Fungal Biology 123 (2019) 51–58

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

Characterization of phytotoxin and secreted proteins identifies of *Lasiodiplodia theobromae*, causes of peach gummosis

Zhi Li [a], He Zhang [b], Guohuai Li [b, *]

[a] State Key Laboratory of Crop Stress Biology in Arid Areas, College of Horticulture, Northwest A&F University, Yangling, Shaanxi, 712100, China
[b] Key Laboratory of Horticultural Plant Biology, Ministry of Education, Huazhong Agricultural University, Wuhan, 430070, China

ARTICLE INFO

Article history:
Received 3 May 2018
Accepted 6 November 2018
Available online 14 November 2018

Nicholas P. Money

Keyword:
Extracellular proteins
Gene expression
Prunus persica
Secretome

ABSTRACT

Lasiodiplodia theobromae, a phytopathogenic fungus that causes peach gummosis, is considered one of the major constraints to peach production worldwide. Here, we report the characteristics of toxic metabolites and the proteomics investigation of the secreted proteins of *L. theobromae* from its *in vitro* culture. The phytotoxins of *L. theobromae* from the culture filtrate of Richard's liquid medium showed high toxicity on peach current year shoots with large lesion diameter and high gum weight. The toxicity measurement showed that 23.6 and 21.2 mg gum were induced from peach shoots by solvent fractions of ethyl acetate and dichloromethane, respectively, with significant differences from other organic solvents. A total of 23 proteins were identified by liquid chromatography–mass spectrometry from the *in vitro* secretome of *L. theobromae*. Sequence analysis indicated that 14 proteins were extracellular proteins based on signal peptides and localization. The expression profiles of the analyzed fungal genes were significantly upregulated from 1 day postinoculation (dpi) to 2 dpi, indicating that the early stage is an important stage for the infection of *L. theobromae*. The present study has provided insights into the extracellular phytotoxins and secreted proteins that are possibly associated with pathogenicity of the peach gummosis.

1. Introduction

Botryosphaeria spp. are common plant pathogens and saprobes detected in economically important woody species (Srivastava et al., 2013), such as peach gummosis (Li et al., 2014), apple ring rot (Tang et al., 2012), grape canker disease (Auger et al., 2004), and mango dieback (Rodríguez-Gálvez et al., 2017). Peach gummosis is one of the most important and damaging diseases of peach in China, Japan, and the United States (Reilly and Okie, 1982; Wang et al., 2011). Three species of *Botryosphaeria*, i.e., *Lasiodiplodia theobromae*, *Botryosphaeria obtusa*, and *Botryosphaeria dothidea*, have been reported in cases of peach gummosis (Britton and Hendrix, 1982), the symptom of which is gum exudates from cracked warts on the trunk and major branches of peach (Biggs and Britton, 1988). Among these three species, *L. theobromae* was the most virulent (Wang et al., 2011). However, little is known about the virulence factors of *L. theobromae*.

Fungal metabolites are one of the important virulence factors during infection of plant cells (Brock, 2009). Among the fungal metabolites, phytotoxic metabolites of fungi play a key role in infection and virulence, such as inhibiting the activity of enzymes, altering the gene expression levels, disrupting the biosynthesis of crucial metabolites, and undermining the membrane integrity of host cells (Möbius and Hertweck, 2009). In recent years, a large number of novel phytotoxins have been isolated and structurally characterized in *B. obtusa* (anamorph *Diplodia seriata*) and *B. dothidea* (anamorph *Fusicoccum aesculi*). Several phytotoxins, namely, mullein, 4-hydroxymellein, 5-hydroxymellein, tyrosol, and 4-hydroxybenzaldehyde, were identified from the culture filtrate of *B. obtusa* (Djoukeng et al., 2009; Venkatasubbaiah and Chilton, 1990). A total of 18 metabolites have been identified from the culture filtrate of *B. dothidea*, and some metabolites exhibited strong cytotoxicity against cancer cells (Xiao et al., 2014). Only one phytotoxin of *L. theobromae* was identified, i.e., (3S,4R)-3-carboxy-2-methylene-heptan-4-olide, which caused black spots on the peel of bananas (He et al., 2004). However, different cultural conditions corresponded to various methods for the culture filtrates. For example, *B. obtusa* from grape was inoculated in Czapek–Dox

* Corresponding author. Fax: +27 87287735.
E-mail address: liguohuai@mail.hzau.edu.cn (G. Li).

https://doi.org/10.1016/j.funbio.2018.11.001
1878-6146/

medium for 14 d (Martos et al., 2008), whereas *B. obtusa* from apple was inoculated in potato dextrose broth (PDB) medium for 21 d (Venkatasubbaiah et al., 1991). Phytotoxicity also showed various virulence factors using different organic solvents for extraction (Vikrant et al., 2006; Zheng et al., 2010). Our previous study showed that the culture filtrate of *L. theobromae* can also cause obvious symptoms of peach gummosis (Li et al., 2016). However, the characteristics of toxic metabolites are still unknown.

Several extracellular proteins have also been identified as virulence factors by secreting and translocating into host cells (Petre and Kamoun, 2014). Identifying the secretome of phytopathogenic fungi may provide evidence for the infection mechanism and provide information for the development of disease management strategies. Historically, the genome, transcriptome, and proteomics of phytopathogenic fungi have been used to screen the secreted proteins. For *D. seriata*, 75 secreted proteins were identified from liquid cultures using two-dimensional gel electrophoresis, and some of these proteins have been identified as virulence factors in other phytopathogenic fungi (Cobos et al., 2010). Exposure of *Vitis* cells to secreted proteins of *D. seriata* produced stilbene, reactive oxygen species (ROS), and defense responses (Stempien et al., 2017). To date, no detailed secretome analysis has been reported for *L. theobromae*.

The aim of our work is to explore the virulence factors of *L. theobromae* by investigating the characteristics of toxic metabolites and identifying secretory proteins of *L. theobromae* by mass spectrometry (MS). These findings will extend our knowledge on the pathogenic mechanism of *L. theobromae* in peach shoots.

2. Materials and methods

2.1. Fungal cultures and toxin production

Following the methods of Abdou et al. (2010), *L. theobromae* strain JMB-122 was grown on potato dextrose agar (PDA) medium at 26 °C for 3 d. For toxin production, six 4 mm-diameter mycelial plugs from PDA cultures were transferred into 500 mL flask containing 200 mL of Czapek's medium (Czapek), PDA liquid medium (PDA), Fries' basic medium (Fries), potato sucrose agar liquid medium (PSA), or Richard's liquid medium (Richard). The cultures were then incubated at 26 °C on a water bath shaker (70 rpm) for 21 d. Culture filtrates were obtained through four layers of cheesecloth and Whatman no. 1 filter paper and centrifuged at 4000×*g* for 15 min. The supernatant was filtered using 0.45 μm sterile microfilters. Unless otherwise stated, Richard culture was used for all subsequent tests.

2.2. Solvent extraction

Extraction assay was conducted according to the methods of Zheng et al. (2010). Then, 800 mL of the culture filtrate was extracted thrice with half volumes of chloroform, ethyl acetate, tetrachloride, petroleum ether, diethyl ether, and dichloromethane using a separatory funnel. The water and solvent fractions were evaporated at 55 °C. The dry fraction was dissolved in sterile distilled water and adjusted to a concentration of 300 μg/mL solution. Each solvent extraction was repeated thrice. Their toxicity was measured subsequently using the peach shoot inoculation assay.

2.3. Effect of treatments on toxin activity

The solvent fraction (300 μg/mL) of ethyl acetate was used for the treatment assays. For protease treatment, the final concentration with 50 μg/mL proteinase K (Aladdin, Shanghai, China) and 250 μg/mL trypsin (Aladdin) were used to treat 50 μL of the solvent fractions for 1 h at 37 °C. Two nucleotidases, DNase I (5 U/μL; Takara, Dalian, China) and RNase H (5 U/μL; Takara), were also used to treat the solvent fractions for 30 min at 37 °C. For the thermostability of toxins, each solvent extraction was subjected to 60 °C, 80 °C, 100 °C, and 121 °C for 30 min in a water bath or autoclave. Each treatment was performed thrice. The biological activities of the treatments were measured subsequently using the peach shoot inoculation assay.

2.4. Shoot inoculation assay

Biological activity assay was performed according to the method of Zheng et al. (2010). Culture filtrates, water and organic solvent extractions, and treated extractions were filtered with a 0.22 μm filter. Biological activities were determined by peach current year shoots (cv. Spring Snow) by a detached assay (Li et al., 2016). The shoots were cut into 15 cm-long segments. The segments were surface sterilized with 70 % alcohol for 10 s, rinsed thrice with sterile water, and wounded at the middle point using a sterilized needle. All of the solutions were adjusted to a concentration of 300 μg/mL. Then, 10 μL of each solution was inoculated in the wound sites. The shoots were placed in a plastic box and covered with a plastic film. Inoculated shoots were incubated in a chamber at 26 °C under 12 h light/12 h dark photoperiod. The lesion diameter and gum weight were measured at 4 d postinoculation (dpi). A total of 10 shoots were used in each test, and the tests were repeated thrice. Inoculated and uninoculated culture filtrates served as the controls.

2.5. Protein preparation

Culture supernatants of *L. theobromae* were collected from Richard 15 dpi by passage through four layers of cheesecloth and centrifuged at 4000×*g* for 5 min (4 °C). The supernatant was filtered using 0.45 μm sterile microfilters and concentrated using a 30 kDa centrifugal filter unit (Millipore, Germany) at 4000 rpm (4 °C). Extracellular protein extraction was based on trichloroacetic acid (TCA)–acetone as described by Cobos et al. (2010). The final concentration with 25 % (w/v) TCA was added to the concentrated protein solutions, incubated at −10 °C for 2 h, and centrifuged at 15000×*g* for 10 min (4 °C) to move the supernatant. Precipitated proteins were washed thrice with 80 % (v/v) acetone, and residual acetone was air-dried. Then, the protein pellet was resuspended in 300 μL 8 M urea (100 mM Tris–HCl, pH 8.0) and stored at −80 °C after determining the protein concentration using the 2-D Quant Kit (Amersham, Piscataway, NJ, USA), according to the manufacturer's instructions.

2.6. MS and peptide identification

Proteins were diluted to 2 M urea, added to 1 mM $CaCl_2$, and digested with trypsin overnight at 37 °C (Fonslow et al., 2013). The digested fractions were desalted using C18 spin columns (Varian, Lake Forest, CA, USA), quantified with the Quantitative Colorimetric Peptide Assay Kit (Pierce; Thermo Scientific), and redissolved with 0.1 % formic acid at a concentration of 0.2 μg. Liquid chromatography (LC; EASY-nLC 1000; Thermo Scientific) and MS (Orbitrap Fusion; Thermo Scientific) were used for the analysis of peptide fractions. Acclaim PepMap C18 column (75 μm × 500 mm; Thermo Scientific) was loaded and used to separate the peptides. The parameters for LC-MS were determined, as described previously (Cooper et al., 2016). The acquired MS/MS spectra were searched against Dothideomycetidae in the National Center for Biotechnology Information (NCBI) nonredundant protein database using

the MaxQuant software (ver. 1.5.3.30; Max-Planck Ins., Germany). The search parameters, i.e., parent ion mass tolerance (±10 ppm), variable modifications (methionine, M), mass values (mono-isotopic), and false discovery rate (<0.01), were fixed. At least two unique peptides were required to assign a confident protein (Wang et al., 2013). The peptide sequences and matching proteins are provided (Supplementary Table 1; Supplementary List 1).

2.7. Sequence analysis

The identified proteins in this study were subjected to multiple bioinformatics tools to predict secretory proteins. Signal peptides were predicted using SignalP 4.1 (Petersen et al., 2011) and TargetP 1.1 (Emanuelsson et al., 2000). Extracellular proteins were determined using SecretomeP 1.0 (Bendtsen et al., 2004), TargetP 1.1, and WoLF PSORT (https://www.genscript.com/wolf-psort.html). Conserved functional domains were annotated using the PFAM database and CD Search of NCBI (Finn et al., 2016; Marchler-Bauer et al., 2016).

2.8. Gene expression profiling

The transcripts levels of the secreted proteins were determined by qRT-PCR to investigate whether the identified secretory proteins are expressed by *L. theobromae* during its infection in peach shoots. The inoculation procedure was conducted according to the methods used in a previous report (Li et al., 2014). Samples were collected at 1, 2, 3, 4, and 5 dpi, and the total RNA was extracted using an EASY Spin Plus RNA Kit (Aidlab, Beijing, China). mRNA samples isolated from uninoculated peach shoots were included as negative controls (C1), and mRNA samples isolated from 3-day-old fungal mycelium on PDA were included as controls (C2). cDNA synthesis and qRT-PCR were also performed as described by Li et al. (2016). Primers were designed based on coding sequences from the NCBI database (https://www.ncbi.nlm.nih.gov/) and listed in Supplementary Table 2. Glyceraldehyde-3-phosphate dehydrogenase gene of *L. theobromae* (GenBank accession number GU251384) was used as internal control for normalization. Relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). qRT-PCR results were expressed as the mean ± SD of three independent biological replicates.

2.9. Statistical analysis

Student's *t* tests were used for the statistical analysis of the lesion diameter, gum weight, and gene relative expression level. Statistically significant differences were assessed by testing for the least significant difference ($P < 0.05$).

3. Results

3.1. Toxin production and characterization of L. theobromae

Five different liquid media were used to examine the toxicity on peach current year shoots to obtain the optimal liquid media. Culture filtrates of *L. theobromae* from 21-day-old PSA, PDA, Fries, Czapek, and Richard resulted in lesion diameters of 3.1, 4.8, 8.1, 4.5, and 5.0 mm, respectively (Fig. 1A and B). However, visible peach gum was only induced by the culture filtrates of Richard and Czapek, and symptoms similar to those of peach gummosis were caused by the fungal organisms. Notably, the culture filtrates of Richard significantly produced more gum than filtrates from Czapek (Fig. 1C). Therefore, we selected Richard for the subsequent tests.

Then, the filtrates of Richard were extracted by six organic solvents, namely, petroleum ether, diethyl ether, dichloromethane, chloroform, carbon tetrachloride, and ethyl acetate, and the toxins were detected in water and solvent fractions (Supplementary Table 3). Visible lesions were observed in the shoot inoculation assay after treatment with most of the fractions, but the solvent fraction of petroleum ether and carbon tetrachloride caused no lesion. The toxicity measurement showed that 23.6 and 21.2 mg gum were induced from peach shoots by solvent fractions of ethyl acetate and dichloromethane, respectively (Fig. 2A), whereas their water fraction caused no peach gum. Filtrates from uninoculated Richard caused no lesions on peach shoots. Hence, all subsequent tests were conducted with ethyl acetate extraction.

The toxicity of *L. theobromae* was not affected by proteinases (proteinase K and trypsin) and nucleases (Fig. 2B). The thermal stability presented in Fig. 2C showed that the toxicity of *L. theobromae* did not change and was stable at 60 °C, 80 °C, and 100 °C but unstable at 121 °C.

3.2. Detection of extracellular secreted proteins of L. theobromae

The *in vitro* secretome of *L. theobromae* was obtained by the culture filtrate proteins from liquid Richard inoculated with the JMB-122 isolate at 15 d. A total of 23 proteins that were identified by EASY-nLC-MS/MS analyses are numbered and listed in Table S1 and showed a sequence coverage of 2.4 %–24.7 % and scores between 11.5.3 and 323.3 by MS/MS peptide fragment fingerprinting. The 23 proteins were matched to the annotated proteins of three species of *Botryosphaeria* (Supplementary List 1), namely, *D. seriata* (13), *Macrophomina phaseolina* (5), and *Neofusicoccum parvum* (5). Fourteen of the proteins visualized in the secretome were predicted to be secreted as determined by the SignalP 4.1 and TargetP tools. Those proteins were also predicted by SecretomeP, TargetP, and WoLF PSORT and indicated in the extracellular location (Table 1). The functional distribution of the secreted proteins of *L. theobromae* (LtSP) is consonant to what was described for the best-matched proteins of other filamentous fungi. Those proteins included carbohydrate metabolism (LtSP1, LtSP4, LtSP7, LtSP12, LtSP14, and LtSP15), protein metabolism (LtSP11 and LtSP13), oxidoreductase (LtSP3, LtSP6, and LtSP9), GPI-anchored cell wall organization protein (LtSP2), FAD-linked oxidase (LtSP10), and putative F5/8 type C domain protein (LtSP8).

3.3. Secreted proteins of L. theobromae with homologies to proteins known to be involved in plant pathogenesis

BLAST searches indicated that four LtSPs contain conserved functional domains in fungal proteins known to be involved in plant pathogenesis (Supplementary Table 4). The identified secreted protein GMC oxidoreductase (LtSP9) contained a conserved GMC-oxred-C domain with homologies to GGOX, which generated H_2O_2 and encoded a glucose oxidase (GOX) of *Aspergillus niger* (Wu et al., 1995). Aspartic proteinase (LtSP11) was determined to be homologous to the eukaryotic aspartyl protease domain as represented by secreted aspartic proteinase from *Botrytis cinerea*, which caused extensive cell death in carrot tissue and carrot suspension cells (Movahedi and Heale, 1990). Peptidase s8/s53 subtilisin (LtSP13) contained a conserved peptidase S8 domain with homologies to Mp1 of *Magnaporthiopsis poae* known to be involved in pathogenesis (Sreedhar et al., 1999). LtSP15 contained a conserved lactonase domain with homologies to AltA, a fungal protein from *Penicillium oxalicum*, which has been shown as an efficient supplement of cellulolytic enzyme system for lignocellulose biodegradation (Peng et al., 2017).

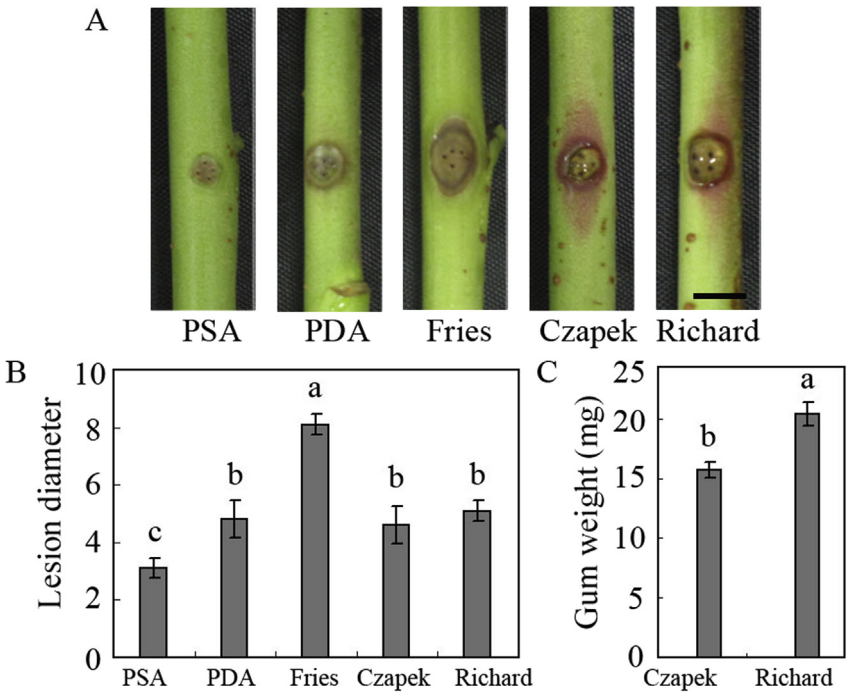

Fig. 1. Effect of different culture filtrates of *L. theobromae* on the pathogenicity of peach shoots. (**A**) The symptoms of peach shoots after inoculation with five culture filtrates of *L. theobromae* at 4 d postinoculation (dpi). Bar = 5 mm. (**B**) Lesion diameter on inoculated peach shoots after treatment with five culture filtrates of *L. theobromae* at 4 dpi. (**C**) Gum weight harvested from diseased shoots at 4 dpi after inoculation with Czapek and Richard culture filtrates of *L. theobromae*. Different letters above the columns indicate statistically significant differences. Significant differences ($P < 0.05$) were determined by Student's *t* test.

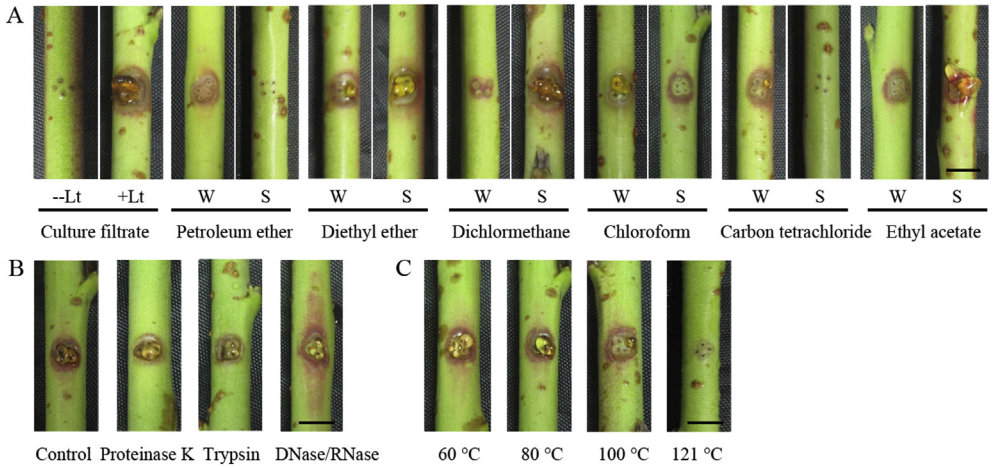

Fig. 2. Effect of different solvent extractions, enzyme treatments, and heat treatments on the toxin activity of *L. theobromae*. (**A**) Phytotoxicity on peach shoots of crude toxin extracted with various organic solvents from the culture filtrates of *L. theobromae* at 4 dpi. (**B**) Toxin activity on peach shoots treated with protease and nucleotidase at 4 dpi. (**C**) Toxin activity after treatment at 60 °C, 80 °C, 100 °C, and 121 °C for 30 min. Bar = 5 mm.

3.4. Expression patterns in peach shoots

The expression patterns of four secreted proteins of *L. theobromae* were analyzed in infected peach shoots from 1 dpi to 5 dpi using qRT-PCR to determine whether the *in vitro* secreted proteins of *L. theobromae* are expressed during its dynamic interaction with peach (Fig. 3). The results of expression profiling showed that GMC oxidoreductase (LtSP9) was significantly upregulated at the early stages (1 and 2 dpi) (Fig. 3A). Aspartic proteinase (LtSP11), peptidase s8/s53 subtilisin kexin sedolisin (LtSP13), and hypothetical protein (LtSP14) were consistently upregulated from 1 dpi to 5 dpi with a peak at 2 dpi, and the expression gradually decreased after 3 dpi throughout the infection stages (Fig. 3B, C, and D). The expression level of GPI-anchored cell wall organization protein ecm33 (LtSP2) was upregulated at 1 dpi but apparently downregulated at later stages (3 dpi to 5 dpi) (Supplementary Fig 1). These results indicate that *L. theobromae* might preferentially express and regulate the expression of these secreted proteins, which appeared to correlate with the development of peach gummosis.

4. Discussion

The production of fungal phytotoxin depended on the culture conditions, such as the type of medium, days of culture, and methods of extraction. For the *Botryosphaeria* species, the

Table 1
Prediction of signal peptides and localization of the identified *in vitro* secreted proteins of *L. theobromae*.

Protein	Signal peptides		Localization prediction			Pfam domain[f]	Protein description
	SignalP[a]	TargetP (SP)[b]	SecretomeP[c]	TargetP (Loc)[d]	WoLF PSORT[e]		
LtSP1	0.906	0.948	Extr = 0.833 S	Extr = 27		Glycosyl hydrolases family 35 (PF01301)	Glycoside hydrolase family 35
LtSP2	0.533	0.459	Extr = 0.935 S	Extr = 27		no	Gpi-anchored cell wall organization protein ecm33
LtSP3	0.761	0.790	Extr = 0.910 S	Extr = 25		Catalase (PF00199)	Mycelial catalase cat1
LtSP4	0.738	0.717	Extr = 0.942 S	Extr = 26		Glycosyl hydrolases family 17 (PF00332)	Glycoside hydrolase family 17
LtSP6	0.680	0.803	Extr = 0.810 S	Extr = 16		Glutathione S-transferase (PF13409)	Ser/thr protein phosphatase
LtSP7	0.760	0.823	Extr = 0.937 S	Extr = 26		no	Cell wall protein
LtSP8	0.716	0.962	Extr = 0.953 S	Extr = 26		F5/8 type C domain (PF00754)	Putative f5/8 type c domain protein
LtSP9	0.820	0.938	Extr = 0.949 S	Extr = 26		GMC_oxred superfamily (PF00732)	GMC oxidoreductase
LtSP10	0.931	0.936	Extr = 0.537 S	Extr = 23		FAD binding domain (PF01565)	FAD linked oxidase
LtSP11	0.758	0.950	Extr = 0.934 S	Extr = 26		no	Aspartic proteinase
LtSP12	0.758	0.793	Extr = 0.954 S	Extr = 25		Glycosyl hydrolases family 16 (PF00722)	Putative cell wall
LtSP13	0.729	0.896	Extr = 0.955 S	Extr = 24		Subtilase family (PF00082)	Peptidase s8/s53 subtilisin kexin sedolisin
LtSP14	0.478	0.640	Extr = 0.907 S	Extr = 24		Lactonase (PF10282)	Extracellular aldonolactonase protein
LtSP15	0.741	0.877	Extr = 0.902 S	Extr = 26		Cellulose-binding domain (PF00734)	Cellulose-binding domain protein

[a] Signal peptide probability computed by SignalP 4.1.
[b] Signal peptide predicted with TargetP 1.1. The numbers represent probability of signal peptide.
[c] Predicting the secretome with programs of SecretomeP 2.0. Extr = Extracellular.
[d] Subcellular location of eukaryotic proteins predicted with TargetP 1.1. S indicates secreted protein.
[e] Predicting the subcellular localization with WoLF PSORT. Extr = Extracellular.
[f] PFAM domains were annotated using PFAM database (e-value <0.1).

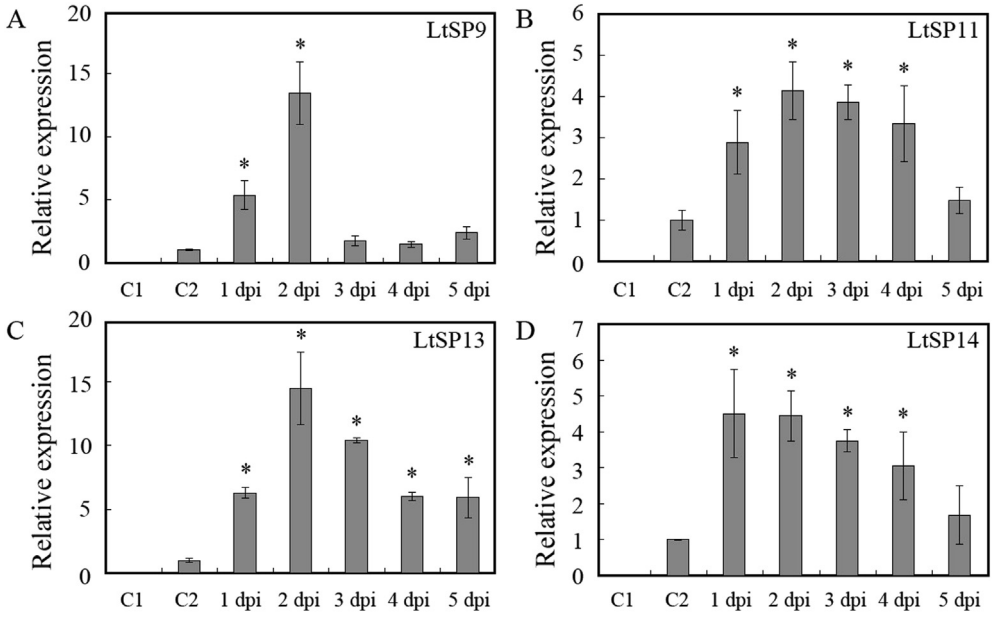

Fig. 3. In planta expression patterns of four *L. theobromae*-secreted proteins (**A**, LtSP9; **B**, LtSP11; **C**, LtSP13; **D**, LtSP14) were determined using quantitative RT-PCR. The relative gene expression levels of uninoculated peach shoots (C1) were considered the negative control and those of 3-day-old fungal mycelium on PDA (C2) were considered the control. Data values are presented as the mean ± SD from three independent experiments. Asterisks indicate significant differences ($P < 0.05$, Student's t test).

phytotoxicity of *B. dothidea* reached a maximum after 14 d, whereas *B. obtusa* exhibited maximum phytotoxic damage after 21 d in Czapek—Dox broth (Martos et al., 2008). PDB medium was the most common medium for liquid culture of *B. dothidea* and *B. obtusa* in many reports (Djoukeng et al., 2009; Venkatasubbaiah and Chilton, 1990; Xiao et al., 2014). In this study, although all five kinds of media caused necrosis in peach shoots, culture filtrates showed the highest phytotoxicity after 21 d in liquid cultures of Richard. Our results support previous reports that the ethyl acetate extract showed significantly higher toxicity than other organic solvents (Singh et al., 2010; Zheng et al., 2010). Dichloromethane also exhibited high phytotoxicity in peach shoots as a candidate for solvent extraction. Extracellular proteins and nucleic acids have been reported as virulence factors (Ahn and Lee, 2001; Bénard-Gellon et al., 2015). Proteases and nucleic acidases were used to treat the phytotoxin of *L. theobromae* to exclude the potential effects of extracellular proteins and nucleic acids. The results showed that the phytotoxin of *L. theobromae* was unchanged after the treatments. Notably, the lesion diameter reached 2 cm after inoculation with the mycelium of *L. theobromae* in our previous work (Li et al., 2014), but only 7.6 mm of lesion diameter was observed after inoculation with the phytotoxin of *L. theobromae* in this study. Thus, in addition to the phytotoxin, other pathogenic factors, such as secreted extracellular proteins of *L. theobromae*, may exist.

Fourteen extracellular proteins of *L. theobromae* were identified in this study by secretome analysis (Table 1). Among the proteins identified, three proteins involved in plant cell wall degradation were identified, including glycosyl hydrolase family 35 (LtSP1),

extracellular aldonolactonase protein (LtSP14), and cellulose-binding domain protein (LtSP15). Two proteins were involved in fungal cell wall modification: glycosyl hydrolase family 17 (LtSP 4) and cell wall protein (LtSP7). Catalase (LtSP3) and GMC oxidoreductase (LtSP9) were involved in the detoxification and generation of ROS. Aspartic proteinase (LtSP11) and peptidase s8/s53 subtilisin kexin sedolisin were involved in degrading plant antimicrobial proteins. In addition to those secreted proteins, we also identified two pathogenicity-related proteins, GPI-anchored cell wall organization protein ecm33 (LtSP2) and Ser/Thr protein phosphatase (LtSP6). For example, CaEcm33p, a GPI-anchored protein of *Candida albicans*, has been reported to be required for virulence (Martinez-Lopez et al., 2004). Ser/Thr protein phosphatase also played a role in regulating the signaling pathways of the infected host cell (Wehenkel et al., 2008). The functional distribution of the extracellular proteins of *L. theobromae* is consonant to proteins that were known to be involved in plant pathogenesis from other filamentous fungi, such as cell wall degradation proteins, peptidases, and anti-oxidative/detoxifying proteins and virulence-associated proteins.

Pathogens secreted numerous proteins to degrade the cell wall of the host during the early infection processes and under nutrient starvation. In this study, glycosyl hydrolase family 35 (LtSP1) was detected through secretome analysis and involved in the degradation of hemicellulose and pectin of plant cell wall (Zhao et al., 2013). The identified LtSP15 also contained a cellulose-binding domain, which was required for cellulosome complexes to degrade the cellulose (Doi and Kosugi, 2004), indicating its role in degrading plant cell wall. Although the biochemical function of the lactonase domain protein is still unknown, a fungal protein AltA from *P. oxalicum* has been shown to be an efficient supplement of the cellulolytic enzyme system for lignocellulose biodegradation (Peng et al., 2017). Our results indicated that the lactonase-domain-containing LtSP14 is upregulated from 1 dpi to 4 dpi (Fig. 3B). This protein may play an auxiliary role in the degradation of peach cell wall. Interestingly, two fungal cell wall degradation proteins, LtSP4 and LtSP12, were also detected in our results based on the conserved domain (Zhao et al., 2013) and seemed to protect the fungus from other fungi. However, LsSP12 was determined to be homologous to the glycosyl hydrolase family 16 as represented by Crh1p and Crh2p proteins, which are required for the cross-linking of chitin to β(1–6)glucan in the fungal cell wall of *Saccharomyces cerevisiae* (Cabib et al., 2007). Our previous results showed that the relative expression level of chitinase increased in the inoculated peach shoots (Li et al., 2014). Thus, those proteins may play an important role in repairing the cell wall of *L. theobromae* with exposure to chitinase of peach.

Fungal pathogens secrete proteinases to degrade plant antimicrobial proteins and promote disease development. Two proteinase proteins, aspartic proteinase (LtSP11) and peptidase s8/s53 subtilisin (LtSP13), were also identified from the *L. theobromae in vitro* secretome and significantly expressed in peach shoots from 1 dpi to 5 dpi (Fig. 3C). Similarly, secreted aspartic proteinase was reported as a virulence factor of *C. albicans* during its infections (Hube et al., 1997). Another peptidase s8/s53 subtilisin (LtSP13) contained a conserved subtilase family, which has been associated with the pathogenicity of fungi to plant. For example, Mp1 protein from *M. poae* is known to be involved in the pathogenesis of grass (Sreedhar et al., 1999). Hence, it is possible that the secrete proteinases identified in our study could be involved in infecting and developing of *L. theobromae* both in the *in vitro* and in planta.

ROS is known to be required for the cross-linking of a hypersensitive response that resulted in the death of cells against biotrophic pathogens, whereas ROS is beneficial to necrotrophic pathogens (Heller and Tudzynski, 2011), such as *L. theobromae*. In this study, the identified secreted protein LtSP9 (GMC oxidoreductase) contained a conserved GMC-oxred-C domain with homologies to GGOX, which generated H_2O_2 and encoded a GOX of *A. niger* (Wu et al., 1995). GOX catalyzes the oxidation of β-D-glucose to H_2O_2 and D-glucono-1,5-lactone, which hydrolyzes spontaneously to gluconic acid (Kiess et al., 1998). In addition to the generation of ROS, gluconic acid of *Penicillium expansum* exhibited pathogenicity in apple (Hadas et al., 2007). In our results, the expression profile of the secreted protein LtSP9 was significantly increased from 1 dpi to 2 dpi. Similarly, induced expression of a secretory glucose oxidase of *Sporisorium scitamineum* was reported in sugarcane at 1 dpi (Barnabas et al., 2017). This protein may play important roles in the interactions of *L. theobromae* with peach. Although the identified LtSP10 contained a FAD-binding domain that is a sequence region of GMC oxidoreductase (Kiess et al., 1998), no evidence showed that it is involved in virulence. A catalase (LtSP3) was also detected in its secretome, indicating that fungal catalase protects the fungus from the ROS of the host. A previous report has shown that detoxification of hydrogen peroxide by the catalase of *Aspergillus fumigatus* was proposed as a method to overcome this host response (Paris et al., 2003). However, catalase has also been shown to be a virulence factor during the process of bacterium–host interaction (Jittawuttipoka et al., 2009; Xu and Pan, 2000). In this study, the identified secreted proteins are perhaps involved in producing and detoxifying ROS to balance redox for the infection process of *L. theobromae*.

The expression of selected secretory proteins in peach shoots confirms the relevance of the approach for the identification of fungal proteins involved in the interaction with peach. The expression profiles of the analyzed fungal genes were significantly increased from 1 dpi to 2 dpi (Fig. 3), indicating that the early stage is an important stage for the infection of *L. theobromae*. By combining the secreted phytotoxins with the functional classification of secreted proteins and its expression profiles, the results have

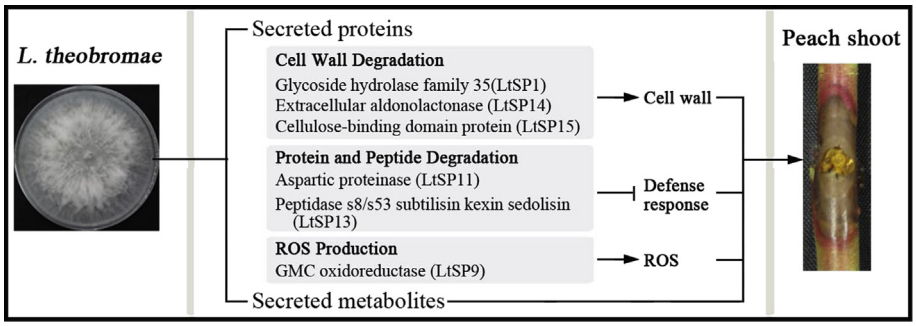

Fig. 4. A predicted model of the phytotoxin and secreted proteins of *L. theobromae* involved in the infection strategies on peach shoots. Proteins of cell wall degradation, protein and peptide degradation, and reactive oxygen species (ROS) detoxification were highly accumulated *in vitro* and in peach shoots.

been brought together to develop a working model for the secretome of *L. theobromae* (Fig. 4). *L. theobromae* secreted extracellular proteins to degrade the cell wall of peach shoots and plant antimicrobial proteins and generate ROS. Extracellular phytotoxins also promote disease development.

In conclusion, the data presented in this study provide important clues for the virulence factors of *L. theobromae*. The phytotoxin and extracellular proteins of *L. theobromae* contributed to the development of peach gummosis. We investigated the characterization of phytotoxin and identified 14 extracellular proteins, which may play important roles in pathogenesis. Further studies are necessary to isolate the key mycotoxins and confirm the function of those pathogenesis-like proteins.

Conflicts of interest

Authors declare no conflict of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31501740 and 31471840) and China Agriculture Research System (Grant No. CARS-30-2-05).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2018.11.001.

References

Abdou, R., Scherlach, K., Dahse, H.M., Sattler, I., Hertweck, C., 2010. Botryorhodines A–D, antifungal and cytotoxic depsidones from *Botryosphaeria rhodina*, an endophyte of the medicinal plant *Bidens pilosa*. Phytochemistry 71, 110–116.

Ahn, I.P., Lee, Y.H., 2001. A viral double-stranded RNA up regulates the fungal virulence of *Nectria radicicola*. Mol. Plant Microbe Interact. 14, 496–507.

Auger, J., Esterio, M., Ricke, G., Pérez, I., 2004. Black dead arm and basal canker of *Vitis vinifera* cv. Red Globe caused by *Botryosphaeria obtusa* in Chile. Plant Dis. 88, 1286–1286.

Barnabas, L., Ashwin, N.M.R., Kaverinathan, K., Trentin, A.R., Pivato, M., Sundar, A.R., Malathi, P., Viswanathan, R., Carletti, P., Arrigoni, G., Masi, A., Agrawal, G.K., Rakwal, R., 2017. *In vitro* secretomic analysis identifies putative pathogenicity-related proteins of *Sporisorium scitamineum*—The sugarcane smut fungus. Fungal Biol 121, 199–211.

Bénard-Gellon, M., Farine, S., Goddard, M.L., Schmitt, M., Stempien, E., Pensec, F., Laloue, H., Mzaet-Kieffer, F., Fontaine, F., Larignon, P., Tarnus, C., Chong, J., 2015. Toxicity of extracellular proteins from *Diplodia seriata* and *Neofusicoccum parvum* involved in grapevine *Botryosphaeria* dieback. Protoplasma 252, 679–687.

Bendtsen, J.D., Jensen, L.J., Blom, N., Von Heijne, G., Brunak, S., 2004. Feature-based prediction of non-classical and leaderless protein secretion. Protein Eng. Des. Sel. 17, 349–356.

Biggs, A.R., Britton, K.O., 1988. Presymptom histopathology of peach trees inoculated with *Botryosphaeria obtusa* and *B. dothidea*. Phytopathology 78, 1109–1118.

Britton, K.O., Hendrix, F.F., 1982. Three species of *Botryosphaeria* cause peach tree gummosis in Georgia. Plant Dis. 66, 1120–1121.

Brock, M., 2009. Fungal metabolism in host niches. Curr. Opin. Microbiol. 12, 371–376.

Cabib, E., Blanco, N., Grau, C., Rodríguez-Peña, J.M., Arroyo, J., 2007. Crh1p and Crh2p are required for the cross-linking of chitin to β (1-6) glucan in the *Saccharomyces cerevisiae* cell wall. Mol. Microbiol. 63, 921–935.

Cobos, R., Barreiro, C., Mateos, R.M., Coque, J.J.R., 2010. Cytoplasmic-and extracellular-proteome analysis of *Diplodia seriata*: a phytopathogenic fungus involved in grapevine decline. Proteome Sci. 8, 46.

Cooper, B., Campbell, K.B., Beard, H.S., Garrett, W.M., Islam, N., 2016. Putative rust fungal effector proteins in infected bean and soybean leaves. Phytopathology 106, 491–499.

Djoukeng, J.D., Polli, S., Larignon, P., Abou-Mansour, E., 2009. Identification of phytotoxins from *Botryosphaeria obtusa*, a pathogen of black dead arm disease of grapevine. Eur. J. Plant Pathol. 124, 303–308.

Doi, R.H., Kosugi, A., 2004. Cellulosomes: plant-cell-wall-degrading enzyme complexes. Nat. Rev. Microbiol. 2, 541–551.

Emanuelsson, O., Nielsen, H., Brunak, S., Von Heijne, G., 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300, 1005–1016.

Finn, R.D., Coggill, P., Eberhardt, R.Y., Eddy, S.R., Mistry, J., Mitchell, A.L., Potter, S.C., Punta, M., Qureshi, M., Sangrador-Vegas, A., Salazar, G.A., Tate, J., Bateman, A., 2016. The Pfam protein families database: towards a more sustainable future. Nucleic Acids Res. 44, 279–285.

Fonslow, B.R., Stein, B.D., Webb, K.J., Xu, T., Choi, J., Park, S.K., Yates III, J.R., 2013. Digestion and depletion of abundant proteins improves proteomic coverage. Nat. Methods 10, 54–56.

Hadas, Y., Goldberg, I., Pines, O., Prusky, D., 2007. Involvement of gluconic acid and glucose oxidase in the pathogenicity of *Penicillium expansum* in apples. Phytopathology 97, 384–390.

He, G., Matsuura, H., Yoshihara, T., 2004. Isolation of an α-methylene-γ-butyrolactone derivative, a toxin from the plant pathogen *Lasiodiplodia theobromae*. Phytochemistry 65, 2803–2807.

Heller, J., Tudzynski, P., 2011. Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. Annu. Rev. Phytopathol. 49, 369–390.

Hube, B., Sanglard, D., Odds, F.C., Hess, D., Monod, M., Schäfer, W., Brown, A.J.P., Gow, N.A., 1997. Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2, and SAP3 of *Candida albicans* attenuates virulence. Infect. Immun. 65, 3529–3538.

Jittawuttipoka, T., Buranajitpakorn, S., Vattanaviboon, P., Mongkolsuk, S., 2009. The catalase-peroxidase KatG is required for virulence of *Xanthomonas campestris* pv. campestris in a host plant by providing protection against low levels of H_2O_2. J. Bacteriol. 191, 7372–7377.

Kiess, M., Hecht, H.J., Kalisz, H.M., 1998. Glucose oxidase from *Penicillium amagasakiense*. FEBS J. 252, 90–99.

Li, Z., Fan, Y., Gao, L., Cao, X., Ye, J.L., Li, G.H., 2016. The dual roles of zinc sulfate in mitigating peach gummosis. Plant Dis. 100, 345–351.

Li, Z., Gao, L., Wang, Y.T., Zhu, W., Ye, J.L., Li, G.H., 2014a. Carbohydrate metabolism changes in *Prunus persica* gummosis infected with *Lasiodiplodia theobromae*. Phytopathology 104, 445–452.

Li, Z., Wang, Y.T., Gao, L., Wang, F., Ye, J.L., Li, G.H., 2014b. Biochemical changes and defence responses during the development of peach gummosis caused by *Lasiodiplodia theobromae*. Eur. J. Plant Pathol. 138, 195–207.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402–408.

Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C.J., Lu, S., Chitsza, F., Derbyshire, M.K., Geer, R.C., Gonzales Marc Gwadz, N.R., Hurwitz, D.I., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Geer, L.Y., Bryant, S.H., 2016. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic Acids Res. 45, 200–203.

Martinez-Lopez, R., Monteoliva, L., Diez-Orejas, R., Nombela, C., Gil, C., 2004. The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in *Candida albicans*. Microbiology 150, 3341–3354.

Martos, S., Andolfi, A., Luque, J., Mugnai, L., Surico, G., Evidente, A., 2008. Production of phytotoxic metabolites by five species of *Botryosphaeriaceae* causing decline on grapevines, with special interest in the species *Neofusicoccum luteum* and *N. parvum*. Eur. J. Plant Pathol. 121, 451–461.

Möbius, N., Hertweck, C., 2009. Fungal phytotoxins as mediators of virulence. Curr. Opin. Plant Biol. 12, 390–398.

Movahedi, S., Heale, J.B., 1990. The roles of aspartic proteinase and endo-pectin lyase enzymes in the primary stages of infection and pathogenesis of various host tissues by different isolates of *Botrytis cinerea* Pers ex. Pers. Physiol. Mol. Plant Pathol. 36, 303–324.

Paris, S., Wysong, D., Debeaupuis, J.P., Shibuya, K., Philippe, B., Diamond, R.D., Large, J.P., 2003. Catalases of *Aspergillus fumigatus*. Infect. Immun. 71, 3551–3562.

Peng, S., Cao, Q., Qin, Y., Li, X., Liu, G., Qu, Y., 2017. An aldonolactonase AltA from *Penicillium oxalicum* mitigates the inhibition of β-glucosidase during lignocellulose biodegradation. Appl. Microbiol. Biotechnol. 101, 3627–3636.

Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.

Petre, B., Kamoun, S., 2014. How do filamentous pathogens deliver effector proteins into plant cells? PLoS Biol. 12 e1001801.

Reilly, C.C., 1982. Distribution in the Southeastern United States of peach tree fungal gummosis caused by *Botryosphaeria dothidea*. Plant Dis. 66, 158–160.

Rodríguez-Gálvez, E., Guerrero, P., Barradas, C., Crous, P.W., Alves, A., 2017. Phylogeny and pathogenicity of *Lasiodiplodia* species associated with dieback of mango in Peru. Fungal Biol 121, 452–465.

Singh, J., Quereshi, S., Banerjee, N., Pandey, A.K., 2010. Production and extraction of phytotoxins from *Colletotrichum dematium* FGCC# 20 effective against *Parthenium hysterophorus* L. Braz. Arch. Biol. Technol. 53, 669–678.

Sreedhar, L., Kobayashi, D.Y., Bunting, T.E., Hillman, B.I., Belanger, F.C., 1999. Fungal proteinase expression in the interaction of the plant pathogen *Magnaporthe poae* with its host. Gene 235, 121–129.

Srivastava, P., Andersen, P.C., Marois, J.J., Wright, D.L., Srivastava, M., Harmon, P.F., 2013. Effect of phenolic compounds on growth and ligninolytic enzyme production in *Botryosphaeria* isolates. Crop Protect. 43, 146–156.

Stempien, E., Goddard, M.L., Leva, Y., Bénard-Gellon, M., Laloue, H., Farine, S., Kieffer-Mazet, F., Tarnus, C., Bertsch, C., Chong, J., 2017. Secreted proteins produced by fungi associated with *Botryosphaeria* dieback trigger distinct defense responses in *Vitis vinifera* and *Vitis rupestris* cells. Protoplasma 1–16.

Tang, W., Ding, Z., Zhou, Z.Q., Wang, Y.Z., Guo, L.Y., 2012. Phylogenetic and pathogenic analyses show that the causal agent of apple ring rot in China is *Botryosphaeria dothidea*. Plant Dis. 96, 486–496.

Venkatasubbaiah, P., Chilton, W.S., 1990. Phytotoxins of *Botryosphaeria obtusa*. J. Nat. Prod. 53, 1628–1630.
Venkatasubbaiah, P., Sutton, T.B., Chilton, W.S., 1991. Effect of phytotoxins produced by *Botryosphaeria obtusa*, the cause of black rot of apple fruit and frogeye leaf spot. Phytopathology 81, 243–247.
Vikrant, P., Verma, K.K., Rajak, R.C., Pandey, A.K., 2006. Characterization of a phytotoxin from *Phoma herbarum* for management of *Parthenium hysterophorus* L. J. Phytopathol. 154, 461–468.
Wang, F., Zhao, L., Li, G., Huang, J., Hsiang, T., 2011. Identification and characterization of *Botryosphaeria* spp. causing gummosis of peach trees in Hubei Province, central China. Plant Dis. 95, 1378–1384.
Wang, Y., Kim, S.G., Wu, J., Huh, H.H., Lee, S.J., Rakwal, R., Agrawal, G.K., Park, Z.Y., Kang, K.Y., Kim, S.T., 2013. Secretome analysis of the rice bacterium *Xanthomonas oryzae* (Xoo) using in vitro and in planta systems. Proteomics 13, 1901–1912.
Wehenkel, A., Bellinzoni, M., Graña, M., Duran, R., Villarino, A., Fernandez, P., Andre-Leroux, G., England, P., Takiff, H., Cervenansky, C., Cole, S.T., Alzari, P.M., 2008. Mycobacterial Ser/Thr protein kinases and phosphatases: physiological roles and therapeutic potential. BBA-Proteins Proteom. 1784, 193–202.
Wu, G., Shortt, B.J., Lawrence, E.B., Levine, E.B., Fitzsimmons, K.C., Shah, D.M., 1995. Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell 7, 1357–1368.
Xiao, J., Zhang, Q., Gao, Y.Q., Tang, J.J., Zhang, A.L., Gao, J.M., 2014. Secondary metabolites from the endophytic *Botryosphaeria dothidea* of *Melia azedarach* and their antifungal, antibacterial, antioxidant, and cytotoxic activities. J. Agric. Food Chem. 62, 3584–3590.
Xu, X.Q., Pan, S.Q., 2000. An *Agrobacterium* catalase is a virulence factor involved in tumorigenesis. Mol. Microbiol. 35, 407–414.
Zhao, Z., Liu, H., Wang, C., Xu, J.R., 2013. Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. BMC Genomics 14, 274.
Zheng, L., Lv, R., Huang, J., Jiang, D., Hsiang, T., 2010. Isolation, purification, and biological activity of a phytotoxin produced by *Stemphylium solani*. Plant Dis. 94, 1231–1237.