyridine (8:2:0.1) on silica TLC plates. These fractions were scraped, and subjected to antifungal and conidial germination bioassays. Only the 11th (R_f : 0.25 g, 0.0021 g) fraction had antifungal activity (Fig. 7C) and inhibited germination of *P. lilacinus* conidia (data not shown). One spot was detected by I_2 vapour in the 11th fraction when re-fractioned on silica and developed in chloroform–methanol–water (65:25:4). The R_f value was 0.45. It was ninhydrin negative, but positive to TDM reagent. A white spot formed when the plate was sprayed with water.

Mass spectrometry analysis

The positive FAB mass spectra of the active 11th fraction showed homologous ion peaks differing by 14 mass units at *m/z* 1,043, 1,057 and 1,071 (Fig. 8A). The difference of 22 mass units in the ion peaks at *m/z* 1,065 and 1,043, and peaks at 1,057 and 1,079, indicated the presence of a sodium ion, presumably as NaCl. In order to identify the molecular ion peaks, an aqueous mixture of NaCl and KCl was added to matrices and the result is shown in Fig. 8B. The FAB mass spectra of the active fraction showed the homologous ion peaks at *m/z* 1,043, 1,057 and 1,071, while other adjacent peaks were $[M + Na]^+$ (1,065, 1,079, 1,093) and $[M + K]^+$ (1,081, 1,095, 1,109), respectively (Fig. 8B). The data indicated that the homologous ion peaks at *m/z* 1,043, 1,057 and 1,071 were protonated molecular ions $[M + H]^+$. These results indicated that there were at least three compounds in the active fraction and that their molecular weights were 1,042, 1,056 and 1,070, respectively.

Discussion

A wide range of soil microorganisms can produce antifungal compounds (Behal, 2000). Several apparent or plausible mechanisms for biocontrol organisms or antifungal compounds involved in the antagonism of pathogenic fungi have been proposed. These mechanisms include interference with conidial germination and germ tube elongation (Silo-Suh et al., 1994; Thrane, Tronsmo, & Jensen, 1997); inhibition as a resulting unusual hyphal swelling (Lim, Kim, & Kim, 1991); lysis and complete degradation of the hyphal tip

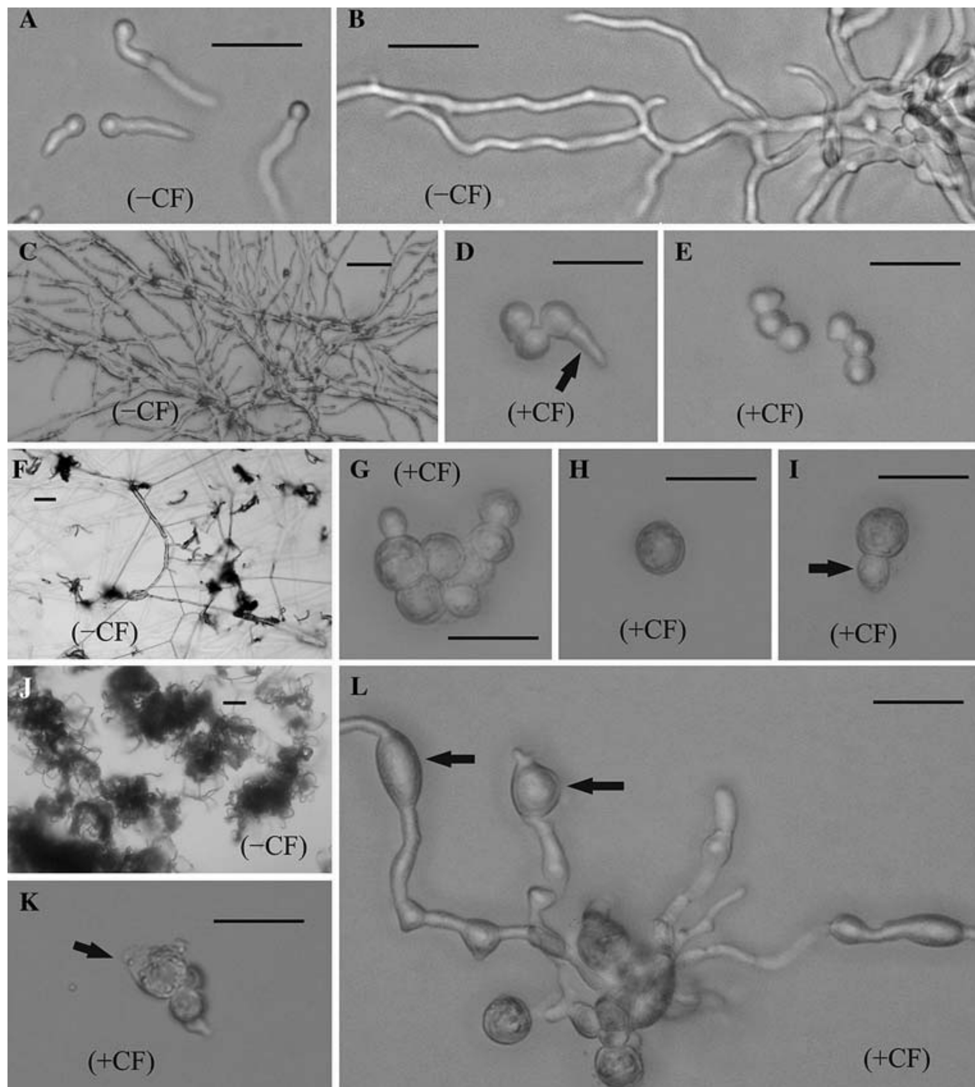

Fig. 5 Effect of the culture filtrate of *Bacillus* sp. strain H6 on morphology of *P. lilacinus* (**A**) Conidia of *P. lilacinus* germinated after incubation for 10 h on PDA in the absence of the culture filtrate of strain H6 (-CF); (**B**) Normal mycelia of *P. lilacinus* cultured on PDA (-CF) for 15 h; (**C**) Bushy hyphae of *P. lilacinus* cultured on PDA (-CF) for 24 h; (**D**, **E**) Swollen conidia and germ tube (arrow) of *P. lilacinus* incubated for 24 h on the PDA mixed with the culture filtrate of strain H6 (+CF); (**F**) *P. lilacinus* cultured on PDA (-CF) for 3 days producing sparse conidia; (**G**, **H**, **I**) Swollen conidia and germ tube (arrow) of *P. lilacinus* incubated for 3 days on the PDA (+CF); (**J**) *P. lilacinus* cultured on PDA (-CF) for 7 days producing abundant conidia; (**K**) Breakdown of spore (arrow) of *P. lilacinus* incubated for 7 days on the PDA (+CF); (**L**) Swollen hyphae (arrows) of *P. lilacinus* incubated for 7 days on the PDA (+CF). The scale bars are 20 μm

(Lim et al., 1991); and suppression by the competition for nutrients (Liesbet, Sofie, & Monica, 2003). Our study revealed that the culture filtrate of *Bacillus* sp. strain H6 appeared to inhibit the growth of *P. lilacinus* predominantly through inducing unusual swelling in the conidia and the germ tubes. This inhibitory mechanism is different from the above-reported mechanisms. Apparently in the culture filtrate of *Bacillus* sp. strain H6 there are active compounds that have a negative effect on fungal conidial survival and germination.

Most previous studies on microbial antifungal activity have concentrated on volatile fungistatic

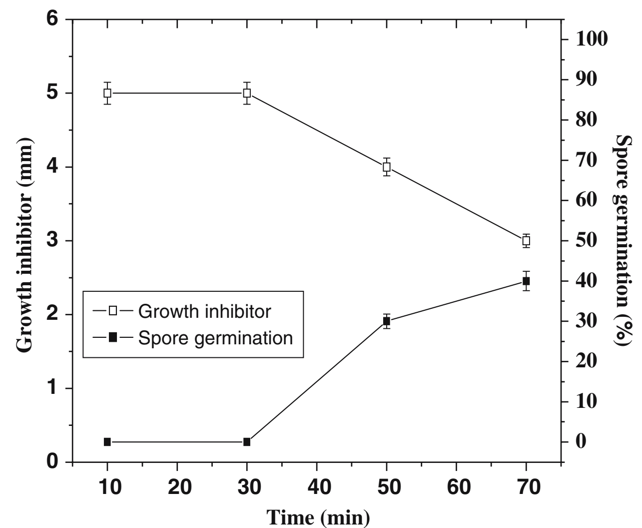

Fig. 6 Inhibition of spore germination and colony growth of the culture filtrate of *Bacillus* sp. strain H6 against *P. lilacinus* after the culture filtrate was treated at 100°C for various times. Curves show mean values with standard error bars

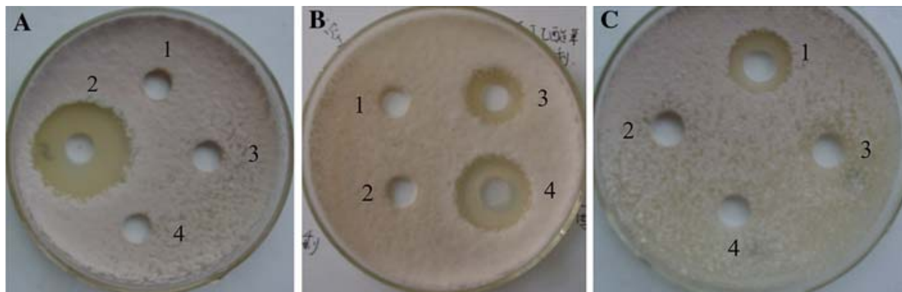

Fig. 7 Antifungal activities of the supernatant and the partial fractions of the supernatant of *Bacillus* sp. strain H6 on the growth of *P. lilacinus*: (**A**) Inhibition zone of the *n*-butanol extract and the residual fraction of supernatant that was extracted by *n*-butanol (RSF): spot 1, 4: control; spot 2: *n*-butanol extract; spot 3: RSF; (**B**) Inhibition zones of the residual fraction of the *n*-butanol extract that was extracted by EtOAc (RBF) and EtOAc extract: spot 1, 2: control; spot 3: RBF; spot 4: EtOAc extract; (**C**) Inhibition zones of different fractions that were scraped from silica TLC plates in the EtOAc extract developed in chloroform–methanol–pyridine (8:2:0.1) with TLC: spot 1: 11thF; spot 2: control; spot 3: 12thF; spot 4: 13thF

compounds with a low molecular weight. These known volatile compounds in soils include ethylene, ammonia and methyl vinyl ketone (Herington, Craig, & Sheridan, 1987). However, the results of Liebman and Epstein (1992) indicated that water-soluble and possibly nonvolatile fungistatic compounds occurred in a variety of soils. In our study the inhibitory substances in the culture filtrate of *Bacillus* sp. strain H6 were not volatile and they were partly water-soluble (data not shown). Furthermore the inhibitory substances produced were heat stable and active over a wide range of pH.

Bacillus species are known to naturally produce small molecular antibacterial and antifungal compounds, including peptides, lipopeptides, aminoglycosides, kanosamine, zwittermicin, etc (Silo-Suh et al., 1994). In this study the bioactive 11th fraction isolated from EBE fraction was ninhydrin negative, thus ruling out two fungistatic compounds, kanosamine and zwittermicin, produced by *B. cereus* (Silo-Suh et al., 1994). The 11th active fraction was also positive to TDM reagent, which indicated the presence of peptide bonds in the compounds. A white spot formed when the plate was sprayed with water, indicating that the compound was lipophilic (Brückner & Przybylski, 1984). Thus, it could be presumed that the active compounds were either peptides or lipopeptides. A study (Chitarra et al., 2003)

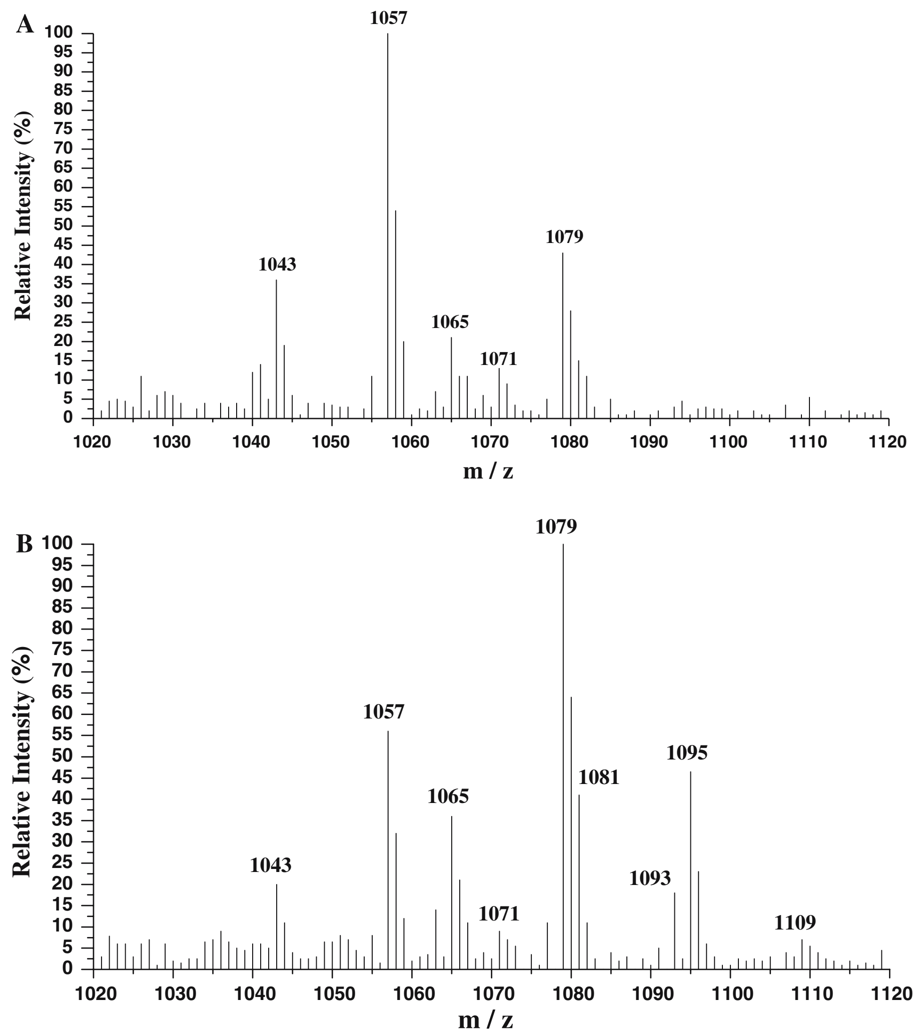

Fig. 8 Positive FAB mass spectra of (**A**) active 11th fraction from *Bacillus* sp. strain H6 and (**B**) active 11th fraction with added aqueous mixture of NaCl and KCl

showed that iturin A-like compounds, related to lipopeptides, produced by *B. subtilis* YM10-20 might permeabilize *Penicillium roqueforti* conidia to inhibit their germination. Iturins are a group of antifungal, cyclic lipopeptides, produced by *B. subtilis* and *B. amyloliquefaciens* RC-2, including iturin A-E, bacillomycin D, F and L, and mycosubtilin (Hiradate, Yoshida, Sugie, Yada, & Fujii, 2002). In nature, iturin A is produced as a mixture of up to eight isomers, named iturin A_1–A_8 (Hiradate et al., 2002). Asaka and Shoda (1996) confirmed that *B. subtilis* RB14 could produce iturin A in a sterilized vermiculite-soil when they studied the bacterium's ability to suppress damping-off of tomato seedlings caused by *Rhizoctonia solani*. Mass spectrometric analysis was used to rapidly identify iturins from a variety of mixtures (Hiradate et al., 2002). In our study the measured molecular weights of the active compounds obtained by a positive FAB mass spectrometer were consistent with iturin A_2 (1,042), A_3 (1.056), A_4 (1,056), A_5 (1,056), or A_6 (1,070), suggesting that the inhibitory compounds produced by *Bacillus* sp. strain H6 showed similarity to the iturin A group.

Although, as shown by fluorescence staining and SEM analysis, iturin A-like compounds were found to permeabilize the conidiospores of

P. roqueforti, and no germination was observed (Chitarra et al., 2003), it is not known if the conidia treated by iturin A-like compounds were markedly swollen during subsequent incubation. The action mechanism of iturin A is that penetration into the lipid bilayer of the cytoplasmic membrane by the hydrophobic tail is followed by auto-aggregation to form a pore that causes cellular leakage (Maget-Dana, Ptak, Peypoux, & Michel, 1985). Although the real mechanism of conidial swelling induced by iturin A-like compounds needs further investigation, it is possible that iturin A damages the cytoplasmic membrane of conidia, causing water to enter into the conidia, resulting in abnormal swelling.

Antibiotic production by bacteria including iturin A in soil is modulated by a wide range of endogenous and exogenous factors (Asaka & Shoda, 1996; Chin-A-Woeng, Bloemberg, & Lugtenberf, 2003; Raaijmakers et al., 2002). Indeed, the population density of bacteria is in itself an important factor that influences antibiotic production (Chin-A-Woeng et al., 2003; Haas, Keel, & Reimmann, 2002). Although *Bacillus* sp. strain H6 was one of the dominant bacteria in soils fungistatic to nematophagous fungi and its population was recorded up to 4.2×10^6 cells g^{-1} soil in our previous study (Xu, 2004), it was difficult to estimate the threshold population density necessary to show significant fungistasis of nematophagous fungi. Furthermore, even if the cells were producing iturin A in situ, they might not have been present at the sites of nematophagous fungi, and loss of iturins could have occurred due to quick diffusion and dilution into the nutrient solution, or degradation. So it is difficult to answer the question as to whether the *Bacillus* sp. strain H6 can produce the amount of iturin A sufficient to inhibit the germination or growth of nematophagous fungi in situ. However, the production of antifungal compounds has been described for a wide range of soil microorganisms including bacteria (Behal, 2000), and many bacteria have been demonstrated to produce antibiotics in the spermosphere and rhizosphere (Raaijmakers et al., 2002). Many lipopeptides produced by soil microorganisms have the same antifungal mechanism as iturin A, e.g., syringopeptin and syringomycin produced by *Pseudomonas syringae* pv. *syringae* (Serra et al., 1999), viscosinamide produced by *Pseudomonas fluorescens* DR54 (Thrane, Olsson, Nielsen, & Sorensen, 1999), and the sufactin and iturinic group (Maget-Dana et al., 1985; Maget-Dana & Ptak, 1995). According to the study of Wietse et al. (2003), bacterial community composition other than a single bacterium is an important factor determining soil fungistasis. Thus, it is reasonable that these kinds of compounds or other unknown compounds that have the same action mechanism as iturin A may be fungistatic compounds present in soil. But most of these antifungal compounds have been tested only against mycelial growth and very little information is available about their effect on fungal spore survival and germination. In order to understand the relationship between fungistatic soils and these kinds of compounds, and factors that can influence production of these compounds, further studies, including identification of the inhibitory compounds produced by *Bacillus* sp. strain H6, its action mechanism and quantification of it in fungistatic soils, are necessary and are now under investigation.

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