The Dual Roles of Zinc Sulfate in Mitigating Peach Gummosis

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Abstract

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Peach gummosis, caused by *Lasiodiplodia theobromae*, is one of the most prevalent diseases that affects peach production. In this study, we investigated the effect of zinc sulfate on inoculated peach shoots, as well as on the growth, morphology, and pathogenicity of *L. theobromae* in vitro, in the laboratory. Zinc deficiency was detected in diseased peach shoots by micronutrient analysis (Cu, Mn, and Zn) and confirmed by the measurement of transcript levels of zinc transporters (*ZIP4*, *HMA4*, and *ZAT*). The zinc was transferred from the diseased peach shoots to the peach gum. Applying zinc sulfate to the diseased peach shoots reduced the severity of peach gummosis, showing significantly reduced lesion size and gum weight, as well as downregulation of cell wall

degradation-related gene (*PG* and *PME*) compared with the control. Zinc sulfate also specifically controlled peach gummosis under *L. theobromae* phytotoxin stress and induced the expression of defense-related genes (*PR4*, *CHI*, *PAL*, *PGIP*, and *GNS3*). In addition, in vitro mycelial growth of *L. theobromae* was significantly inhibited by zinc sulfate compared with the control. Zinc sulfate caused abnormal hyphae at 25 mM and swelling hyphal tips at 50 mM. Exposure of *L. theobromae* to zinc sulfate for 20 min inhibited the ability of the pathogen to cause peach gummosis. Our physiological and molecular data demonstrated that zinc sulfate has a dual function by reducing susceptibility in the host and by direct inhibition of the pathogen.

Peach gummosis is one of the most prevalent and damaging peach diseases in China, Japan, and the United States (Reilly and Okie 1982; Wang et al. 2011). *Lasiodiplodia theobromae* causes disease on a number of hosts including gummosis in peach and apricot (Li et al. 1995), canker disease in grapevine (Urbez-Torres et al. 2008), fruit rot disease in banana (Mortuza and Ilag 1999), inflorescence blight in mango (Serrato-Diaz et al. 2013), and black branch dieback in cashew (Cardoso et al. 2002). The common symptoms of peach gummosis include necrotic lesions on the bark and gum formation on the trunks, branches, and fruits (Biggs and Britton 1988). The occurrence of this disease results in earlier defoliation, twig die back, reduced vigor, and decreased fruit production (Li et al. 1995).

Historically, chemical control has been used widely to suppress plant diseases caused by *L. theobromae*. For peach gummosis, disease severity significantly decreased after treatment with captafol on susceptible peach cultivars (Beckman et al. 2003). In mango, 2,4-dichlorophenoxyacetic acid (2,4-D) reduced the incidence of stem end rot of fruit caused by *L. theobromae* (Kobiler et al. 2001). Bester et al. (2007) observed that fenarimol was effective fungicide against *L. theobromae*. In Japan, gummosis of apricot caused by *L. theobromae* was reportedly suppressed by the application of thiophanate-methyl (Li et al. 1995). In contrast to fungicides, pretreatment of banana fruits with *Trichoderma viride* effectively controlled storage rots caused by *L. theobromae* (Mortuza and Ilag 1999).

The effect of micronutrients on reducing the severity of diseases can be attributed to their involvement in physiology, and the expression of disease resistance in the plant (Dordas 2008). Reduction in disease severity has been reported in cucumber plants after foliar application of CuSO_4 , which provided protection against powdery mildew (Reuveni et al. 1997). Mn has an important role in lignin biosynthesis, phenol biosynthesis, and photosynthesis. Mn fertilization can also suppress a number of pathogenic diseases, such as

downy mildew, powdery mildew, and potato scab (Graham and Webb 1991; Simoglou and Dordas 2006). Fe is a component of peroxidase, which stimulates other enzymes involved in the biosynthetic pathway. Previous studies have shown that iron deficiency contributes to *Arabidopsis thaliana* soft rot disease symptoms (Kieu et al. 2012). Zn is also an essential micronutrient for plant growth and a catalytic component of over 300 enzymes belonging to six major enzyme classes, including hydrolases, transferases, oxidoreductases, lyases, ligases, and isomerases (Broadley et al. 2007; Grotz et al. 1998). Zinc has shown various effects on plant susceptibility to disease including both suppression and enhancement of the host pathogen interaction (Graham and Webb 1991; Grewal et al. 1996). Several genes are involved in zinc transport within plants. HMA4 is a ATPase that transports Zn and Cd; a ZIP4 is expressed in shoots in response to Zn deficiency (Grotz et al. 1998; Verret et al. 2004), and ZAT is a Zn efflux gene that belongs to a cation diffusion facilitator family (Guerinot and Eidet 1999).

The current study aims to (1) evaluate the effects of zinc sulfate on the development of peach gummosis; (2) investigate the antifungal effects of Zn on mycelial growth, hyphal morphology, and pathogenicity of *L. theobromae*; and (3) observe the potential control of peach gummosis by zinc sulfate treatments.

Materials and Methods

Plant and fungus materials. Approximately 900 three-year-old peach trees with low incidence of peach gummosis were cultivated in an experimental field at Huazhong Agricultural University in Wuhan. No symptom was observed on the current-year peach shoots. For all inoculation experiments, current-year shoots were randomly collected from 120 plants of *Prunus persica* (L.) ‘Springsnow’ from June 2012 to August 2013. The plants were grafted on wild peach rootstock. *L. theobromae* strain JMB-122 was originally isolated from diseased peach orchards in Hubei Province, China (Wang et al. 2011) and stored on dry filter paper at -20°C .

Host inoculation and measurement of mineral content. The inoculation procedure was performed according to the method of Li et al. (2014a). Current-year peach shoots were surface sterilized with 70% alcohol for 10 s, rinsed three times with sterile water, and cut into 15 cm length segments. The shoot segments were wounded with a sterilized needle, and an actively growing isolate of strain JMB-122 on a mycelial agar plug (4 mm in diameter) was placed over the wound. Incubation conditions were 12 h light/12 h dark photoperiod at 26°C with 90% relative humidity. For mineral analysis, host tissue

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samples were collected from 10 inoculated peach shoots and 10 non-inoculated shoots. Collected tissues included epidermal cells, phloem, and parenchyma (excluding xylem) and located within 0.5 to 1.0 cm of a lesion. Non-inoculated shoots were inoculated with sterile PDA plugs as control. Peach gum was also obtained from 10 diseased peach shoots at 3, 4, and 5 days post-inoculation (dpi). Three independent replicates were performed on the inoculated and control shoots. Each replicate comprised 10 current-year shoots.

To measure the mineral content of the shoot and gum samples, 0.5 g fresh host shoot tissue and gum samples were ground with liquid nitrogen and placed in a porcelain crucible. The furnace temperature was gradually increased from room temperature to 500°C within 30 min (Tüzen 2003). The samples were burned for approximately 8 h until a white ash residue was obtained. The residue was dissolved in 5 ml of 1 M hydrochloric acid. The levels of Cu, Mn, and Zn were determined by an atomic absorption spectrometer

Table 1. Gene-specific primer sequences of peach used in this study

Gene name	Accession number	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon (bp)	Description
<i>CHI</i>	DY641594 ^x	TTCTGATTTTCGGTCGGTTCG	GCTGTTTCCAGCAGCTCCAT	232	Chitinase IV
<i>GNS3</i>	AF435089 ^x	TCAAGCCCTCAGACTCCTATGC	AACGGCGATCTGTTGTCTACTA	217	Beta-1,3-glucanase 3
<i>PAL</i>	AF206634 ^x	AGCTTCACCCCTCTAGGTTCTGT	AGCCTGGCACTTTCAACTTCTTT	253	Phenylalanine ammonia lyase
<i>PG</i>	DQ340809 ^x	AATGCCGCAGACCTAACCTACA	CCCCTTCACATGCTCAAACAAG	288	Endo-polygalacturonase
<i>PGIP</i>	EF409977 ^x	GGGGCTGACCCAAGTGGATTT	AGCCTGTCTAGCACTGGTGGC	256	Polygalacturonase-inhibiting protein
<i>PME</i>	AB231903 ^x	CCTCAGTCAACTCCACACCCAC	AGTCACCATTCCACTCAAGCCA	126	Pectin Methylesterase 1
<i>PR4</i>	AF362989 ^x	GGTGACAAACACGGGCACAGGAG	AAGAAGCGATCCCACTTTGAAGT	251	Pathogenesis-related protein 4
<i>HMA4</i>	ppa000656m ^y	TTGAAGGTCGTAAGGGTGGG	TGCTGCGGATGACTATCTC	173	Cadmium/zinc-transporting ATPase
<i>ZAT</i>	ppa006401m ^y	AAGCCTGAGTGAAGATAGTCGA	CACTTCATCCATCTCCAGCAAC	171	Zinc transporter
<i>ZIP4</i>	ppa006989m ^y	TGCCACGTTGCTCGTTGACT	CCTTCCCATTCCGATCCTTTT	158	Zinc transporter 4
<i>TEF2^z</i>	JQ732180 ^x	AGCAAGTCACCCAACAAGCATA	CCAACCAAATCTTCAGCCAAT	147	Translation elongation factor 2

^x Accession number from GenBank.

^y Sequence from peach genome database at phytozome (<http://www.phytozome.net/peach.php>).

^z Peach housekeeping normalization gene.

Fig. 1. Level of micronutrients in peach infected shoots compared with the control shoots and the relative expression of Zn-transporter genes after inoculation with *Lasiodiplodia theobromae*. **A**, Levels of Cu, Mn, and Zn in diseased and control shoots of peach. **B**, Zn content in peach gum at 3, 4, and 5 days post-inoculation. **C**, Expression levels of zinc transporter 4 (*ZIP4*), cadmium/zinc-transporting ATPase (*HMA4*), and zinc transporter (*ZAT*) in comparison with that of peach *TEF2* were determined by qRT-PCR. Significant differences in relative transcript levels between inoculated and control shoots were estimated by performing Student's *t* test on individual $\Delta\Delta\text{CT}$ values (Yuan et al. 2006). Data and relative values are the means $\pm$ SD of three biological replicates. Asterisks indicate a significant difference (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

(Spectr AA 220, Varian, Palo Alto, CA). Mineral content was expressed as $\mu\text{g/g}$ fresh weight. The relative expression of *ZIP4*, *HMA4*, and *ZAT* was analyzed by quantitative real time (qRT)-PCR to investigate zinc transport during the development of peach gummosis (see below and Table 1).

Application of Zn sulfate solution on peach inoculated shoots. Strain JMB-122 was cultured on potato dextrose agar (PDA) media for 3 days prior to inoculation. Current-year peach shoots were inoculated with *L. theobromae* strain JMB-122. The inoculation procedure also was executed as described by Li et al. (2014a). For Zn sulfate treatments, the inoculated peach shoots were treated with 10 ml of Zn sulfate solution at concentrations of either 25 and 50 mM for 24 h at 12 h intervals. Control shoots were sprayed only with sterile water. The treated and control shoots were then inoculated and incubated with a 12 h light/12 h dark photoperiod at

26°C with 90% relative humidity. Three independent replicates were performed on the inoculated and control shoots. Each replicate comprised 10 current-year shoots. Disease severity was evaluated by measuring the lesion diameter and gum weight at 1, 2, 3, and 4 dpi. Tissue samples (0.5 g) for cell wall degradation-related gene (*PG* and *PME*) expression analysis by qRT-PCR were collected in the proximity of the inoculation sites as described above, frozen in liquid nitrogen, and stored at -80°C (see below and Table 1).

Collection of phytotoxins produced by *L. theobromae*. *L. theobromae* strain JMB-122 was grown on PDA medium at 26°C for 3 days according to the methods of Abdou et al. (2010). For phytotoxin production, six 4-mm mycelial plugs from PDA cultures were placed in a 500-ml flask containing 200 ml of modified Richard's liquid medium (3% sucrose, 1% KNO_3 , 0.5% KH_2PO_4 , 0.25% MgSO_4). The cultures were then incubated at 26°C in a water bath

Fig. 2. Effect of zinc sulfate treatments on the symptoms of peach gummosis and transcript level of cell wall degradation related-genes. **A**, Symptoms of gummosis in peach shoots with or without *Lasiodiplodia theobromae* and inoculated shoots after applying zinc sulfate at 2 and 4 days post-inoculation (dpi). Bar, 5 mm. **B**, Lesion diameter on untreated inoculated shoots and inoculated shoots with zinc sulfate (25 and 50 mM) treatment. Relative values are the means $\pm$ SD of three biological replicates. For each time point, bars with different letters are significantly different ($P < 0.05$). **C**, Gum weight harvested from diseased shoots at 4 dpi. Bars with different letters are significantly different ($P < 0.05$). **D**, The transcripts level of endo-polygalacturonase (*PG*) and pectin methylesterase (*PME*) in inoculated shoots at 3 dpi. Significant differences in relative transcript levels between inoculated shoots with zinc sulfate (25 and 50 mM) treatment and untreated inoculated shoots were estimated by Student's *t* test on individual ΔCT values (Yuan et al. 2006). Data and relative values are the means $\pm$ SD of three biological replicates. Asterisks indicate a significant difference (** $P < 0.01$ and *** $P < 0.001$).

Fig. 3. Symptoms of gummosis in peach shoots after inoculation with *Lasiodiplodia theobromae* phytotoxins (LT-toxin) and the combination of phytotoxins and Cu sulfate, Fe sulfate, Mn sulfate, and Zn sulfate at 4 days post-inoculation. Scale bar, 5 mm.

shaker (70 rpm) for 21 days. Extraction assay was conducted according to the methods of Singh et al. (2010) with minor modifications. Approximately 800 ml of culture filtrate was extracted three times with half volumes of ethyl acetate using a separatory funnel. Organic solvent fractions were evaporated to dryness at 55°C. After weighing, the residues were dissolved in 5 ml sterile distilled water and adjusted to a concentration of 100 µg/ml phytotoxin.

Effect of micronutrients on peach gummosis caused by *L. theobromae* phytotoxin. To determine the effect of micronutrients on peach gummosis, we tested the toxicity of *L. theobromae* phytotoxin amended with sulfate salts of Cu, Fe, Mn, or Zn. Mineral salts were dissolved in sterile distilled water and filtered through a 0.22 µm filter. Wounds on shoots were inoculated with 6 µl of a mixture of mineral salts and phytotoxin at a ratio of 1:5 (v/v). The tested concentrations of Zn sulfate were 5, 10, and 20 mM; Cu sulfate, Fe sulfate, and Mn sulfate were tested at 20 mM. Phytotoxin without mineral salt was the control.

The peach shoots were cut into 15-cm segments for the inoculation assay. These segments were surface sterilized with 70% ethanol for 10 s, rinsed three times with sterile water, and wounded at the middle with a sterilized needle. Approximately 6 µl of the phytotoxin/mineral solution was inoculated on the wounded sites. After drying of the phytotoxin treatment, sterile water was sprayed on the shoots to maintain humidity at 12 h interval. The shoots were placed in plastic boxes, covered with a clear plastic film, and incubated in a chamber at 26°C under a 12 h/12 h light/dark photoperiod. The inoculated and control shoots each had three independent replicates. Each replicate comprised 10 current-year shoots. As described above, tissue samples were collected

from within a 0.5 to 1.0 cm from a lesion at 12, 24, 48, and 72 h post-inoculation (hpi), frozen in liquid nitrogen, and stored at -80°C for defense-related gene (*PR4*, *CHI*, *PAL*, *PGIP*, and *GNS3*) expression analysis by qRT-PCR (see below and Table 1).

Effect of Zn sulfate on *L. theobromae* growth, morphology, and pathogenicity. Active mycelia of *L. theobromae* strain JMB-122 were transferred to plates of PDA amended with 0, 5, 10, 25, and 50 mM Zn sulfate. Inoculation assays were performed with three biological replicates; each replicate comprised three plates. The plates were incubated at 26°C with a 12 h/12 h light/dark photoperiod. Colony diameter of *L. theobromae* strain JMB-122 was recorded and observed from 1 to 5 dpi. To observe the hyphal morphology, three plates were selected from Zn-treated and control cultures of *L. theobromae* strain JMB-122. In each plate, three hyphae were randomly selected and observed. The hyphae were sampled from the edge of the colony using a sterile inoculation needle and subsequently placed on slides. The specimens were observed using an Eclipse 90i microscope (Nikon, Tokyo, Japan) under bright-field light at 40× magnification. For the infectivity assay, strain JMB-122 was grown on a PDA medium at 26°C for 3 days. Mycelial plugs (4 mm in diameter) from PDA cultures were placed in a 1.5 ml tube containing different Zn sulfate concentrations (10, 25, and 50 mM) for 20 min. The plugs were subsequently inoculated on wounded peach shoots. Inoculation procedure was performed as described above (Li et al. 2014a).

RNA extraction and qRT-PCR for gene expression. For collected peach tissue samples, total RNA was extracted from 0.5 g of frozen current-year shoot tissues using an EASYspin Plus RNA kit

Fig. 4. Effects of Zn sulfate on the expression of defense-associated genes of peach (A, *PR4*; B, *CHI*; C, *PAL*; D, *PGIP*; E, *GNS3*). The transcripts level of defense-related genes between 12 and 72 h post-inoculation in peach shoots inoculated with phytotoxins of *Lasiodiplodia theobromae* with or without Zn sulfate. Significant differences in relative transcript levels between shoots inoculated with phytotoxins amended with Zn sulfate (10 and 20 mM) and phytotoxin alone were estimated by Student's *t* test on individual Δ CT values (Yuan et al. 2006). Relative values are the means $\pm$ SD of three biological replicates. Asterisks indicate a significant difference (***P* < 0.01 and ****P* < 0.001).

(Aidlab, Beijing, China). DNase I (TaKaRa, Dalian, China) was added to remove genomic DNA. RNA yield and purity were analyzed with NanoDrop 2000 (Thermo, Wilmington, DE), and RNA integrity was measured by electrophoresis in 1.5% agarose gel. First-strand cDNA was synthesized from total RNA with oligo(dT) primers according to the instructions on the PrimeScript RT reagent kit (TaKaRa, Dalian, China).

qRT-PCR was performed in a LightCycler 480 Sequence Detection System (Roche, Basel, Switzerland) with intercalation dye SYBR Green as the fluorescent reporter. Reactions were conducted at a final volume of 15 μ l, with 30 ng of cDNA, 10 μ mol of each primer, and 7.5 μ l of 2 $\times$ SYBR Green PCR master mixes (TaKaRa, Dalian, China), according to instructions of the manufacturer. PCR primers were designed based on the peach cDNA sequences published in the GenBank database and described in Table 1. Thermal cycling conditions comprised 2 min of denaturation at 95°C, followed by 40 cycles of amplification (95°C for 15 s, 60°C for 15 s, and 72°C for 30 s). Translation elongation factor 2 (*TEF2*) of peach was used as reference, as proposed by a previous report (Tong et al. 2009). Relative expression levels were calculated by the $\Delta\Delta$ CT method (Livak and Schmittgen 2001). qRT-PCR results were expressed as mean $\pm$ SD of three independent biological replicates.

Statistical analysis. Student's *t* tests were used for statistical analysis of the content of elements and gene relative expression level. For the suppression assay, colony diameter and lesion diameter were compared with multiple comparison tests. Statistically significant

differences were assessed by testing for least significant difference ($P < 0.05$).

Results

Zn deficiency in diseased peach shoots. The Zn content was significantly lower ($P < 0.01$) in inoculated peach shoot tissues than the control shoots. Similarly, the Mn level was only lower than control shoots at 3 dpi. In contrast, the Cu level significantly increased in the inoculated shoots at 3 and 4 dpi (Fig. 1A). Zinc was detected in the peach gum (Fig. 1B). To investigate the regulatory mechanism underlying the effect of Zn content in the host tissue, we examined the transcript abundance of *ZIP4*, *HMA4*, and *ZAT* in the control and diseased peach shoots. The transcript levels of *ZIP4* and *HMA4* were significantly increased in diseased shoots compared with the control shoots at 5 dpi. Conversely, *ZAT* was significantly down-regulated in diseased shoots compared with the control shoots at 3, 4, and 5 dpi (Fig. 1C).

Application of zinc sulfate alleviates the development of peach gummosis. In comparison with the control shoots, the Zn-treated shoots displayed reduced symptoms of peach gummosis (Fig. 2A). Lesion diameters were smaller and gum weights were significantly ($P < 0.05$) reduced in shoots treated with 25 and 50 mM zinc sulfate compared with control shoots (Figs. 2B and C). We did not observe evident changes in lesion diameter after application of copper sulfate and manganese sulfate compared with the control (Supplementary Fig. S1). The expression of endo-polygalacturonase

Fig. 5. Effects of zinc sulfate on mycelial growth, morphology, and pathogenicity of *Lasiodiplodia theobromae* on peach shoots. **A**, Mycelia cultured on potato dextrose agar with 0, 5, 10, 25, and 50 mM zinc sulfate at 26°C for 1, 3, and 5 days. **B**, Effect of zinc sulfate on mycelial morphology of *L. theobromae*. Mycelia were cultured in PDA medium with 5, 10, 25, and 50 mM zinc sulfate or without zinc sulfate (control) at 26°C for 1, 3, and 5 days. H, hypha; SH, septa hypha. Black arrows represent abnormal hyphae, and white arrows represent swelling hyphal tips. Scale bar = 10 μ m. **C**, Effect of zinc sulfate treatment of *L. theobromae* plugs on the pathogen's ability to infect peach shoots. Mycelium plugs (4 mm) were treated with 10, 25, and 50 mM zinc sulfate for 20 min or left untreated prior to inoculation on peach shoots. Scale bar = 5 mm.

Table 2. Impact of zinc sulfate on colony diameter of *Lasiodiplodia theobromae* on PDA and lesion diameter of peach inoculated shoots by treated *L. theobromae* with zinc sulfate^x

Zinc sulfate Treatments	Colony diameter (cm) ^y			Lesion diameter (cm) ^z		
	1 d	3 d	5 d	1 d	2 d	3 d
0 mM	4.36 $\pm$ 0.15a	9.00 $\pm$ 0.00a	9.00 $\pm$ 0.00a	0.77 $\pm$ 0.04a	1.03 $\pm$ 0.08a	1.98 $\pm$ 0.15a
10 mM	2.32 $\pm$ 0.11b	5.60 $\pm$ 0.10b	7.93 $\pm$ 0.21b	0.71 $\pm$ 0.04b	0.95 $\pm$ 0.05a	1.71 $\pm$ 0.10b
25 mM	1.63 $\pm$ 0.15c	2.67 $\pm$ 0.15c	3.45 $\pm$ 0.22c	0.41 $\pm$ 0.04c	0.50 $\pm$ 0.02b	0.53 $\pm$ 0.08c
50 mM	0.60 $\pm$ 0.05d	1.10 $\pm$ 0.10d	2.17 $\pm$ 0.15d	0.00 $\pm$ 0.00d	0.00 $\pm$ 0.00c	0.00 $\pm$ 0.00d

^x Mean values followed by different letters differ significantly within a row according to multiple comparison tests (least significant difference, $P < 0.05$).

^y Colony diameter of *L. theobromae* on PDA with zinc sulfate at different concentrations.

^z Lesion diameter on current-year peach after inoculation with *L. theobromae*, which was treated for 20 min with 0, 10, 25, and 50 mM zinc sulfate.

(PG) significantly decreased by 53 and 81% in the shoots treated with 25 and 50 mM zinc sulfate, respectively, at 5 dpi. The expression of pectin methylesterase (PME) was also downregulated in Zn treated shoots at 25 mM relative to the control (Fig. 2D).

Zn sulfate inhibits virulence of *L. theobromae* phytotoxin and induces defense gene expression. Peach current-year shoots exhibited symptoms resembling peach gummosis when treated with *L. theobromae* phytotoxin. In contrast, the addition of Zn sulfate to the phytotoxin inhibited expression of the gummosis symptom, and phytotoxin-induced lesions were significantly ($P < 0.05$) smaller as a result of the zinc amendment (Fig. 3).

To verify that Zn sulfate increased the resistance of peach shoots to the phytotoxin, the expressions of five defense-related genes were examined in peach shoots inoculated with the phytotoxin with or without Zn sulfate treatment. The expressions of *PR4* and *CHI* were significantly upregulated in the shoots simultaneously treated with Zn sulfate and inoculated with phytotoxin at 24 and 72 hpi, but the expressions were downregulated at 48 hpi (Figs. 4A and B). The expressions of *PAL* and *PGIP* in the treated shoots were significantly greater than that in the control shoots at 12, 24, and 48 hpi. The expression of *GNS3* was significantly increased at 12 and 24 hpi. (Figs. 4C, D, and E).

Effects of zinc sulfate on mycelial growth, morphology, and pathogenicity of *L. theobromae*. Mycelial growth of *L. theobromae* was significantly ($P < 0.05$) inhibited by zinc sulfate at 25 and 50 mM; however, 50 mM zinc sulfate did not completely inhibit mycelial growth (Fig. 5A). Compared with the control, at 3 dpi, colony diameter of *L. theobromae* on PDA amended with zinc sulfate at 25 or 50 mM was reduced 3.37-fold and 8.18-fold, respectively (Table 2). *L. theobromae* normally forms smooth, white filamentous hyphae. However, abnormal hyphae and swollen hyphal tips of *L. theobromae* were observed in PDA media supplemented with 25 and 50 mM zinc sulfate (Fig. 5B). In contrast to the control, the black septate hyphae increased with increasing zinc sulfate concentrations. As shown in Figure 5C, treatments with 25 and 50 mM zinc sulfate significantly inhibited the development of peach gummosis. Lesion diameter was significantly ($P < 0.05$) inhibited by Zn treatments at 10, 25, and 50 mM (Table 2). Gum formation was observed at 3 dpi in the control, whereas peach gum production was controlled by 50 mM Zn treatment after inoculation with *L. theobromae*.

Discussion

In this study, Zn deficiency was shown to be an important factor in the expression of symptoms of peach gummosis. Bolle-Jones and Hilton (1956) also observed that Zn deficiency in *Hevea brasiliensis* increased disease severity after infection by *Oidium* spp. Our previous study found that PG is a cell wall degrading enzyme that is produced in peach shoots during *L. theobromae* infection (Li et al. 2014b). After treatment with zinc sulfate, however, the expression of cell wall degradation-related genes (PG and PME) significantly decreased in inoculated peach shoots. To compensate for pathogen-induced Zn deficiency in diseased shoots, we applied zinc sulfate solutions to peach shoots. Our data displayed that zinc sulfate alleviates the development of peach gummosis, reducing both lesion development and gum formation. One possible explanation for this result is that Zn is important in maintaining the integrity or stability of biological membranes (Graham and Webb 1991). In addition, Zn could be an essential component of finger proteins that are involved in the amelioration of biotic stresses (Guo et al. 2009).

Zn sulfate also inhibited the development of peach gummosis caused by the mycelia of *L. theobromae* that had been treated with 50 mM zinc sulfate for 20 min. Previous studies reported that Zn has antifungal activity against *Pycnoporus cinnabarinus* and *Pleurotus ostreatus* (Malachova et al. 2011), and that Zn represses the production of the phytotoxin fusaric acid, which is a *Fusarium oxysporum* virulence factor (Duffy and Defago 1997). In addition, in *Botrytis cinerea*, Zn stress resulted in a decrease in the production of numerous cell wall degrading enzymes (Cherrad et al. 2012). Therefore, the effects of zinc sulfate on hyphal morphology, development, and phytotoxin production of *L. theobromae* may contribute in alleviating symptoms of peach gummosis.

In this study, zinc sulfate significantly inhibited in vitro mycelial growth of *L. theobromae* and caused the formation of abnormal hyphae and swelling of hyphal tips. These findings are consistent with those in previous reports, which stated that Zn significantly reduced the fungal biomass of *F. oxysporum* and ericoid mycorrhizas (Duffy and Defago 1997; Lanfranco et al. 2002). Chitin is the main cell wall polysaccharide. The abnormal hyphal morphology in the presence of zinc could have caused chitin deposition, as reported by Lanfranco et al. (2002). The mode of action of Zn sulfate against fungal pathogens is not well understood; however, ions of Zn at 50 mM reportedly destroy the internal hydrostatic pressure of the cell and inhibit elongation of the hyphal tip of *L. theobromae* (Lew 2011).

Phytotoxins of pathogens can be used as experimental models to investigate the host responses (Mase et al. 2013). In order to eliminate the effect of zinc on the pathogen, we used toxin of *L. theobromae* with zinc sulfate to understand the effect of zinc on peach shoots. Our data showed zinc sulfate induced the expression of defense-related genes and inhibited expression of the gummosis symptom. Previous reports had found systemic acquired resistance may be involved in the suppression of plant diseases by micronutrients, such as B, Mn, and Cu (Reuveni et al. 1997, 2000). Zinc sulfate may have similar function based on the protection against *L. theobromae*.

This study suggests that zinc sulfate has a dual function, namely enhancing peach resistance and inhibiting the pathogen. Zinc sulfate mitigated peach gummosis disease caused by *L. theobromae*. Our results showed that Zn was detected in the peach gum after infection. Previous reports demonstrated that peach gum was only soluble in water at high temperatures (Simas et al. 2008). Therefore, it is difficult for the plant to remobilize the zinc in peach gum. Based on mineral measurements and the measured expression levels of Zn transporter genes, *L. theobromae* was apparently a contributing factor in loss of Zn from the diseased peach shoots during peach gummosis development. Consequently, the application of zinc to diseased orchards may serve as a potential method to compensate zinc loss and mitigate peach gummosis. For example, application of Zn to the soil reduced infections of *Fusarium graminearum* (Grewal et al. 1996). As described by Cripps et al. (1983), foliar application with 3 g/liter zinc sulfate on peach completely inhibited growth of *Trametes versicolor* and *Stereum strigosozonatum*. However, excessive zinc can result in toxicity symptoms that include reduced yields and stunted growth, although zinc toxicity in crops is far less common than Zn deficiency. Toxicity symptoms usually become visible at a range of 100 to 300 mg Zn per 1 kg of leaf dry weight for most crops (Broadley et al. 2007). Therefore, more tests are need in the field for peach gummosis, such as effective zinc concentration, zinc toxicity range, and application times, among others.

Overall, our study contributed to the understanding of the mechanisms underlying how zinc sulfate improves the defense of peach against *L. theobromae* and provided new insights into host responses mediated by Zn. Further study is necessary to confirm the effect of zinc sulfate on peach gummosis in the field.

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