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Isolation and Identification of Antifungal Compounds Produced by *Bacillus* Y-IVI for Suppressing Fusarium Wilt of Muskmelon

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Abstract

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The *Bacillus* strain Y-IVI was used in the biocontrol of muskmelon Fusarium wilt. It was identified as *Bacillus subtilis*. The antifungal compounds from the culture filtrate were purified by high performance liquid chromatography. Two series of homologous ion peaks were analysed by liquid chromatography-electrospray ionisation-mass spectrometry, one with molecular weights of 1028.7, 1042.7, and 1056.7 and the other with molecular weights of 1463, 1477, and 1491. The compounds were ascribed to iturin A and fengycin, respectively. The maximum production of iturin by Y-IVI was 89.75 mg/l. In conclusion, we provided biochemical evidence that strain Y-IVI was able to produce antifungal compounds and thus holds great potential for use in the biocontrol of Fusarium wilt disease.

Keywords: antifungal substance; *Bacillus subtilis*; biological control; *Cucumis melo* L.

Fusarium wilt caused by isolates of *Fusarium oxysporum* is responsible for extensive economic losses in many crops, such as muskmelon, watermelon (De Cal *et al*. 2009), cucumber (Wu *et al*. 2009), pepper (Chung *et al*. 2008), chickpea, and cumin (Tawfic & Allam 2004). Controlling plant diseases through the overuse of chemical pesticides or fungicides is of concern to the environment and human health. Thus, alternate solutions to replace chemicals in crop disease management are encouraged. Biological control through the application of antagonistic microbes of plant pathogens is one of the most promising options (Zhao *et al*. 2011).

Many antagonistic microbes that produce inhibitory substances have been recently reported (Yuan *et al*. 2012; Song *et al*. 2013; Rautela *et al*. 2014). For example, at least two active compounds produced by *Penicillium oxalicum* PY-1 suppressed the growth of the phytopathogen *Sclerotinia sclerotiorum* (Yang *et al*. 2008); calbistrin A and calbistrin B produced by *Penicillium striatisporum* Pst10 showed strong activity against *Phytophthora capsici* (Ma *et al*. 2008); peptide and glycosidic compounds excreted by *Paenibacillus lentimorbus* WJ5 can inhibit the growth of several fungal phytopathogens (Lee *et al*. 2008).

The production of antibiotics by many *Bacillus* species is widely emphasised to explore their potential in biological control of plant diseases (Chung *et al*. 2008). A broad spectrum of active compounds such as active lipopeptides (iturins, surfactins, fengycins) are produced by *Bacillus* species (Ongena & Jacques 2008; Ma & Hu 2014). These lipopeptides are synthesised ribosomally in the early growth cycle or non-ribosomally after growth has ceased (Mizumoto & Shoda 2007). The iturin groups include iturin A, C, D, and E, bacillomycin D, F, and L, bacillopeptin,

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and mycosubtilin (Peypoux *et al*. 1986; Besson & Michel 1987; Kajimura *et al*. 1995). They exhibit strong antifungal activity against many yeast and fungal species (Li *et al*. 2014). Fengycins show strong fungitoxic activity, particularly against filamentous fungi (Vater *et al*. 2002; Williams *et al*. 2002). Additionally, bacterial lipopeptides are important industrial materials and attract biotechnological and pharmaceutical interests (Bie *et al*. 2009).

The *Bacillus* spp. strain Y-IVI has shown strong antagonistic activity against *Fusarium oxysporum* in *in vitro* antagonistic activity tests, and it was used to effectively control muskmelon Fusarium wilt under greenhouse and field conditions (Zhao *et al*. 2011, 2013). We hypothesised that the antagonistic substances produced by strain Y-IVI might be linked to the biocontrol mechanism. The objectives of this research were to characterise, optimise, identify, and quantify the antifungal substances produced by strain Y-IVI to improve our understanding of its biocontrol mechanism.

MATERIAL AND METHODS

Bacterium and phytopathogens. The bacterium strain Y-IVI was originally isolated from healthy muskmelon rhizosphere soil (Zhao *et al*. 2011). Phytopathogens of *Fusarium oxysporum* f.sp. *melonis* (FOM), *Fusarium oxysporum* f.sp. *cubense*, *Fusarium oxysporum* f.sp. *cucumerinum*, *Fusarium oxysporum* f.sp. *vasinfectum*, *Fusarium oxysporum* f.sp. *niveum*, *Phytophthora parasitica* var. *nicotianae*, *Phytophthora capsici*, *Verticillium dahilae*, and *Rhizoctonia solani* AG4, which are used for broad-spectrum antagonistic activity analysis, were obtained from the Jiangsu Provincial Key Lab of Organic Solid Waste Utilization at Nanjing Agricultural University.

Identification of strain Y-IVI. The bacterium Y-IVI was identified by partial sequencing of the 16S rDNA gene. Bacterial genomic DNA was isolated using standard procedures (Sambrook & Russell 2001). The DNA fragment was amplified by PCR using universal 16S eubacterial primers 27F and 1492R (Dubey *et al*. 2003). PCR was carried out under the following conditions: initial denaturation at 94°C for 2 min, then 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min, and finally an extension at 72°C for 10 minutes. The PCR product was purified by PCR Purification Kit (Axygen Biosciences, Union City, USA) using procedures recommended by the manufacturer. The purified PCR product was cloned into PMD18-T easy vector and ligated into *Escherichia coli* DH5α cells. The cloned 16S rDNA gene was sequenced by Invitrogen Company (Shanghai, China). The sequence homology was analysed using the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree was constructed using Neighbour-Joining and the Clustal-X program.

Antifungal compounds production. The strain Y-IVI was cultured in an optimized medium (Jourdan *et al*. 2009) at 30°C with constant shaking at 170 rpm for 8 hours. The seeding culture was transferred into optimised medium at a ratio of 5% (v/v) and separately incubated at 20, 25, 28, 30, 35, and 40°C at 170 rpm for 28 h to choose the most suitable temperature for antagonistic substance production. The effect of pH on antagonistic substance production was measured from pH 4 to pH 10. Furthermore, the flasks were incubated on a rotary shaker under a suitable antifungal compound production pH and temperature for 48 hours. Samples were collected every 3 h for cell density measurement and antifungal activity analysis. Samples for antifungal activity analysis were centrifuged at 10 000 *g* at 4°C for 15 min, passed through a 0.22-μm membrane to obtain cell free filtrate, and finally stored at 4°C before use.

Preparation and characterisation of Y-IVI culture filtrate. The Y-IVI strain was incubated on a rotary shaker under the optimum antifungal compounds production conditions (pH 7 for 30 h at 30°C). The cell-free filtrate was obtained as described above.

Proteinase K (39.0 U/mg at 37°C; Sigma-Aldrich, Shanghai, China) and pepsin (3000–3500 U/mg; Solarbio, Shangai, China) were separately dissolved in 25mM phosphate buffer (pH 7.0) with a concentration of 1 mg/ml. The cell-free filtrate and enzymes were mixed at a 1 : 1 ratio (v/v) and incubated at 37°C for 1 h, before residual antimicrobial activity measurements were taken using agar diffusion assays against FOM.

The cell-free filtrate was diluted to 5, 10, 25, 50, and 100% by optimised medium or concentrated to 500% by freeze-drying. The medium was used as a control. The cell-free filtrates in different concentrations were used for *in vitro* antagonistic activity analysis. In addition, the heat and pH stability of cell-free filtrates were tested. The filtrate was exposed to heat treatments at 4, 20, 40, 60, 80, and 100°C for 2 h or autoclaved at 121°C for 20 min for heat stability analysis. The pH of filtrate samples was adjusted from 2 to 12 with 1 mol/l NaOH or

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1 mol/l HCl, and the samples were stored at 4°C for 24 h and then readjusted to pH 7. These treated samples were stored at 4°C for antifungal activity analysis using the method described below.

In vitro antifungal activity analysis. Agar diffusion assays were performed following the method of Li *et al.* (2007), with modifications. FOM phytopathogens were activated, and the fresh mycelia were incubated in PDA Petri plates for 5 days. Sterile distilled water was poured into the Petri plates to collect FOM spores. The spore suspension was filtered through three layers of sterile gauze, and the concentration was counted with a hemacytometer (Shanghai nuocai Co. Ltd., Shanghai, China). The concentration of FOM spores was adjusted to 10^4 colony forming units (CFU) per ml and stored at 4°C until use.

An aliquot (100 µl) of FOM spore suspension was spread evenly onto the surface of PDA Petri plate (90 mm in diameter). Wells (8 mm in diameter) were produced using a sterilised pontil-borer. The culture broth samples (50 µl) were added to each well. The plate was incubated at 28°C, and the diameter of the inhibition zone was measured after 5 days. The medium was used as control. The experiments were repeated thrice.

Extraction of antifungal compounds and measurement of inhibitory concentration to phytopathogens. The cell-free supernatant was obtained as described above. Antifungal substances were extracted using the method of Hsieh *et al.* (2008) with minor modifications. Active substances were extracted twice with an equal volume of *n*-butyl alcohol. The extract was concentrated and the residues were dissolved in 50 ml distilled water. The pH of the sample was adjusted to 2 with concentrated HCl. The acidic mixture was stored at 4°C for 7 h to precipitate completely and then centrifuged at 12 000 *g* at 4°C for 10 minutes. The precipitates were added to HPLC-grade methanol, and the pH was adjusted to 7. The samples were vortexed thoroughly and stored at 4°C for 5 h and then centrifuged as mentioned above. The supernatant was collected and recognised as crude antifungal compounds. The crude antifungal compounds were filtrated through a 0.22-µm membrane and stored at –20°C for further use.

The antagonistic activity analysis of crude antifungal compounds against phytopathogens was performed. Four equidistant wells (8 mm in diameter) were made in each PDA Petri plate. A fresh phytopathogen mycelium (8 mm in diameter) was loaded onto the centre of the PDA Petri plate, and 50 µl samples were then injected into each well (*Verticillium dahilae* Petri plates were incubated for 7 days before the injection of crude antifungal compounds). HPLC-grade methanol was used as control. The Petri plates were incubated at 28°C for 4 days to observe the crude antifungal compounds inhibitory ability. This experiment was repeated thrice.

Purification of antifungal compounds. To purify the antifungal compounds, HPLC (Agilent 1200 series) was used. A reverse-phase HPLC column (ZORBAX, SB-C18, 9.4 × 150 mm, 5-µm particle; Agilent, Santa Clara, USA) was used to separate and collect the active peaks. A linear gradient solvent A of 0.05% formic acid and solvent B of 100% acetonitrile over 70 min was used to separate the antifungal substance. The column flow rate was 0.8 ml/min, and the peaks were monitored at 254 nm. The peaks were collected in clean glass tubes based on the different retention time intervals. Seven samples were collected, freeze-dried, and then dissolved in HPLC-grade methanol for *in vitro* antagonistic tests. The eluted solutions of acetonitrile and 0.05% formic acid mixed at a volume ratio of 1 : 1 were also freeze-dried for their use as controls. Samples showing antagonistic activity were further separated, collected, and tested with the methods described above.

Determination of the antagonistic compounds by LC-ESI-MS. Liquid chromatography-electrospray ionisation-mass spectrometry (LC–ESI-MS) (Agilent 1100 series, LC/MSD; Agilent, Santa Clara, USA) analysis was carried out to determine the molecular weight of the antagonistic compounds. The ion polarity mode was positive, and the electrospray ionisation dry temperature was 350°C.

Data analysis. The data were statistically analysed using the statistical program SPSS for MS Windows, Version 19 (SPSS, Inc., Chicago, USA). Data were subjected to Duncan's analysis of variance (ANOVA), and the means were compared by Duncan's tests at $P \leq 0.05$.

RESULTS

Identification of antagonistic strain Y-IVI. The 16S rDNA gene fragment of strain Y-IVI (1430 bp) showed 100% homology with *Bacillus subtilis* (GenBank accession numbers BG-B28 and xm-1). Consequently, the strain Y-IVI was identified as *Bacillus subtilis* (Figure 1).

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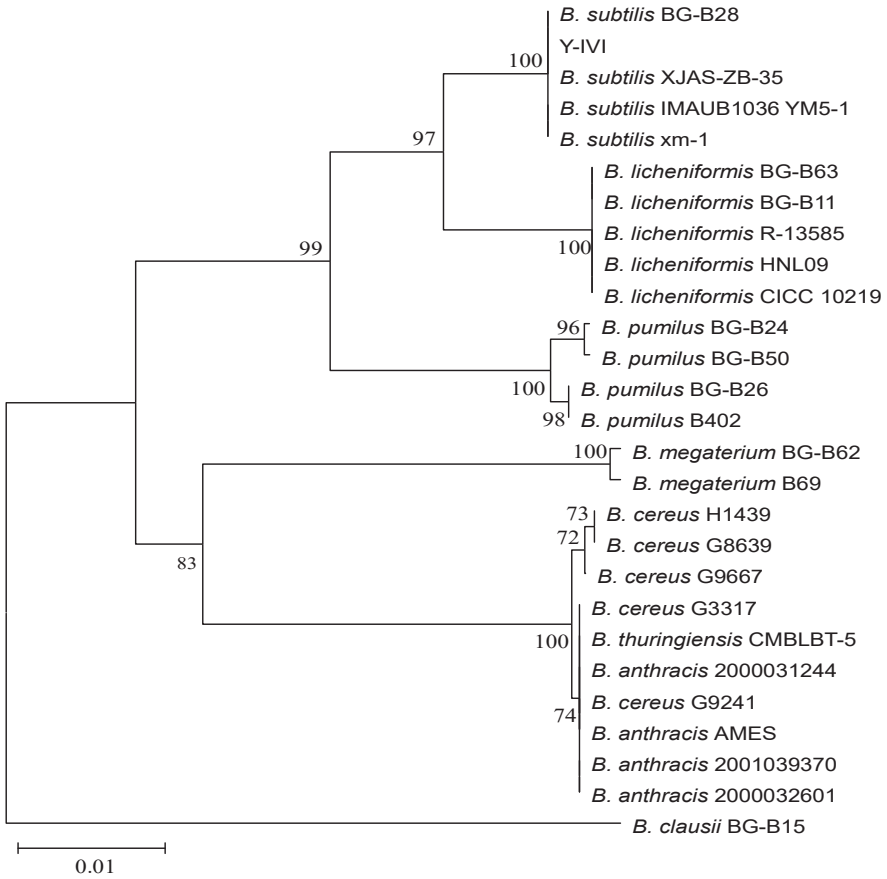

Figure 1. Phylogenetic tree of strain Y-IVI, constructed using Neighbour-Joining and the Clustal-X program

The scale bar of 0.01 stands for nucleotide substitution per sequence position

Production of antifungal compounds. Figure 2A shows that the maximum amount of antifungal compounds was produced at pH 7. Incubation in acidic or alkaline culture mediums had negative effects on strain Y-IVI antifungal compounds production. Figure 2B shows that the most suitable temperature range for antifungal compounds production was 28–32°C. The culture filtrate began to show antifungal activity

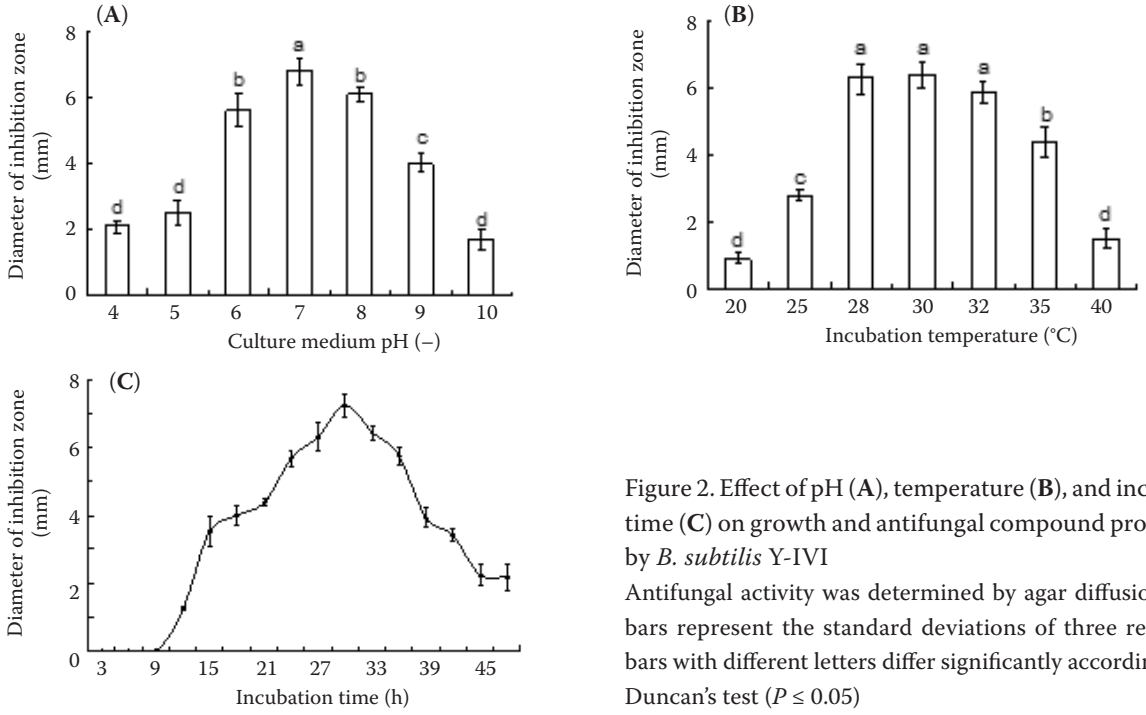

Figure 2. Effect of pH (**A**), temperature (**B**), and incubation time (**C**) on growth and antifungal compound production by *B. subtilis* Y-IVI

Antifungal activity was determined by agar diffusion assay; bars represent the standard deviations of three replicates; bars with different letters differ significantly according to the Duncan's test ($P \leq 0.05$)

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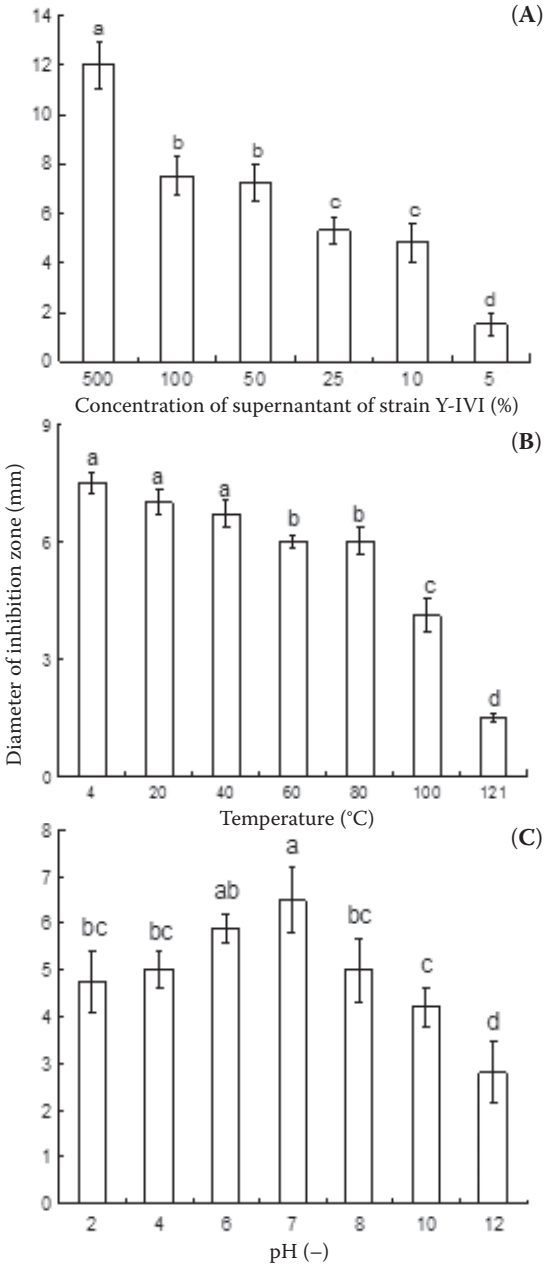

Figure 3. Antagonistic activities of *B. subtilis* Y-IVI culture filtrates against *Fusarium oxysporum* f.sp. *melonis* after being subjected to different concentrations (**A**), temperatures (**B**), and pH (**C**) treatments

Vertical boxes ± bars are the mean ± standard deviation of three replicates; bars with different letters differ significantly according to the Duncan's test ($P \leq 0.05$)

after incubation for 12 h; the maximum antifungal activity was obtained at 30 h, and then growth and antifungal activity decreased gradually. The culture filtrates still showed antifungal activity at 48 h (Figure 2C).

Characterization of antifungal compounds. The culture filtrate of strain Y-IVI suppressed FOM growth and produced a clear inhibition zone. Figure 3A shows that the extent of inhibition decreased with dilution of the culture filtrate. For example, the diameter inhibition zones were 12, 7.5, and 5.3 mm when the culture filtrate concentrations were 500,

Table 1. Broad-spectrum antagonistic activities of antifungal compounds extracted from culture filtrates inoculated with *B. subtilis* Y-IVI in optimised medium

Phytopathogen	Diameter of inhibition zone (mm)
Fusarium oxysporum f.sp. *melonis*	8.2 ± 0.8
Fusarium oxysporum f.sp. *niveum*	7.7 ± 1.5
Fusarium oxysporum f.sp. *vasinfectum*	7.9 ± 1.2
Fusarium oxysporum f.sp. *cucumerinum*	5.4 ± 0.6
Fusarium oxysporum f.sp. *cubense*	5.6 ± 2.1
Rhizoctonia solani AG4	4.3 ± 0.6
Verticillium dahilae	3.1 ± 1.2
Phytophthora capsici	none
Phytophthora parasitica var nicotianae	none

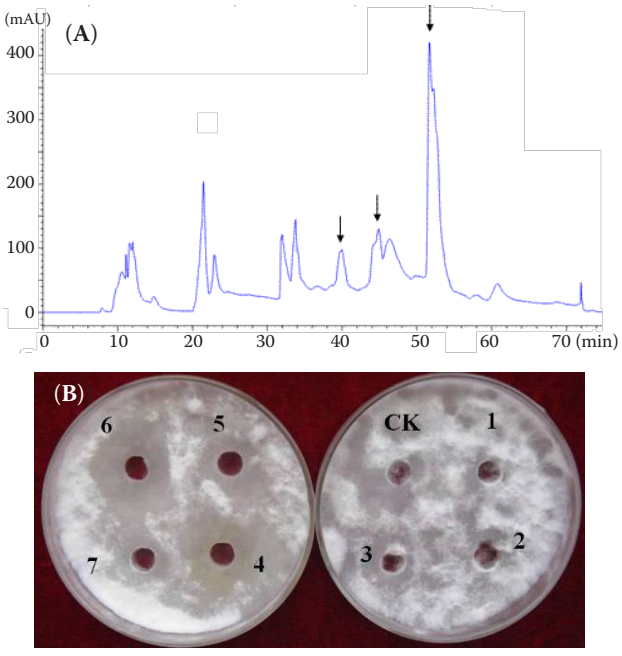

Figure 4. Antifungal compounds produced by *B. subtilis* Y-IVI against *Fusarium oxysporum* f.sp. *melonis* were separated and purified by HPLC

Peaks with antifungal activity were marked by arrows; Petri plate spots 1, 2, 3, 4, 5, 6, and 7 were compounds from peaks collected from 7 to 15, 15 to 25, 25 to 30, 30 to 41, 41 to 51, 51 to 59, and 59 to 68 min, respectively

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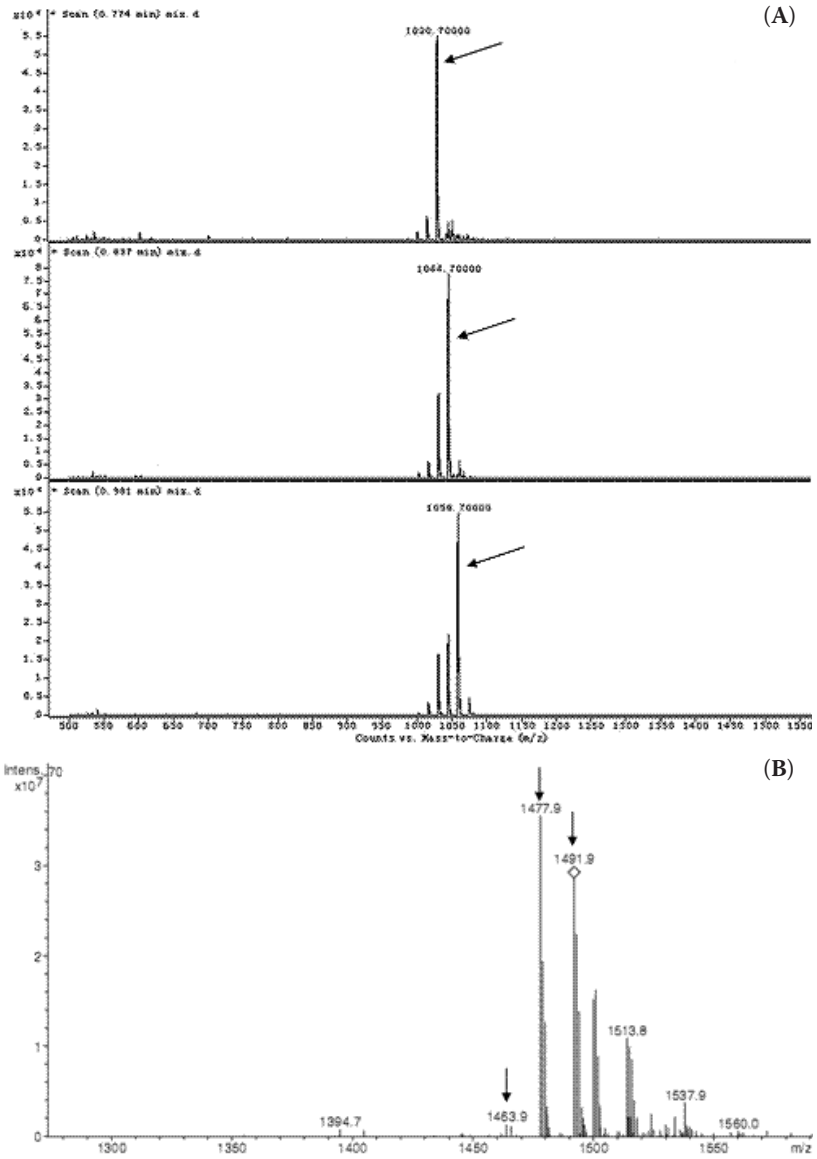

Figure 5. The mass spectrum of the purified antifungal compounds produced by *B. subtilis* Y-IVI. Mass spectra of peaks with antagonistic activity against *Fusarium oxysporum* f.sp. *melonis* were marked by arrows

100, and 25%, respectively. However, the diameter was only 1.5 mm in the presence of 5% of the culture filtrate. No inhibition zone was detected in the presence of optimised medium (control not shown). In addition, no loss of activity was observed after exposure to Proteinase K and pepsin treatment at 37°C for 1 hour.

The active metabolites in the culture filtrate were relatively thermostable. The diameter of the inhibition zone of the Y-IVI culture filtrate treated at 100°C for 2 h was significantly decreased compared with that treated at 4°C for 2 h (Figure 3B). The filtrate still showed inhibitory activity after being autoclaved at 121°C for 20 min, but the diameter of this inhibition zone was by 80% smaller than that treated at 4°C for 2 hours. The antagonistic activity of the culture filtrates treated at pH 12 for 2 h was significantly lower than that of other treatments. The culture filtrate treated at pH 7 for 2 h produced the greatest inhibition zone (Figure 3C).

Broad-spectrum antagonistic activity of antifungal compounds extracted from culture filtrate. The active compounds extracted from the culture filtrate of strain Y-IVI showed antifungal activity against several phytopathogens (Table 1). The substances produced by strain Y-IVI strongly inhibited the mycelia growth of FOM and other species in the genus of *Fusarium* on PDA plates. The diameters of the inhibition zones were 8.2, 7.9, and 5.4 mm in the case of FOM, *Fusarium oxysporum* f.sp. *vasinfectum,* and *Fusarium oxysporum* f.sp. *cucumerinum*, respectively. In addition, the substances produced

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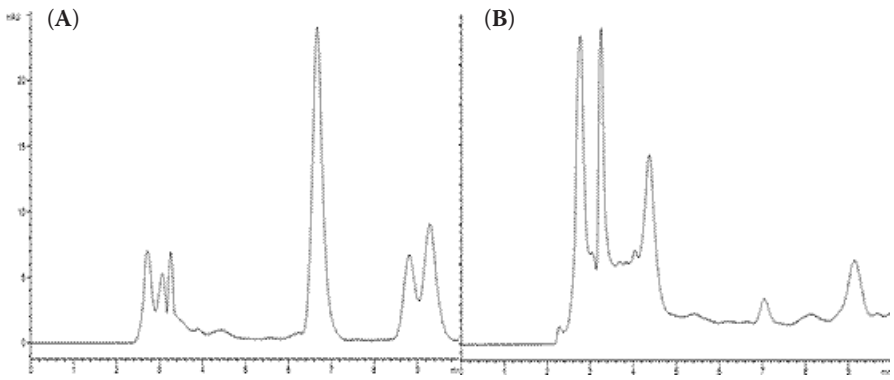

Figure 6. HPLC chromatograms: methanolic solution of authentic iturin (Sigma Aldrich, USA), 1 mg/ml (**A**) and iturin in methanolic solution of lipopeptides extracted from culture filtrate inoculated with *B. subtilis* Y-IVI in optimised medium (**B**)

by strain Y-IVI showed antagonistic activity against *Rhizoctonia solani* AG4 and *Verticillium dahilae*, forming 4.3- and 3.1-mm diameter inhibition zones, respectively. However, no inhibition zone was observed in the presence of *Phytophthora capsici* and *Phytophthora parasitica* var *nicotianae*.

Purification of antifungal substances. FOM was used as the target phytopathogen in *in vitro* antagonistic tests of the purified compounds. Figure 4 shows that the peaks collected from the retention time of 30 min to 60 min inhibited FOM mycelia growth. The antifungal activities of the peaks collected from 51 min to 62 min were higher than those collected from 30 min to 41 min and 41 min to 51 minutes. Furthermore, the three interval peaks were separately purified with HPLC by altering their mobile phase ratios. After several cycles of separation and purification, two peaks with stronger antifungal activity were detected by mass spectrometry.

Mass spectrometry analysis. Mass spectrometry was carried out to determine the molecular weights of the antifungal compounds produced by *B. subtilis* Y-IVI. The mass spectrum showed protonated homologous ion peaks at *m/z* of 1030.7, 1044.7, and 1058.7 $[M+2H]^+$, differing by 14 mass units, respectively (Figure 5A); their molecular weights were 1028.7, 1042.7, and 1056.7, respectively. This result indicated that the compounds of *m/z* 1028.7, 1042.7, and 1056.7 belonged to iturin A. Figure 5B shows the mass spectrum of another antifungal peak. The protonated homologous ion peaks were at *m/z* 1463.9, 1477.9, and 1491.9 $[M+H]^+$, with an ion peak at *m/z* 1513.8 $[M+Na]^+$, and their molecular weights were 1463, 1477, and 1491, respectively. These compounds belonged to fengycin-type lipopeptides.

Quantification of antifungal lipopeptides by HPLC. The authentic iturin (Sigma Aldrich) was determined by HPLC with 40% acetonitrile and 60% trifluoroacetic acid (0.1%) as elute solutions at 280 nm. Four chromatogram peaks of authentic iturin at retention times of 3.3, 6.6, 8.8, and 9.2 min were detected (Figure 6A). The calibration curves at a retention time of 8.8 min are not shown (Figure 6B). After calculation, the concentrations were 73.42, 1.52, and 14.81 mg/l, respectively. The total concentration of iturin produced by *B. subtilis* Y-IVI incubated in optimised medium was 89.75 mg/l.

DISCUSSION

The antagonistic bacterium strain Y-IVI was identified as *Bacillus subtilis* based on 16S rDNA sequencing. *Bacillus subtilis* has been widely used as a biological control agent. This bacterium was reported to produce a plethora of low molecular weight antibacterial and antifungal substances, such as surfactin, iturin, and fengycins (Nagorska *et al.* 2007). Antagonistic compounds are suppression factors and play a major role in biocontrol of soil-borne diseases. Many studies have reported that *Bacillus* spp. can produce a broad range of metabolites with antifungal and/or antibacterial activities (Chung *et al.* 2008; Huang *et al.* 2014; Li *et al.* 2014).

Incubation conditions have significant effects on bacteria growth and metabolic products such as temperature, pH, and incubation time and metal ions (Raza *et al.* 2009; Huang *et al.* 2014). It is essential to know the optimum temperature, pH, and incubation time to produce antifungal compounds. The maximum amount of antifungal compounds produced by strain Y-IVI was obtained via incubation at pH 7 at 30°C for 30 hours. The strain can produce antifungal compounds, even when it is incubated at 20°C. This temperature is close to the soil temperature, suggesting that strain Y-IVI could be used as a biocontrol agent in melon-growing regions (Beatty & Jensen 2002).

The antifungal compounds produced by strain Y-IVI were relatively heat stable and aciduric. The

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filtrate showed antagonistic activities even after being diluted to 10%. These results are in agreement with those of Lee *et al* (2008) and Zhang *et al* (2008). The enzymes Proteinase K and pepsin were unable to degrade antifungal compounds. These results suggest that the compounds may be N- and C-terminally blocked or composed of unnatural amino acids. Cyclic peptides were stable to protease degradation because their cyclic structure renders them relatively inflexible. The cyclic nature also leads to inaccessible cleavage sites because of steric hindrance (Eckart 1994). Moreover, the antifungal compounds were successfully extracted from Y-IVI culture filtrate, which shows broad-spectrum antagonistic activities against *Fusarium oxysporum*, *Rhizoctonia solani* AG4, and *Verticillium dahilae*.

LC–ESI-MS analysis of the purified active compounds revealed two sets of peaks that had different iturin and fengycin homologues and were detected by comparing their molecular weights with those obtained by Vanittanakom and Loeffler (1986) and Kowall *et al.* (1998). Iturin and fengycin are reported to synthesise in a synergistic manner (Romero *et al*. 2007). Fengycin shows strong antagonistic activity against fungi but is not effective against yeast and bacteria (Vanittanakom & Loeffler 1986). In this study, *Bacillus subtilis* Y-IVI could coproduce iturin and fengycins inoculated in optimised medium. Its broad-spectrum antifungal activity could be due to these iturins and fengycins.

In this study, an effective biocontrol agent of *Bacillus* Y-IVI was identified by 16S rDNA gene sequencing. We also optimised its incubation conditions to increase the antifungal compounds production. Antifungal compounds of iturin A and fengycin were detected in its culture filtrate. Lipopeptides production was considered as possible mechanism underlying the biological control of Fusarium wilt by *Bacillus subtilis* Y-IVI.

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