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Identification of lipopeptides produced by *Bacillus subtilis* Czk1 isolated from the aerial roots of rubber trees

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ABSTRACT. We obtained a strain of *Bacillus subtilis*, which we named Czk1, from the aerial roots of rubber trees. This bacterial isolate exhibits strong antagonistic activity against *Ganoderma pseudoferreum*, *Phellinus noxius*, *Helicobasidium compactum*, *Rigidoporus lignosus*, *Sphaerostilbe repens*, and *Colletotrichum gloeosporioides*. Our earlier research has shown that the antagonistic activity of a fermentation supernatant Czk1 isolate produces a complex mixture of lipopeptides. In this study, we used methanol to extract crude lipopeptides, purified them using a Sephadex G-25 column, cloned the lipopeptide genes, and

analyzed purified fractions by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) to identify the lipopeptides from *B. subtilis* strain Czk1. The cloned lipopeptide genes included those that encode the enzymes lpa, ituD, sfp, and fenB. The crude lipopeptides were purified and found in five fractions. Further analysis revealed that five fractions of the purified composition contained members of the surfactin, iturin, fengycin, and bacillomycin families of antibiotics. This suggests that these lipopeptides from strain Czk1 have potential as plant disease biocontrol agents.

Key words: *Bacillus subtilis*; Antifungal activity; Lipopeptide; *Colletotrichum gloeosporioides*; MALDI-TOF-MS

INTRODUCTION

The rubber tree (*Hevea brasiliensis*) is a wild tropical angiosperm that belongs to the Euphorbiaceae family (Dornelas and Rodriguez, 2005). It is considered an important perennial crop species because it is the source of natural rubber. However, rubber trees are often affected by various diseases, including root diseases (Oghenekaro et al., 2014), *Colletotrichum* leaf disease (CLD) (Jayasinghe et al., 1997; Cai et al., 2013), powdery mildew (Mitra and Mehta, 1938; Limkaisang et al., 2005), *Corynespora* leaf fall (CLF) (Chee, 1990), etc. (Jayasinghe et al., 1997; Cai et al., 2013; Oghenekaro et al., 2014). CLD is one of the major causes of the decline in rubber yields in Asia (Thambugala and Deshappriya, 2009). *Colletotrichum gloeosporioides* causes the CLD that affects rubber (Cai et al., 2013; Yong et al., 2014). This pathogen can attack any of the green parts of the plant, including young leaves, shoots, and pods. In its most severe form, CLD causes secondary leaf fall in rubber trees; as a consequence, huge economic losses have been recorded (Wastie, 1967). In China, root diseases and CLD are a severe problem, and are considered limiting factors that seriously affect rubber production. For example, huge yield losses attributed to CLD have been observed in Hainan, Guangdong, and Yunnan provinces when weather conditions are favorable for disease outbreaks (Cai et al., 2009). CLD is usually controlled by chemical fungicides such as benomyl, maneb, chlorothalonil, and mancozeb (Mahoney and Tattar, 1980). However, the increased use of chemical fungicides has led to the development of fungicide-resistant fungal strains (Howarth, 1991; Peres et al., 2004). Therefore, biocontrol agents may be a more efficient and environmentally friendly alternative to fungicides for the control of rubber diseases.

Bacillus spp are often regarded as optimum candidates for the control of plant diseases because they can secrete antifungal compounds (Pathak and Keharia, 2014; Yang et al., 2015). The majority of *Bacillus* strains can produce antimicrobial compounds. For instance, *Bacillus* spp can generate antimicrobial peptides including various classes of bacteriocins (Klaenhammer, 1993), and antimicrobial surface-active biosurfactants such as lipopeptides, glycopeptides, and non-ribosomally synthesized cyclic peptides (Mukherjee et al., 2006; Rodrigues et al., 2006). Lipopeptides comprise peptides with 7-10 amino acids cyclized via a lactone ring to β-hydroxy fatty acids of varying chain length (Baindara et al., 2013). These lipopeptides mainly differ in their amino acid sequences and fatty acid branches; they are produced by various *Bacillus* spp and can be further divided into three families: iturin (Delcambe et al., 1977); fengycin (Vanittanakom et al., 1986); and surfactin (Arima et al., 1968). The βAA

lengths of iturin members vary from 14 to 17 carbon atoms (Pathak and Keharia, 2014). Iturins are also classified on the basis of the variation of amino acids in their corresponding peptide moieties: iturin A, iturin C, iturin D, iturin E, bacillomycin D, bacillomycin F, bacillomycin L, bacillomycin Lc, and mycosubtilin (Winkelmann et al., 1983; Gong et al., 2006; Romero et al., 2007; Pecci et al., 2010). Iturin A is the most promising antifungal lipopeptide because it is secreted by most *Bacillus* strains and is characterized by strong and broad-spectrum antifungal activity (Romero et al., 2007; Pecci et al., 2010). Surfactin contains a β-hydroxy fatty acid with 13 to 15 carbon atoms (Koumoutsi et al., 2004). The members of the fengycin family comprise β-hydroxy fatty acids with 13 to 15 carbon atoms (Koumoutsi et al., 2004). They have unusual properties such as the presence of ornithine in the peptide portion and antifungal activity, and they are more specific to filamentous fungi than to other fungal types (Steller et al., 1999; Vanittanakom et al., 1986). The lipopeptides belonging to the surfactin family are β-hydroxy heptacyclic depsipeptides with Ala, Val, Leu, or Ile variations at positions 2, 4, and 7 in the cyclic depsipeptide moieties, and C13 to C16 variations in the β-hydroxy fatty acid chains (Peypoux et al., 1994; Kowall et al., 1998; Hue et al., 2001). Different *Bacillus* strains also exhibit diversity in the production of cyclic lipopeptides; for example, most *Bacillus* strains produce lipopeptides belonging to only one family, whereas a few strains co-produce lipopeptides belonging to two or three families (Vater et al., 2002; Nagórska et al., 2007; Romero et al., 2007; Pecci et al., 2010).

Previously, we have isolated *Bacillus subtilis* Czk1 from the aerial roots of rubber trees; *B. subtilis* Czk1 exhibits *in vitro* antifungal activity against several plant pathogens: *Ganoderma pseudoferreum*, *Phellinus noxius*, *Helicobasidium compactum*, *Rigidoporus lignosus*, *Sphaerostilbe repens*, and *Colletotrichum gloeosporioides* (Zhao et al., 2011). We found that the antifungal activity of *B. subtilis* Czk1 supernatants is tolerant of high temperatures and is not sensitive to ultraviolet radiation or the action of protease K (Fan et al., 2013). Other researchers have reported that the antifungal activity of *B. subtilis* Czk1 supernatants is stable at high temperatures (40°-130°C) and is resistant to enzymatic degradation (proteinase K); these characteristics are typically associated with the lipopeptide antibiotics produced by *Bacillus* spp (Stein, 2005; Geetha et al., 2010). In the current study, we aimed to determine the antibiotic category of *B. subtilis* Czk1 by detecting the specific markers of several enzymes involved in antimicrobial peptide synthesis. Those enzymes were: ituD (ituD synthetase D); lpa (lipopeptide antibiotic iturin A); sfp (4'-phosphopantetheinyl transferase for surfactin synthesis); and fenB (fengycin synthetase B for fengycin B synthesis). We also analyzed the obtained lipopeptide fractions using a Sephadex G-25 column and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) to identify the putative compounds responsible for the antifungal activity of the supernatants. This is the first report of a *B. subtilis* strain from *Hevea brasiliensis* co-producing so many variants of fengycins, surfactins, and iturins.

MATERIAL AND METHODS

Bacterial strain and design of primers

B. subtilis Czk1 was isolated from the aerial roots of a rubber tree (He et al., 2012). The isolates were checked for purity and preserved at -70°C for future studies. The indicator strain used in this study was *C. gloeosporioides* isolate RC178 obtained from a rubber tree

stored in the laboratory of the Environment and Plant Protection Institute (Chinese Academy of Tropical Agricultural Sciences, Haikou, China). The specific primers for the target antifungal genes for polymerase chain reaction (PCR) assays were designed on the basis of the nucleotide sequences of *ituD* (the gene that encodes ituD synthetase D), *lpa14* (the gene that encodes the lipopeptide antibiotic iturin A), *sfp* (the gene that encodes 4'-phosphopantetheinyl transferase for surfactin synthesis), and *fenB* (the gene that encodes fengycin synthetase B for fengycin B synthesis). The primers were synthesized under salt-free conditions. The nucleotide sequences of the PCR primers used in this study are listed in Table 1.

Table 1. Amplified genes and corresponding primers sequence.

Primer	Sequence (5'-3')	Target gene	Gene ID	Product length (bp)
*Sfp*F/R	F: ATGAAGATTTACGGAATTTA R: TTATAAAAGCTCTTCGTACG	*Sfp* 4'-Phosphopanteinyl transferase	EU882341.1	675
*FenB*F/R	F: CTATAGTTTGTTGACGGCTC R: CAGCACTGGTTCTTGTCGCA	*fen*B Fengycin synthetase B	JN086144	1401
*lpa-14*F/R	F: ATGAAAATTTACGGAGTATA R: TTATAACAGCTCTTCATACG	*lpa*-14 lpa-14 gene encoding the lipopeptide antibiotic iturin A	D21876.1	675
*ituD*F/R	F: ATGAACAATCTTGCCTTTTT R: TTATTTTAAAATCCGCAATT	*ituD* synthetase D	KF381340	1203

PCR amplification and sequencing

The total DNA of *B. subtilis* Czk1 was extracted using a BioMiGA kit (San Diego, USA) in accordance with the manufacturer instructions. PCR amplifications were performed in a 20 μL reaction mixture containing 20 ng template DNA, 2X EconoTaq PCR SuperMix (10 μL; Beijing TransGen Biotech Co., Ltd.), and 0.5 μL each primer (10 μmol/L); ddH2O was added to obtain the final volume. Templates were amplified in an ABI 9600 thermal cycler under the following cycling conditions: one interval of 94°C for 3 min; 35 cycles of 94°C for 30 s, 50°-60°C for 30 s, and 72°C for 1-2 min; and one cycle of 72°C for 5 min. After the amplified templates had been confirmed by running the samples on 1.0% agarose gel, each PCR amplicon was purified by PCR using version 2.0 of the TaKaRa fragment DNA purification kit (TaKaRa Biotechnology, Dalian, China) and cloned into a pGEM®-T Easy Vector (Promega, Madison, WI, USA) in accordance with the manufacturer instructions. Each amplicon was transformed into *Escherichia coli* DH5á and subsequently sequenced by Sangon Biotech (Shanghai, China) using the primers M13F (ACTGGCCGTCGTTTTAC) and M13R (GTCATAGCTGTTTCCTG). Three colonies were recovered from each ligation reaction and sequenced separately. The sequences were BLASTed using the National Center for Biotechnology Information (NCBI) blastx program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Lipopeptide production and extraction

A colony of *B. subtilis* Czk1 was inoculated into Luria broth (50 mL) in a 250-mL Erlenmeyer flask, and incubated at 28°C and 180 rpm for 24 h. The 24-h-old inoculum was then used to seed 250 mL yeast extract peptone dextrose medium (YPD medium; composition in g/L: 1% yeast extract, 2% peptone, 2% glucose) in a 1000-mL flask to an initial OD_{600} (optical density measured at a wavelength of 600 nm) of approximately 0.05. The inoculum was then incubated at 28°C and 180 rpm for 72 h.

The 72-h-old fermentation broth of *B. subtilis* Czk1 was centrifuged at approximately

10,000 rpm and 4°C for 10 min. The supernatant was collected, and its pH was reduced to 4 using concentrated HCl; it was then incubated at 4°C overnight to precipitate the lipopeptides. The acid precipitates were recovered by centrifugation at approximately 10,000 rpm and 4°C for 10 min, and freeze-dried; the obtained pellet was solubilized in anhydrous methanol (Pathak et al., 2012) and extracted twice. The methanolic lipopeptide extract was further concentrated using a vacuum evaporator (Buchi, Switzerland).

Chromatographic purification and *in situ* acid hydrolysis in Sephadex G-25

The yellowish-brown extract was dissolved in a small quantity of chloroform/methyl alcohol (1:1) and filtered using a 0.45-mm membrane; the dissolved extract was then added to a Sephadex G-25 chromatographic column (1 x 60 cm; Shanghai Biological Engineering Co., Ltd., China). Chloroform/methyl alcohol (1:1) was used as the mobile phase to collect each constituent at a flow rate of 6 drops/min to obtain 20 mL in each tube. Each constituent was merged with ninhydrin/methyl alcohol as a color developing agent and chloroform/methyl alcohol/water (65:25:4) as a developing solvent. One thin layer chromatography (TLC; Qingdao Marine Chemical, China) plate was heated to develop color with 0.5% ninhydrin/ethyl alcohol, and the other TLC plate was placed in a development cylinder with 1 mL concentrated hydrochloric acid for 2 h of *in situ* acid hydrolysis in an airtight and high-temperature (110°C) environment. This TLC plate was removed from the cylinder and transferred to a fume cupboard to complete the volatilization of hydrochloric acid before 0.5% ninhydrin/ethyl alcohol was used for color development. The colorations of the two plates were observed and the same constituent was merged after the *in situ* acid hydrolysis was complete.

Each active constituent was detected using the cylinder plate method, with *C. gloeosporioides* growing on a rubber tree used as the indicator fungus; the constituents were selected via a spore germination experiment and further analyzed.

In the spore germination experiment, the conidium form of *C. gloeosporioides* was washed off the plate with 2% glucose/water solution; under a 10 x 10 microscope, the spore count was adjusted to approximately 100 conidia in the visual field. Next, 20-μL samples of each composition were coated evenly on slides. Another 20 μL of the spore suspension was coated on the sensitive layer once the first layer had formed following organic solvent evaporation. Methyl alcohol/chloroform (1:1) and water were used for contrast, and each treatment was repeated three times. When the germination rate in the control group reached >80% (approximately 10-12 h), the germination of the spores in the differently treated groups was observed. Three random visual fields were observed (repeated three times) for each treatment; the number of germinated spores and the total number of spores were recorded (spore germination was defined as the point at which the length of the germ tube exceeded the short radius).

MALDI-TOF-MS analysis

All the active lipopeptide fractions obtained (20-μL samples) were analyzed by MALDI-TOF-MS detection at Nanjing Agricultural University.

RESULTS

We used the specific PCR primers to detect antimicrobial biosynthetic genes in *B.*

subtilis Czk1. We designed four primer pairs to detect antifungal genes. The expected DNA bands were detected following amplification with the specific primers for the DNA extracted from *B. subtilis* Czk1 (Figure 1). The specific *sfp*F/R, *ituD*F/R, *lpa14*F/R, and *fenB*F/R primers successfully amplified the DNA band from *B. subtilis* Czk1 and produced 675, 1203, 675, and 1401 bp bands in the lanes, respectively (Figure 1).

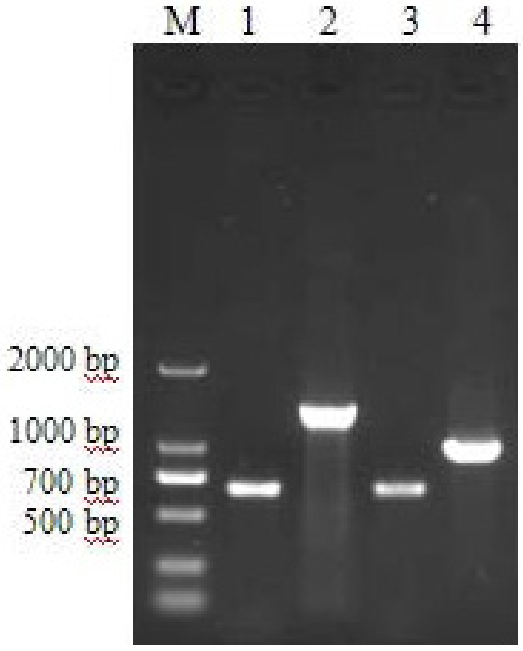

Figure 1. Molecular detection of the genes corresponding to antimicrobial substances. *Lane M*: DNA marker; *lanes 1-4*: *sfp*, *fenB*, *lpa14*, and *ituD*.

Multiple gene nucleotide alignments

To further confirm the genes corresponding to the antimicrobial substances, the gene-specific bands were recovered and cloned for sequencing. The sequence analysis revealed 98% homology between the *B. subtilis* Czk1 *sfp* gene nucleotide sequence (675 bp) and the *sfp* gene of *Bacillus amyloliquefaciens* LX-11 (JN086145.1). Similarly, we found 98% homology between the *B. subtilis* Czk1 *sfp* gene nucleotide sequence and the *sfp* genes related to surfactin synthesis in *B. amyloliquefaciens* FZB42 (CP000560.1). The homology between the fengycin synthesis-related *fenB* sequence (1401 bp) amplified from *B. subtilis* Czk1 and that from the *B. amyloliquefaciens* 96-79 (EU882342.1) bacterial strain was 99%. The fengycin synthesis-related *fenB* sequence was 97% homologous to the genes related to the synthesis of the *B. amyloliquefaciens* LX-11 (JN086144.1) fengycin enzyme. Furthermore, *lpa14* (675 bp), which is related to iturin synthesis, was 98% identical to the gene controlling the synthesis of iturin A (*lpa14*) in *B. subtilis* RP24 (EU797520.1). The *ituD* genes (1203 bp) were 99% identical to *B. subtilis* COGB29 (HQ7116.1) *ituD*. These findings indicate that the genes related to the three major metabolites (surfactin, iturin, and fengycin) exist in the genome of *B. subtilis* Czk1.

Purification of lipopeptides and detection of antifungal activity

The polygene test results initially confirmed the presence of genes related to lipopeptide synthesis in *Bacillus* spp. We carried out an *in situ* acid hydrolysis experiment to identify lipopeptides (Figure 2A). The two TLC plates were compared, and the results revealed that free amino acid residues were released from the closed structure after acid hydrolysis (Figure 2B), as indicated by numerous orange-yellow or salmon dots on the plate dyed by *in situ* acid hydrolysis. Therefore, these residues were identified as lipopeptide antibiotics (Hou et al., 2006). Seven fractions were found after the same fraction was merged following *in situ* acid hydrolysis.

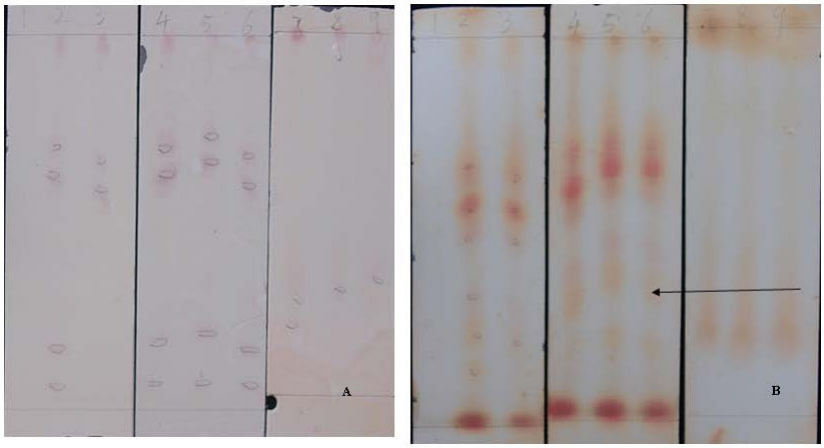

Figure 2. A. Direct dyeing with ninhydrin/ethanol; and **B.** ninhydrin/ethanol after acidolysis.

The activity of *C. gloeosporioides* was detected using all fractions via the cylinder plate method. Only five fractions elicited an inhibitory effect on RC178. Thus, the five fractions were renamed F1, F2, F3, F4, and F5 (Figure 3). Among these fractions, that fractions F3 and F4 exhibiting a strong antimicrobial activity (Figure 3). By contrast, fractions F1, F2, and F5 yielded weak antimicrobial activity on RC178 without an evident inhibition zone (Figure 3). However, the spore germination experiment revealed that the spore germination of RC178 was inhibited by the five fractions (Figure 4). When the germination rate in the control group was >80% (the 1:1 ratio of methyl alcohol/chloroform did not affect germination) (Figure 4A-C), the single spore in the six-fold diluent of fraction F5 germinated with a germination rate of <5% (Figure 4L). The two- or four-fold diluent of fraction F5 and the four- or six-fold diluent of fraction F1 could stop germination in a short period, and the spore did not exhibit malformation or expansion (Figure 4E-F, J-K). Fraction F3 elicited the largest influence on spore germination: its two-, four-, and six-fold diluent could stop the entire spore differentiation by inducing the malformation and expansion of the spore top and by changing the permeability of the cell wall (Figure 4G-I). The two-fold diluents of fraction F1, F2, and F4 could also inhibit the spore germination, and the single spore underwent top expansion or malformation (Figure 4D). However, the four- and six-fold diluents of the three fractions did not affect the spores, and the spores were not germinated.

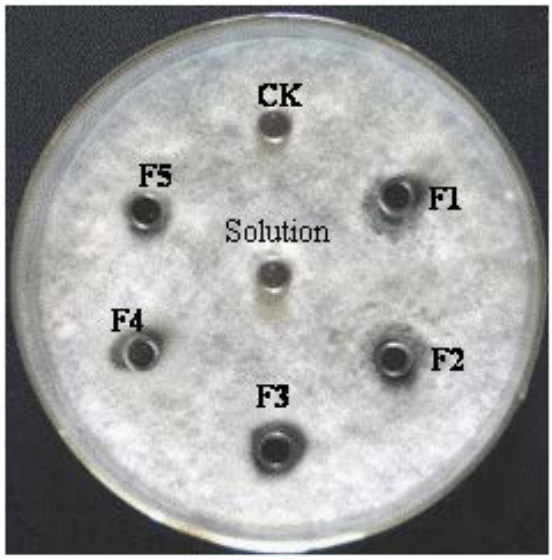

Figure 3. Effect of the five components on *Colletotrichum gloeosporioides* RC178 from a rubber tree.

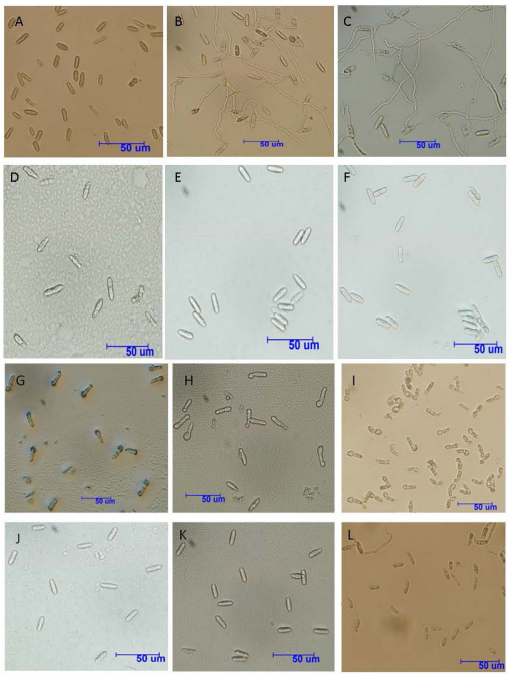

Figure 4. Influence of different diluent concentrations on the conidium germination of *Colletotrichum gloeosporioides* from a rubber tree. **A.** normal spores; **B.** no-treatment control; **C.** methyl alcohol/chloroform (1:1); **D.** two-fold dilution of F1; **E.** four-fold dilution of F1; **F.** six-fold dilution of F1; **G.** two-fold dilution of F3; **H.** four-fold dilution of F3; **I.** six-fold dilution of F3; **J.** two-fold dilution of F5; **K.** four-fold dilution of F5; and **L.** six-fold dilution of F5.

MALDI-TOF-MS analysis

The MALDI-TOF-MS analysis of the Sephadex G-25-purified fractions of crude antifungal extract obtained from the liquid culture-grown supernatant of *B. subtilis* Czk1 revealed the presence of compounds with molecular mass to charge number ratio (m/z) ranges of 994-1065, 1028-1109, and 1421-1566, which we attributed to surfactins, iturins, and fengycins, respectively (Kowall et al., 1998; Vater et al., 2002; Williams and Brodbelt, 2004; Gong et al., 2006; Romero et al., 2007; Pathak et al., 2012). Fractions 1-5 contained molecules with m/z ratios of 1072.6, 1542.6, 1031.4, 1049.4, 1081.5, 1109.5, 1485.7, 1528.7, 1123.5, 1086.6, 1499.7, and 1556.7 (Figure 5). There were two isomers of iturin B (m/z ratios 1072.6 and 1086.6), two isomers of fengycin B (m/z ratios 1542.6 and 1556.7), one isomer of bacillomycin D (m/z ratio 1031.4), one isomer of surfactin (m/z ratio 1049.4), two isomers of iturin A (m/z ratios 1109.5 and 1123.5), and three isomers of fengycin (m/z ratios 1485.7, 1499.7, and 1528.7). We also detected a set of m/z ratios (with a corresponding mass difference of 22 Da) that we putatively ascribed to sodium adducts of bacillomycin D (m/z ratio 1081.5) (He, 2002).

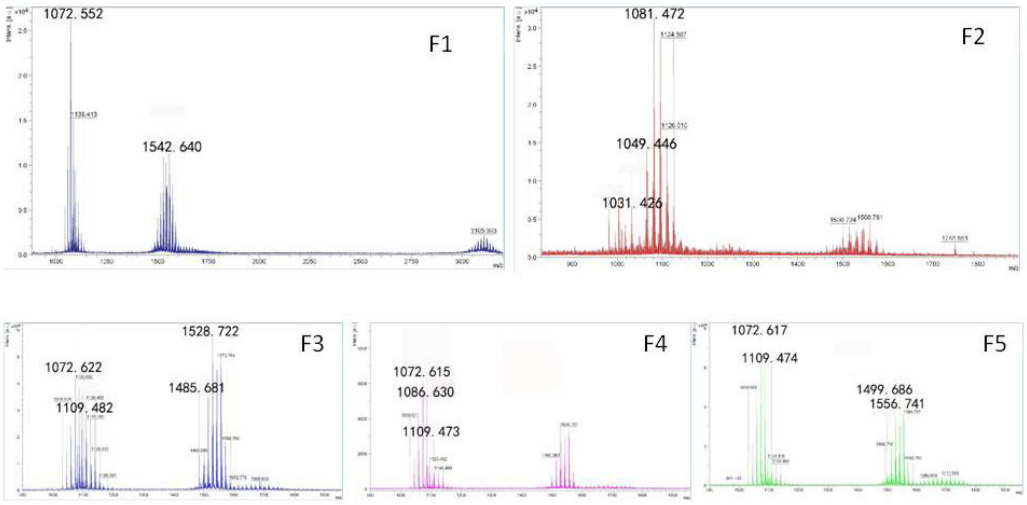

Figure 5. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectra for the five samples: F1, F2, F3, F4, and F5.

DISCUSSION

Plant diseases must be controlled by applying biological preparations to reduce costs, address environmental concerns, and protect health. The efficacy of antagonistic organisms that protect plants from root rot disease has been evaluated. For instance, Chakraborty et al. (2009) reported that *Ochrobactrum anthropi* TRS-2 isolated from tea rhizospheres solubilized phosphates, produced siderophores and indole-3-acetic acid *in vitro*, and exhibited antifungal activity against six test pathogens. *O. anthropi* treatment also decreases the incidence of brown root rot caused by *P. noxius* in tea (Chakraborty et al., 2009). With regard to rubber root disease, *Trichoderma* isolates obtained from rubber-growing areas have antagonistic effects

on *Rigidoporus microporus*, and *Trichoderma harzianum* can also control *R. microporus in vitro* (Jayasuriya and Thennakoon, 2007). The *in vitro* and *in vivo* control of fungal antagonists has been evaluated against *R. lignosus* in *H. brasiliensis* (Willd. ex A. Juss.) Muell. Arg. in a rainforest in Nigeria. The dual inoculations of the fungal antagonists and *R. lignosus* inoculated on the same day differed significantly from the antagonists inoculated 24 h before inoculation with *R. lignosus*. Among the biocontrol agents, *Hypocrea jecorina* is most effective for controlling *R. lignosus*, with a percentage inhibition of 86.83%. *Hypocrea virens*, *Hypocrea jecorina*, *Trichoderma* spp including *Trichoderma spirale* and *Trichoderma* Pers, and *Hypocrea lixii* are also effective against *R. lignosus* (Ogbebor et al., 2015). In the first step of biocontrol, microbes from the environment, such as soil or plants, are isolated and identified; in the second step, pure cultures are examined to determine their antagonistic effects. In this study, we identified peptide-related genes using PCR and antibiotic substances using MALDI-TOF-MS. The *B. subtilis* Czk1 genes related to the synthesis of surfactins, iturins, and fengycins were amplified by PCR, and the signals generated by these substances were detected by MALDI-TOF-MS. We found that *B. subtilis* Czk1 produces lipopeptide antibiotics such as surfactins, iturins, and fengycins. Interesting results can be obtained using PCR and MALDI-TOF-MS. For example, Li (2009) found that the two methods produced different results when applied to the lipopeptides from *B. subtilis* 7Ze3; the genes related to surfactin synthesis were not detected by PCR amplification, but surfactins were detected by MALDI-TOF-MS. *B. subtilis* YB-05 has also been isolated from wheat rhizosphere soil, and the results of analysis revealed that this strain strongly inhibits the wheat take-all pathogen *Gaeumannomyces graminis* var. *tritici*. Seven primer pairs have also been evaluated to detect antifungal genes; four have been detected in *B. subtilis* YB-05 but the other three genes were not found. When *B. subtilis* YB-05 was grown in liquid culture medium under specific conditions, six main types of antifungal substances were detected through analysis by mass spectrometry (Yang et al., 2015). The difference between the number of antifungal genes and the number of the expressed products in the culture supernatant can be attributed to the specific liquid culture growth conditions that may not have permitted the production of the final products of the hyphae (Yang et al., 2015). Therefore, PCR can provide the basis for follow-up to MALDI-TOF-MS analysis. However, false-positive results may be obtained for the identification of peptide-related genes if PCR alone is used. The accuracy of peptide-related gene detection is probably improved by primary identification through PCR and further detection through MALDI-TOF-MS, because the latter process is acutely sensitive and highly efficiency.

The number and type of antimicrobial peptide genes may vary in *B. subtilis* strains. Yang et al. (2015) found that *B. subtilis* YB05 contains at least four antifungal genes (*fenB*, *ituA*, *hag*, and *tas*); the other antifungal genes tested (*spaS*, *sfp*, and *mycB*) may also be present but were not detectable using the primers and PCR conditions used in this study. The antimicrobial peptide (AMP) biosynthetic genes *srfAA* (surfactin), *bacA* (bacilysin), *fenD* (fengycin), *bmyB* (bacillomycin), *spaS* (subtilisin), and *ituC* (iturin) have been examined in 184 *Bacillus* spp isolates obtained from plant environments, including aerial, rhizosphere, and soil environments in Spain (Mora et al., 2011). Most of the strains comprise two or four AMP genes; strains with five genes are rarely detected, and none of the strains contains six genes (Mora et al., 2011). By contrast, *B. subtilis* A1/3 shows exceptionally diverse antibiotic capacity; this strain also comprises six AMP genes, namely, *srf* (surfactin), *bacA*, *fenD*, *bmyB*, *spaS*, and *ituC* (iturin) (Hofemeister et al., 2004). In our study, four primer pairs were designed to detect antifungal genes in *B. subtilis* Czk1. The results showed that four specific

PCR primers, which targeted the flanking region of the gene-coding regions, amplified the predicted fragments from the genomic DNA of *B. subtilis* Czk1. This finding indicates that *B. subtilis* Czk1 carries *sfp*, *fenB*, *ituD*, and *lpa*.

Bacterial strains produce various antimicrobial biosurfactants such as the lipopeptide antibiotics classified as iturins or surfactins (Chen and Hoover, 2003). SK.DU.4, a novel strain identified during the screening of soil bacteria for antimicrobial substance production, produces bacteriocin-like peptide and lipopeptide (Baindara et al., 2013). Three kinds of antibiotics have also been isolated from *B. subtilis* JA, and include the lipopeptide family surfactin, iturin, and fengycin (Chen et al., 2008). However, the co-production of lipopeptides causes purification problems (Akpa et al., 2001). In our study, four main types of antifungal substances were detected by MALDI-TOF-MS after liquid culture of strain Czk1 under specific conditions: isomers of iturin (iturin A, iturin A1, iturin B, and iturin B1), fengycin (fengycin, fengycin1, fengycin2, fengycinB1, and fengycin B2), bacillomycin (bacillomycin D1 and bacillomycin D2), and surfactin. Surfactin, fengycin, and iturin were produced by *B. subtilis* Czk1, and five active fractions were separated. The active fractions were mainly associated with F3; the other fractions had an inhibitory effect on the plate and inhibited *C. gloeosporioides* conidium germination to some extent; at high concentrations, the top of the conidium expanded and became malformed. Therefore, the type and content of bacteriostatic substances should be investigated further because the five fractions detected by MALDI-TOF-MS comprise more than one component. Further investigations are underway to promote *B. subtilis* Czk1 in agricultural applications.

Conflicts of interest

The authors declare no conflict of interest.

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