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Full Length Research Paper

Endophytic *Bacillus subtilis* ZZ120 and its potential application in control of replant diseases

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An endophytic bacterial strain ZZ120 that was isolated from healthy stems of *Prunus mume* (family: *Rosaceae*) was identified as *Bacillus subtilis* based on biochemical and physiological assays and 16s rRNA, rpoB and tetB-yyaO / yyaR genes analysis. Both the culture filtrate and the n-butanol extract of strain ZZ120 showed strong growth inhibition activity *in vitro* against the replant disease phytopathogens *Fusarium graminearum*, *Alternaria alternata, Rhizoctonia solani, Cryphonectria parasitica* and *Glomerella glycines*. The active metabolite in the filtrate was found to be produced 24 h after inoculation and the concentration remained at a high level until 66 h and was quite thermally stable with more than 75% of the antifungal activity even after being held at 121°C for 30 min. In addition, the antifungal activity of the filtrate remained almost unchanged when the culture was exposed to a pH ranging from 1 to 8, but significantly reduced after the filtrate had been exposed to alkali conditions (pH 9 to 14) for 30 min. The antifungal compounds were isolated from n-butanol extract as a mixture of iturins. The strong antifungal activity suggested that the endophytic *B. subtilis* ZZ120 and its bioactive components might provide an alternative agent for the biocontrol of replant diseases.

Key words: Endophytic bacterium, *Bacillus subtilis*, replant pathogens.

INTRODUCTION

Endophytes are microorganisms that grow within plants without causing any obvious symptoms of infection or disease (Bacon and White, 2000). Some of the endophytes are thought to protect their host from being attacked by fungi, insect and mammals by producing secondary metabolites (Zhang, 2007). Among them, endophytic bacteria are thought to interact closely with their host plants, and therefore could be used as biological control agents in sustainable crop production potentially (Sturz and Nowak, 2000; Taechowisan et al., 2003; Zhang et al., 2008). *Bacillus* spp., the most often isolated bacteria from natural environments, has been used widely in agricultural applications. The fungicides Serenade® and Sonata® that are made of *B. subtilis* QST713 and QST2808 have been registered and applied in America (Cao et al., 2010). The poor growth of crops when they were replanted on a site that had previously supported the same, or a closely related species, has been termed "replant disease" (Angelika et al., 2010). It has been reported that replant diseases are responsible for reductions in plant growth, crop yields and production duration in most of the major agriculture regions of the world for pomes, stone fruits and also related ornamentals (Utkhede et al., 2000). Pepper is an important vegetable crop that is widely planted from north to south in China. The major obstacle for pepper cultivation is replant disease that could result in death and even complete loss of production. The disease incidence caused by *Fusarium* spp. is about 15 to 30%, and even higher, up to 70 to 80%, in the pepper cultivation regions in Shaanxi, Gansu, Jilin, Sichuan, Hunan and Guangxi provinces (Liang and Wang, 2002). The changes of soil nutrition and structure (Elizabeth et al., 2009), the release of phytotoxic metabolites during their decomposition or by the support of deleterious microorganisms (Benizri et al., 2005), and accumulation of pathogenic fungi (Liu et al.,

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2008) have been suggested to be the causal factors in the etiology of replant disease. In our previous studies, we have investigated on the pathogenic fungi and their distribution in the main replant soils in several dominant pepper production areas of China. The dominant species were proven to belong to the genera of *Fusarium* (33.3%), *Mortierella* (27.5%) and *Phytophthora* (18.7%), which are unevenly distributed in different areas of China (Liu et al., 2008).

Traditional agricultural practices, such as the use of resistant varieties, crop rotation, intercropping and the enrichment of organic manure have been widely used in the control of replant diseases; however, these traditional methods have not been effective in significantly controlling these diseases. But several disadvantages inherent to the use of these biocides, including difficulty in application, high cost and potential hazards to human health, are undeniable (Marc and Philippe, 2007). Besides, due to the development of resistance mutations and new physiological races of pathogens, many of these synthetic chemicals are gradually becoming ineffective (Ge et al., 2004). There is an emerging need for more effective and safer fungicides, especially those that are with novel modes of actions with disease resistance-breaking properties and biological control potentials.

Biopesticides, whereby a natural organism or its' metabolites are used as the controlling agent, are the material basis and an important means of pesticidal control (Marc and Philippe, 2007). Biopesticides can be divided into several categories according to different sources, such as fungi, actinomycetes and bacteria (Kumar et al., 2008). As a bacteria-derived biopesticide, the most used bacteria in the early study belong to the family of Gram-negative bacteria, mainly *Pseudomonas* spp., *Agrobacterium* spp., *Erwinia* spp. (Du et al., 2000). In recent years, it has been gradually realized that the Gram-positive bacteria, as a group of biological agents also play an important role in the control of plant diseases, such as *Bacillus* spp. (Marc and Philippe, 2007).

In this study, we isolated a potential endophytic antagonist from healthy stems of *Prunus mume* (family: *Rosaceae*) by an *in vitro* screening technique and identified it as *B. subtilis* ZZ120 based on biochemical, physiological examination and 16s rRNA, rpoB, tetB-yyaO / yyaR sequences analysis. Moreover, the strain ZZ120 showed great growth inhibition against several phytopathogenic fungi of pepper replant diseases, including *F. graminearum*, *R. solani*, *A. alternata*, *C. parasitica* and *G. glycines*. The antifungal compounds were isolated from the culture filtrate of the strain and identified primarily to be a mixture of iturins.

MATERIALS AND METHODS

Isolation of endophytic bacteria

Endophytic bacteria were isolated from healthy stems of *P. mume* (family: *Rosaceae*) with the method described previously (Liu et al., 2001). Briefly, the healthy stems were washed with running water, sterilized in 75% ethanol and 40% formaldehyde for 3 min, rewashed with sterile distilled water for 5 times, and then cut into 1 cm long pieces with sterile scissors. Two or three segments were placed on PSA medium (potato 200 g, sucrose 20 g, agar 20 g, water 1 L). After incubation at 30°C for 1 to 2 days, bacteria appeared on the plates and were isolated individually as single colonies on PSA. And another uncut but surface-sterilized stems were placed on the same PSA medium as control.

Identification of strain ZZ120

Total genomic DNA for PCR amplification of 16S rRNA, rpoB and tetB- yyaO / yyaR sequences were extracted from strain ZZ120 according to the described method (Zhao et al., 2010). The specific primers used for PCR amplification targeting 16S rRNA and rpoB genes as well as the sequences between tetB and yyaO / yyaR, respectively are shown in Table 2. The PCR mixtures were prepared in 50 µl volumes containing 0.5 µM of primer, 200 mM of deoxyribonucleotide triphosphate, 5 µl of the 10X PCR buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl; pH 8.3), 1 U of Taq DNA polymerase (Tianwei), and 1 µl of the extracted DNA. DNA amplification was performed in a GeneAmp PCR system 2400 (Perkin Elmer) with an initial denaturation for 2 min at 94°C, followed by 30 cycles of denaturation (0.5 min at 94°C), annealing (1 min at 55°C), and extension (1 min at 72°C), plus a final extension for 7 min at 72°C. The PCR products were purified using a PCR Purification Kit (Tianwei) and identified by horizontal electrophoresis on a 1% agarose gel. PCR products were sequenced with an ABI 3100-Avant Genetic Analyzer (Applied Biosystems). The sequences were compared with similar sequences retrieved from the DNA databases by using the BLAST search program in the National Center for Biotechnology Information (NCBI) and aligned with BioEdit and Mega 4 software.

Biochemical and physiological characteristics (Table 3) were employed to identify the strain ZZ120 according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1986).

Growth curve and antifungal activities

500 ml of PS (Potato Sucrose) medium in a 1-L flask were inoculated with 1% of the overnight culture of strain ZZ120 and incubated at 30°C, 100 rpm for 72 h. During incubation, 1 ml of the culture broth was sampled at time intervals to measure its OD_{600} and to test its antifungal activity against three fungi that give rise to pepper replant diseases, namely *F. graminearum*, *R. solani* and *A. alternate* (Liu et al., 2008).

The effects of pH and temperature on the stability of antifungal filtrate

The culture filtrate of strain ZZ120 was adjusted to various pH values in the range from 1.0 to 14.0 using 2 M HCl or 2 M NaOH and maintained at 4°C for 24 h. Antifungal activity was assayed after the samples were readjusted to pH 7.0. A similar procedure was used to assess the effect of temperature on the antifungal activity of the metabolites produced by strain ZZ120. The culture filtrate was held at temperatures of 25, 80, 100, and 121°C, respectively, for 30 min and was then tested for their antifungal activity after being cooled to room temperature. The relative remaining activity was measured by comparison with that of the samples held at pH 7.0 and room temperature. The data are presented as the mean values of the three replications ± SEM (standard error of the mean).

Table 1. Antagonism of four endophytic bacteria against various phytopathogens.

Strain	*R. solani*	*G. glycines*	*P. capsici*	*F. graminearum*	*B. cinerea*
ZZ120	+	++	+	++	+
ZZ121	+	+	+	NA	+
ZZ129	NA	+	+	NA	NA
ZZ130	++	++	+	++	+

+ and ++ represents relative inhibition rates against mycelial growth of each bacterial colony on the PSA medium to the extent of 30 to 60 and > 60%, respectively. "NA" means Not Applicable.

Isolation and structure elucidation of the antifungal compounds

After centrifugation at 2,900 g at room temperature for 10 min, the cell-free culture filtrate was extracted by n-butanol (filtrate : n-butanol = 1:3). The solution was concentrated using a rotary vacuum evaporator R-124 (Pharmaceutical, China) and the residue was dissolved in n-butanol. Based on *in vitro* evaluation of antifungal activities, the yield of antifungal fractions was 0.41 g L^{-1}.

The antifungal compounds were isolated from the n-butanol extract using *in vitro* antifungal activity guided chromatography on silica gel and Sephadex LH-20, and identified by reverse phase HPLC (Waters Alliance 2695) analysis coupled to a UV (UV Detector: Waters 2996 Photo Diode Array) and MS detector (Mass Spectrometer: ThermoFinnegan LCQ Deca XP Plus Electrospray ionization). Comparison of the results with the data of standard compounds in the Syngenta natural product dereplication database was done. The database consists of reverse phase HPLC retention times, UV spectra and MS spectra, run under a standardized set of chromatographic conditions, and contain data of bioactive compounds isolated from bacteria, fungi or plants. The antifungal activity of each fraction obtained from chromatography or HPLC was determined by the TLC - Bioautography assay as described by Prakash et al. (2009). A drop of each fraction was applied to a TLC plate which was then eluted with an appropriate solvent and allowed to dry naturally. The plate was then sterilized under UV light for 30 min and treated in a petri dish with a suitable amount of PS agar medium (45°C) containing ampicillin (400 µg ml^{-1}) and a spore suspension of *Aspergillus niger* and incubated at 28 to 30°C for 48 h.

Antifungal activity of strain ZZ120

The target phytopathogenic fungi were *C. parasitica, F. graminearum, A. alternate, R. solani* and *G. glycines,* which were isolated from the pepper replant soils in our previous study (Liu et al., 2008), and maintained in our laboratory.

To test for the excretion of antifungal metabolites by strain ZZ120, it was transferred to 4 ml of PS liquid medium in a 15 ml tube and incubated in shake flasks at 100 rpm at 30°C for 2 days. After removal of the cells by centrifugation of the culture broth at 2,900 g for 10 min and filtration through a 0.22 µm membrane filter, 1 ml of the culture filtrate mixed with 14 ml of the PS agar containing ampicillin (400 µg ml^{-1}) was poured into a Petri dish (9 cm in diameter). Once the medium had cooled, discs (7 mm in diameter) of the target fungi, taken from the fresh margin of the mycelium, were spaced equally on the Petri dish, and the dish was incubated at 30°C for 48 h. The inhibitory activity of the filtrate against fungal growth was recorded as the percentage reduction of mycelia growth in comparison with that of the control plates: Growth inhibition (%) = [(length of mycelia in the negative control plate–length of mycelia in the treated plate) / (length of mycelia in the negative control plate)] ×100. The same procedure was used to determine the antifungal activity of the n-butanol extract at different concentrations. Control plates contained same amounts of medium and solvents only. Chlorothalonil (SDS Biotech) was used as the positive control at 0.1 mg ml^{-1}.

The IC_{50} value was defined as the drug concentration that causes 50% growth inhibition compared to the controls (Paolo et al., 2006). The antifungal activities of different concentrations of n-butanol extract were obtained and then using linear regression analysis the IC_{50} value of n-butanol extract to pathogens (*C. parasitica, F. graminearum, A. alternate, R. solani* and *G. glycines*) was determined. The same process was used to investigate the IC_{50} value of culture filtrate and 75% Chlorothalonil.

A simulated *in vivo* disease control assay, which was described by Liu et al. (2007) was used to test the control efficiency of the culture filtrate of ZZ120 against the replant disease pathogens *F. graminearum* and *A. alternate*.

Results of the antifungal activities were analyzed with GraphPad Prism (version 3.02) software to estimate the significance of differences with the positive control ($p < 0.05$) by one-way ANOVA analysis and Dunnett test.

RESULTS

Isolation of antifungal strains from *P. mume*

Twenty isolates of endophytic bacteria were obtained from the healthy stems of *P. mume* and 4 out of them showed a broad and strong *in vitro* antifungal activity against the test phytopathogens. Particularly, strain ZZ120 and ZZ130 exhibited a broad inhibition spectrum to all the test 5 pathogens. Meanwhile, the uncut fragments of the healthy stems did not have any mycelia, indicating that the surface sterilization was effective (Table 1).

Identification of strain ZZ120

Almost entire sequence analysis (1513 bp) of 16S rRNA demonstrated that the strain ZZ120 which was isolated from healthy stems of *P. mume* (family: *Rosaceae*) was most likely to be one of the species including *B. subtilis* (such as strain 168, HOB2, ZM06), *B. amyloliquefaciens* (such as strain A1-1), *B. licheninformis* (such as strain B425, YB915, KIBGE-IB1) and *B. tequilensis* KNUC9029 with > 99.6% similarity (Figure 1a). Obviously, it is impossible to classify strain ZZ120 in the species level only dependent on sequence analysis of 16S rRNA gene.

Table 2. Primers used for PCR amplification in this study.

Primer	Sequence	Target gene	Reference
8f	AGAGTTTGATCCTGGCTCAG	16S rRNA	Martin and Colleen, (1998)
1492r	TACCTTGTTACGACTT	16S rRNA	Martin and Colleen, (1998)
rpoB_r1f	AGGTCAACTAGTTCAGTATGG	rpoB	This work
rpoB_r1r	TAATTCAGCAAGCGGGTTCG	rpoB	This work
rpoB_r2f	ATCGATCATCTTGGAAACCG	rpoB	This work
rpoB_r2r	TCGGCAGTCAGTTCAGTTAC	rpoB	This work
rpoB_r3f	TCGCGATATTCCAAACGTC	rpoB	This work
rpoB_r3r	TTGTTACTACATCGCGTTC	rpoB	This work
YyaO_F	GGAACCAGTCCACAGGGTTGTGG	yyaO	Reva et al. (2004)
YyaR_F	CGATTGAGTGGGCRAAGGAGAATCATTTWTGYGGT	yyaR	Reva et al. (2004)
TetB_R	CCATATAGAGCTGTTCCAATGGAGAAG	tetB	Reva et al. (2004)

Table 3. Physiological and biochemical characteristics of strain ZZ120 and the reference strain of *B. subtilis* 168.

Item	*B. subtilis* 168	Strain ZZ120
Shape	Rod	Rod
Size	(0.7-0.8 μm) ×(2-3 μm)	0.75 μm × 2.1 μm
Endospore	Middle	Middle
Gram stain	Positive	Positive
Catalase	+	+
Indole production	-	-
Nitrate reductase	+	+
Citrate utilization	+	-
Starch hydrolysis	+	+
Methyl red (MR) reaction	+	+
Urease	-	-
Glucose ferment	+	+
Voges-proskauer test	+	+
Growth in pH 5.7	+	+
Growth at 55℃	-	-
Pigment producing	+	+

RpoB is a housekeeping gene encoding the DNA-directed RNA polymerase beta subunit which can be used for bacterial identification (Jang et al., 2009). The entire rpoB sequence (3574 bp) of strain ZZ120 which was amplified with 3 pairs of primers and analyzed phylogeneticaly showed that the strain ZZ120 was likely to be *B. subtilis* 168 with 99.88% similarity (Figure 1b).

It has been reported that *B. subtilis* is very close to *B. amyloliquefaciens*. In order to prove the strain ZZ120 belonging to *B. subtilis* rather than *B. amyloliquefaciens*, two pairs of primers such as tetB_R / yyaR_F (specific for *B. amyloliquefaciens*) and tetB_R / yyaO_F (specific for *B. subtilis*) were used to run PCR amplification and sequence analysis. Results show that the chromosome DNA of strain ZZ120 could be amplified with tetB_R / yyaO_F primers but not tetB_R / yyaR_F, which further indicated that strain ZZ120 was a strain of *B. subtilis.* Besides, biochemical and physiological assays also demonstrated that the strain ZZ120 was most likely to be a strain of *B. subtilis*, which was similar to the reference strain of *B. subtilis* subsp. *subtilis* 168 (Holt et al., 1986; Tye et al., 2002) (Table 3).

a

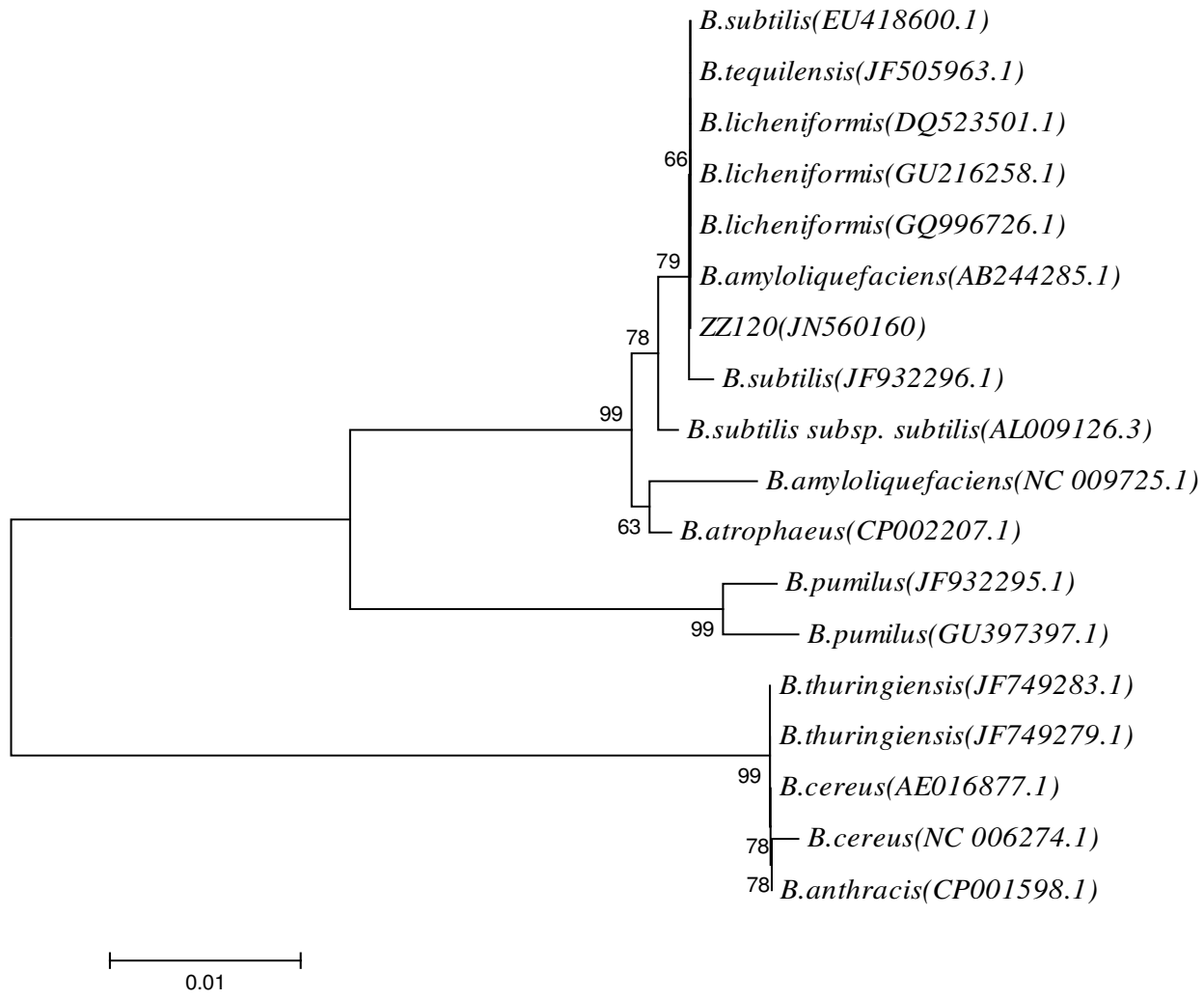

Figure 1. Phylogenetic tree of the strain ZZ120 based on 16S rRNA (a) and rpoB (b) sequences. Numbers in parentheses are the accession numbers of published sequences. Bootstrap values were based on 1000 replicates.

Growth curve of the strain ZZ120 and its production of antifungal metabolites

As shown in Figure 2a, strain ZZ120 grew relatively quick. It reached logarithmic growth phase after 3 h and the stationary phase at 10 h after inoculation. The antifungal activity of the culture filtrate sampled at different time intervals showed that the strain ZZ120 produced active metabolites along with the bacterium growth. The strongest antifungal activity against *A. alternate, R. solani* and *F. graminearum* was obtained 24 h after inoculation and kept in a high level until 66 h (Figure 2b). It may therefore be concluded that under the culture conditions used in this study, the optimal harvest time for the antifungal metabolites of strain ZZ120 was 24 h after inoculation.

Stability of antifungal metabolites

Results shown in Figure 3a demonstrated that the antifungal activity of the filtrate of strain ZZ120 against *R. solani*, *F. graminearum* and *A. alternata* was significantly reduced after the filtrate had been exposed to alkali conditions for 30 min, but remained almost unchanged when the filtrate was exposed to conditions in the pH range from 1 to 8.

Moreover, the antifungal components in the filtrate of strain ZZ120 were quite thermally stable with more than 75% of the antifungal activity of the culture filtrate being

b

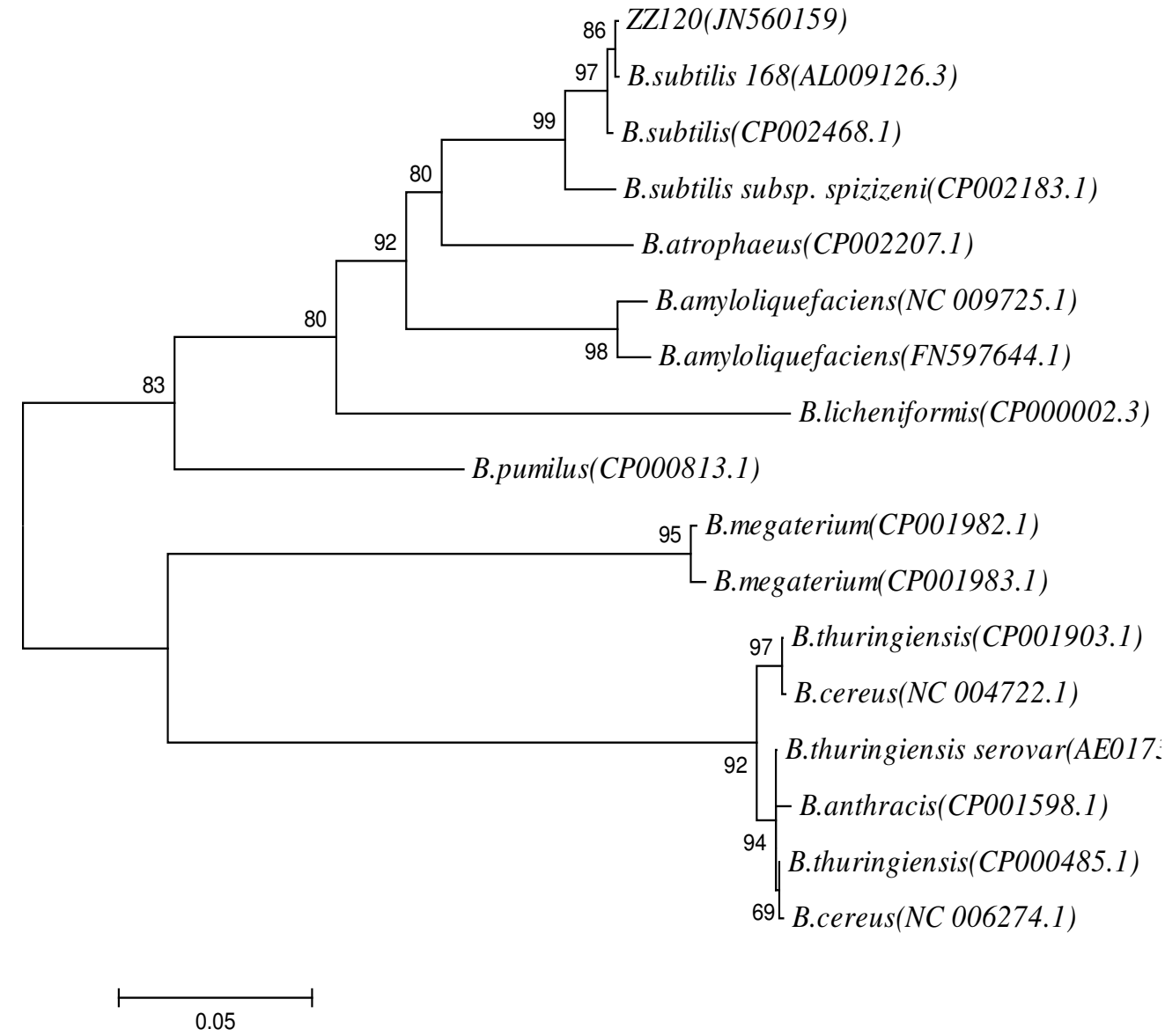

Figure 1. Contd.

retained even after it had been held at 121°C for 30 min, though it was reduced in a limited content, especially for *A. alternate* (Figure 3b).

Antifungal activity of strain ZZ120

The culture filtrate of strain ZZ120 obtained at 48 h after inoculation was evaluated for its *in vitro* antifungal activity against the pepper replant diseases causing fungi. Results in Table 4 showed that the culture filtrate significantly inhibited the growth of the test fungi, especially on *A. alternata*.

To characterize the antifungal compounds in the culture filtrate of strain ZZ120, the n-butanol extract was obtained. The *in vitro* antifungal activity of the n-butanol extract revealed significant growth inhibition of the test fungi which was consistent with the culture filtrate. The inhibition percentage of the extract on the growth of test fungi at the concentration 1 mg ml^{-1} was significantly higher than that of the commonly used fungicides, 75% chlorothalonil at the concentration of 1 mg ml^{-1} (Table 4).

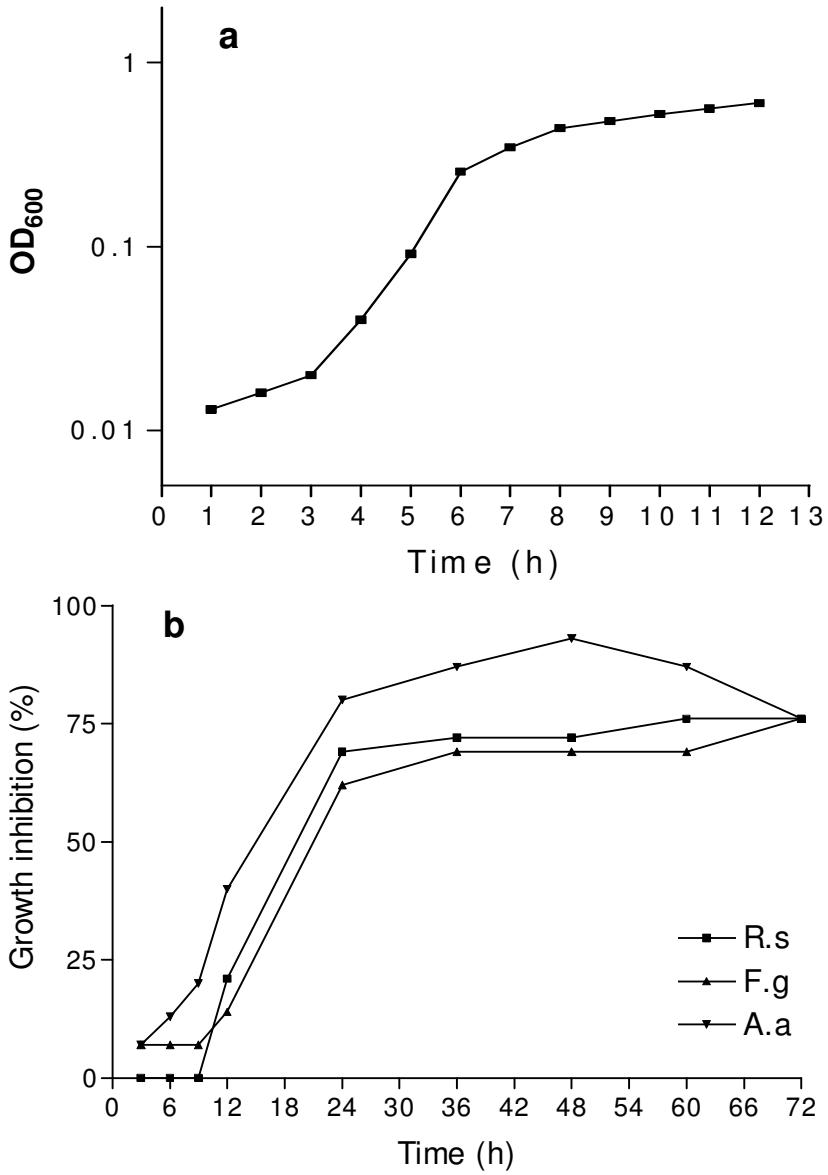

Figure 2. Growth curve (a) and *in vitro* antifungal activity (b) of strain ZZ120. R.s, F.g and A.a represent *R. solani*, *F. graminearum* and *A. alternate*, respectively.

Of the test phytopathogens, *A. alternate, C. parasitica* and *G. glycines* appeared to be the most sensitive species. The results also demonstrated that the anti-fungal activity of the extract has dose-dependent responses.

The broth filtrate and bioactive fractions showed different IC_{50} to the various test phytopathogens (Table 5). The inhibition was most effective on *A. alternate* and *R. solani* which were much better than 75% chlorothalonil.

According to the simulated *in vivo* disease control assay, the culture filtrate displayed a great efficiency in the control of wheat root diseases caused by the infection of *F. graminearum* and *A. alternate*. The highest control efficiency of the culture filtrate was 64.2% against *A. alternata* and 92.9% against *F. graminearum* which were much higher than chlorothalonil treatment (Table 6).

Isolation of the active compounds

By following the antifungal activity tested *in vitro*, the n-Butanol extract of ZZ120 were used for further purification. Since less was known about the composition of the extract, the active compounds were then monitored by a TLC-bioautography method to guide the separation. The inhibition zone of TLC could indicate the location of the active substances, and their Rf values were

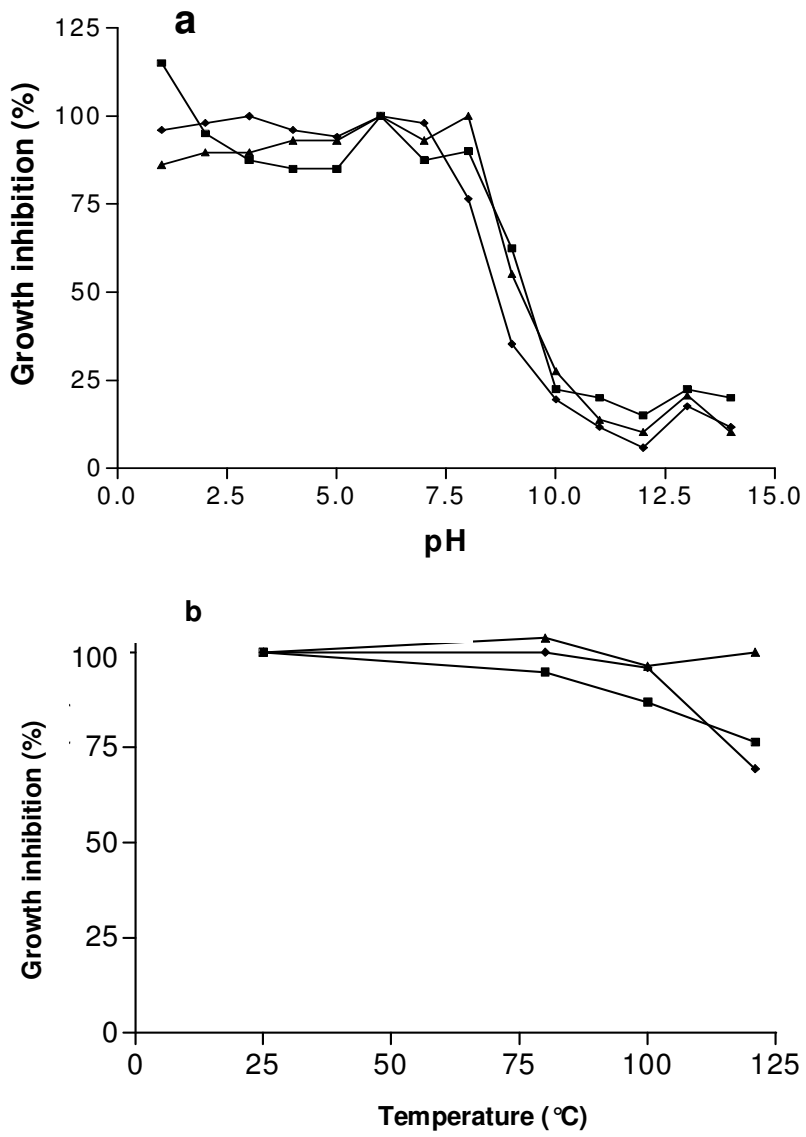

Figure 3. Effects of pH (a) and temperature (b) on the antifungal activity of the *B. subtilis* ZZ120 culture filtrate. ◆, ■ and ▲ indicate the antifungal activity of the culture filtrate against *R. solani*, *F. graminearum* and *A. alternate*, respectively.

Table 4. Antifungal activity of culture filtrate and n-butanol extract from strain ZZ120 against pathogens (%).

Treatment	Concentration (mg ml^{-1})	A. a	C. p	R.s	F. g	G.g
Chlorothalonil	1	60.0 ± 0.6	60.0 ± 0.6	76.3 ± 1.2	83.0 ± 3.4	76.0 ± 0.4
Culture filtrate	1	81.2 ± 1.3**	67.3 ± 3.3	61.4 ± 1.3**	58.0 ± 1.1**	45.5 ± 0.0**
	2	81.8 ± 1.6**	92.0 ± 0.0**	79.7 ± 1.9	73.3 ± 1.7**	84.6 ± 7.7
n-Butanol extract	1	76.9 ± 1.6**	73.3 ± 2.7**	64.1 ± 0.9**	61.4 ± 0.0**	71.8 ± 9.3
	0.4	55.7 ± 2.8	48.0 ± 2.7**	53.8 ± 1.6**	53.4 ± 2.0**	65.3 ± 3.8
	0.2	44.3 ± 1.8**	36.0 ± 4.6**	30.6 ± 1.1**	48.7 ± 1.0**	57.7 ± 3.9*

*,** indicates that the data are significantly different from that of the positive control of chlorothalonil at 5% level. And A.a, C.p, R.s, F.g, G.g represent *A. alternate*, *C. parasitica*, *R. solani*, *F. graminearum*, G.glycines, respectively.

Table 5. The IC_{50} of ZZ120 n-butanol extract and culture filtrate against the phytopathogens.

Treatment	A. a	C. p	R. s	F. g	G. g
75% Chlorothaloni (μg ml^{-1})	36	NA	45	57	NA
Culture filtrate (μl ml^{-1})	8	50	29	209	140
n-Butanol extract (μg ml^{-1})	21	31	25	530	92

"NA", Not applicable. A.a, C.p, R.s, F.g, G.g represent *A. alternate*, *C. parasitica*, *R. solani*, *F. graminearum*, *G.glycines*, respectively.

Table 6. The efficiency of the culture filtrate of the strain ZZ120 in control of the wheat root diseases caused by *A. alternata* and *F. graminearum* (%).

Item	Concentration	*A. alternata*	*F. graminearum*
Broth filtrate (ml/ml)	1.00	64.2 ± 1.5^a	92.9 ± 0.9^a
	0.67	63.5 ± 0.9^a	92.2 ± 0.7^a
	0.33	60.3 ± 0.4^a	66.9 ± 0.9^b
	0.15	46.4 ± 0.7^b	59.7 ± 0.9^c
Chlorothalonil (mg/ml)	1 mg/ml	17.2 ± 0.6^c	NA

Data are the average of three experiments and they were analyzed using one-way ANOVA ($p < 0.05$). The letter "a" indicates the highest value. The same letters (a, b or c) indicate no significant differences among the data in the same column.

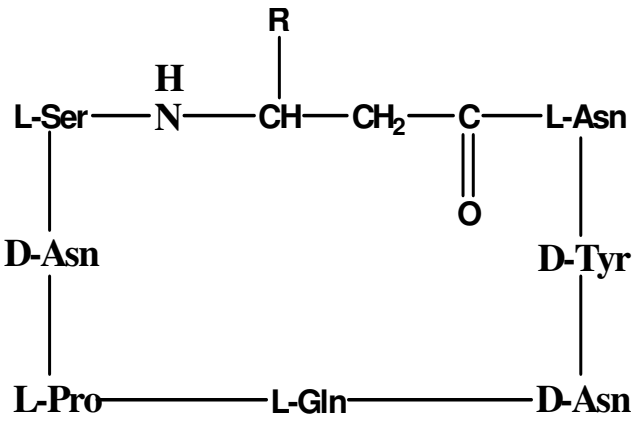

Figure 4. The structure of iturins. R means, the β-amino fatty acid chain with a length of 14 to 17 carbons link to the heptapeptides.

compared with the reference plate. The active compounds were collected and purified through column chromatography and Sephadex LH-20. Then, the antifungal chemicals were determined by reverse phase HPLC analysis of the isolated active fraction coupled to UV and MS detection and comparison of the results with the natural product dereplication database was done. Results indicate that the antifungal chemicals in the n-butanol extract of strain ZZ120 was a mixture of iturins (UV absorption 276 nm; MS: 1043 iturin A2, 1057 iturins A3/A4/A5, 1071 iturins A6/A7) with structures as described in the literature (Marc and Philippe, 2007) (Figure 4).

DISCUSSION

Up to 2007, the growth rate of biopesticides was estimated to be about 10% per annum for the next five years, and is expected to reach a billion dollars by 2010. Among these biopesticides, microorganism-based products (bacteria, fungi, virus, yeasts) represent ~30% of total sales and new products are regularly brought to the market (Marc and Philippe, 2007). Microorganisms

are the largest biological populations with great species diversity in the planet. Many microorganisms can produce a wide range of bioactive compounds. Meanwhile, the production of bioactive compounds also can be regulated by strain selection, genetic manipulation, metabolic regulation, large-scale fermentation and other techniques (Koji et al., 2010). Moreover, in comparison to the synthetic pesticides, microbe derived pesticides are more easily degradable with lower residue and higher environmental compatibility (Makkar et al., 2002). As part of our ongoing research program on the development of endophytic resources, several endophytic bacteria and fungi (data not shown) have been isolated from *P. mume*, *Ilex latifolia*, *Osmanthus fragrans*, *Cinnamomum camphora, Pinus bungeana, Nerium indicum*, *Chimonanthus praecox, Ilex purpurea*, *Cunninghamia lanceolata*, *Cephaltaxus sinensis, Sabia japonica*, *Pittosporum tobira*, *Bambusa multiplex* and *Ulmus pumila* (Liu et al., 2007, 2010; Zhao et al., 2010). Among them, strain ZZ120 isolated from healthy stems of *P. mume* showed strong antifungal activities against several pepper replant diseases causing pathogens such as *F. oxysporum, A. alternate, R. solani*. The culture filtrate and its n-butanol extract of strain ZZ120 exhibited significant inhibition on the growth of the test fungi, and the inhibitory potency was significantly higher than that of the commonly used chemical fungicide Chlorothalonil at the concentrations tested (Table 4). In addition, the strain ZZ120 was identified to be *B. subtilis* based on 16S rRNA and rpoB sequence analysis and biochemical and physiological assays.

B. subtilis is a complex of closely related species, and isolates previously classified as *B. subtilis* are now recognized as *B. atrophaeus* (Nakamura, 1989), *B. mojavensis* (Roberts et al., 1994), *B. vallismortis* (Roberts et al., 1996) and *B. amyloliquefaciens* (Oleg et al., 2004). Many evidences have proved that identification of *B. subtilis* can not rely on the limited information obtained from 16S rRNA gene analysis and biochemical and physiological assays (Heather and Geraldine, 2011; Oleg et al., 2004), which is consistent with our results (Figure 1a). However, the housekeeping rpoB gene encoding the DNA-directed RNA polymerase beta subunit (β), is demonstrated to be more sensitive DNA-sequencing subtyping than 16S rRNA sequencing for a number of bacterial species (Jang et al., 2009). The rpoB gene has been used to identify and classify various bacterial species, including *Mycobacterium* (Richert et al., 2007), *Pseudomonas* (Ait et al., 2005), *Staphylococcus* (Drancourt et al., 2004), *Streptomyces* (Kim et al., 2004) and *Vibrio* (Tarr et al., 2007). Jang et al. (2009) firstly used the rpoB gene for *Bacillus* species identification from marine bacteria. In comparison to 16S rRNA genes, rpoB gene has many advantages: it is homogeneous within cells, contrains relatively long sequences (approximately 3.5 kb in *Bacillus*), and many of the rpoB sequences are available in public databases (Jang et al., 2009). Our result also indicated that rpoB gene is good candidate at sequence analysis for endophytic bacteria indentification. Based on 16S rRNA genes analysis, the strain ZZ120 can not be distinguised among the species of *B. subtilis*, *B. amyloliquefaciens*, *B. licheninformis* and *B. tequilensis* (similarity > 99.6%) (Figure 1a). However, after sequence analysis of rpoB gene, the strain ZZ120 can clearly be identified as *B. subtilis* out of the other species (Figure 1b). Moreover, two pairs of primers of tetB_R / yyaO_F and tetB_R / yyaR_F have been used to identify the strains which were most likely to be *B. subtilis* or *B. amyloliquefaciens* (Oleg et al., 2004). Same primers used in this study showed that the strain ZZ120 could be amplified with primers of tetB_R / yyaO_F, which further indicated that the strain should be a strain of *B. subtilis.* According to our results, many methods have to be employed in identification and classification of endophytic *Bacillus* species.

There is an average of 45% genome of *B. subtilis* devoted to antibiotic synthesis and more than two dozen structurally diverse antimicrobial compounds potentially produced (Stein et al., 2005). Among these antimicrobial compounds, cyclic lipopeptides (LPs) of the surfactin, iturin and fengycin (or plipastatin) families have well-recognized potential uses in biotechnology and biopharmaceutical applications because of their surfactant properties (Marc and Philippe, 2007). In this present study, we showed that the strain ZZ120 had broad and great growth inhibition spectra against several phytopathogenic fungi of pepper replant diseases, including *F. graminearum, R. solani*, *A. alternata*, *C. parasitica* and *G. glycines*. Moreover, the active compounds were isolated from the culture filtrate of the strain and identified primarily belonging to the well-known antifungal lipopeptide iturins (Figure 4). Iturins are a family of cyclic peptide substance mainly produced by *Bacillus* spp. and other close phylogenetic relationship of several species of bacteria in the soil (Marc and Philippe, 2007; Soo, 2003). It was reported that the synthetic capacity of iturins was reduced along with the spore formation of *Bacillus* species (Mohammad et al., 2001). However, at the given fermentation condition, *B. subtilis* ZZ120 showed a continuing production of the bioactive compounds whether the spore formed or not formed. The highest level of antifungal activity was observed during the stationary phase 24 h after inoculation. It has been reported that the synthesis of peptide antibiotics usually starts at the end of the exponential growth of a cell culture, reaching maximum concentration after cell growth has ceased (Marc and Philippe, 2007), but this is not completely in agreement with the production kinetics of strain ZZ120. It seems that the production of bioactive compounds in strain ZZ120 is positively correlated with cell growth. Further research is needed to study the kinetics of this bioactive compounds production and the optimization of medium design and procedures for product recovery. And also the exact structure of this

compound isolated from ZZ120 could be identified deeply. Furthermore, the efficiency of *in vivo* disease control provided by the broth filtrate, the n-butanol extract, and iturin itself obtained from strain ZZ120, should be confirmed in field trials.

In conclusion, an endophytic bacterium ZZ120, isolated from healthy stems of the *P. mume,* was identified as *B. subtilis* by integrate determination including sequence analysis of 16S rRNA and rpoB genes as well as biochemical and physiological characterization. The culture filtrate and the n-butanol extract showed strong *in vitro* inhibition activity against pepper replant disease causing pathogens such as *F. graminearum, R. solani*, *A. alternata*, *C. parasitica* and *G. glycines.* Further study disclosed that the compounds responsible for the antifungal activity in the n-butanol extract of culture filtrate of strain ZZ120 were a mixture of five relatively thermo-stable iturins compounds. The endophytic *B. subtilis* ZZ120 and its bioactive component may provide an alternative resource for the biocontrol of replant diseases.

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