A Novel Sulfone Derivative Controls *Lasiodiplodia theobromae* in Tea Leaf Spot by Reducing the Ergosterol Content

Xingtao Bao,¹ Rui Yang,¹ Shilong Jiang,^{1,2} Jinping Zhao,³ Delu Wang,⁴ Dongxue Li,¹ Xian Wu,¹ Baoan Song,^{1,†} and Zhuo Chen^{1,†}

¹ Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Guizhou University, Guiyang 550025, Guizhou, China

² College of Agricultural, Guizhou University, Guiyang 550025, Guizhou, China

³ Texas A&M University AgriLife Research Center at Dallas, Dallas 75252, Texas, U.S.A.

⁴ College of Forestry, Guizhou University, Guiyang 550025, Guizhou, China

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Diseases caused by fungi can affect the quality and yield of the leaves of tea [*Camellia sinensis* (L.) Kuntze]. At present, the availability of highly effective and safe fungicides for controlling tea plants remains limited. The objectives of this study were to identify novel compounds with antifungal activities and to determine their molecular mechanisms. A series of sulfone compounds containing 1,3,4-oxadiazole were evaluated in China for their antifungal activities against several pathogens causing foliar diseases and high production losses. Transcriptomics and bioinformatics were used to analyze the differentially expressed genes of *Lasiodiplodia theobromae* treated with a representative compound, jiahuangxianjunzuo (JHXJZ). Moreover, the effects of JHXJZ on ergosterol content, membrane permeability, cell structure, and seven key genes involved in the ergosterol biosynthetic pathway were investigated. JHXJZ had a strong antifungal activity against *L. theobromae* in vitro, with an effective concentration giving 50% inhibition of 3.54 ± 0.55 µg/ml, and its curative efficacies on detached tea leaves reached 41.78% at 100 µg/ml. JHXJZ upregulated 899 genes ($P < 0.05$) and downregulated 1,185 genes ($P < 0.05$) in *L. theobromae*. These genes were found to be associated with carbohydrate metabolic processes, which are closely related to steroid biosynthesis in the Kyoto Encyclopedia of Genes and Genomes pathways. Because JHXJZ regulates the key genes of sterol biosynthesis, it decreased the ergosterol content, increased cell-membrane permeability, changed the cellular

structure, enhanced the roughness of the surface of the hyphae, and resulted in degradation of the hyphal nuclei and necrosis of the hyphal cytoplasm. Our study demonstrates that JHXJZ is a fungicide with a novel mechanism of action that differs from that of triazole fungicides. JHXJZ has potential for applications in controlling tea plant diseases.

Keywords: antifungal activity, *Camellia sinensis*, foliar disease, molecular mechanism, steroid biosynthesis, sulfone derivatives, transcriptome

Tea [*Camellia sinensis* (L.) Kuntze)] has been widely grown in Guizhou Province, China, in recent years (Li et al. 2019). The tea-growing area has reached nearly 865,000 acres, and the cultivation area in the region ranks first among all the provinces or cities in China (Bao et al. 2019; Li et al. 2019). Because of the high altitude (from about 3,200 to 5,900 feet above sea level), limited sunlight, higher humidity, and late spring cold spells in this region, the incidence of disease caused by fungal pathogens has been relatively greater than that caused by pests, including anthracnose caused by *Colletotrichum camelliae* and *C. fructicola* (Wang et al. 2016), and gray blight disease caused by *Pestalotiopsis*-like species or *Pseudopestalotiopsis camelliae-sinensis* (Li et al. 2018; Wang et al. 2019). Some tea leaf spots are caused by *Phoma segeticola* var. *camelliae*, *Lasiodiplodia theobromae*, and *Epicoccum sorghinum*, and tea gray blight disease is caused by *P. camelliae-sinensis* in some tea regions in Guizhou province (Bao et al. 2019; Ren et al. 2019; Zhao et al. 2018a and b). Tea leaf spots caused by *L. theobromae* severely affect the quality and production of tea leaves in Guizhou province tea plantations (Li et al. 2019).

In the prevention and control of tea pests, spraying chemical insecticides has been found to be an effective measure. However, at present, new fungicides against fungal diseases mainly contain triazole and methoxyacrylate (He et al. 2019; Sowndhararajan et al. 2013). Tea is a special crop with a high requirement for pesticides, but environmental considerations require lower pesticide residues, lower toxicity levels, and an absence of harm to other organisms in the environment. At present, because fungicides containing triazole and methoxyacrylate have been applied over the long term, there is a risk of pesticide resistance and environmental pollution (Paramasivam et al. 2017). Thus, to prevent tea diseases, it is crucial to develop new pesticides with novel action mechanisms,

†Corresponding authors: B. A. Song; songbaoan22@yahoo.com and Z. Chen; gychenzhuo@aliyun.com

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higher antifungal activity, and higher safety levels for humans and other organisms in the environment. Sulfone derivatives containing a 1,3,4-oxadiazole moiety display strong antibacterial and antifungal activities in crops (Li et al. 2013; Xu et al. 2011, 2012). In previous studies, jiahuangxianjunzuo (JHXJZ), a representative compound of sulfone derivatives containing a 1,3,4-oxadiazole moiety, has strong antibacterial activity against *Xanthomonas oryzae* pv. *oryzae* by inhibiting the production of extracellular polysaccharides (Shi et al. 2015). Moreover, JHXJZ is a relatively safe pesticide, based our research group's evaluation of the toxicity, environmental pollution, and pesticide residues results from the evaluation tests for pesticide registration, thus making it worthy of study for preventing tea diseases. Therefore, we evaluated the antifungal activities of JHXJZ and its analogs against *L. theobromae* and other pathogens infecting tea plants, in vitro and in vivo, and studied the mechanisms of action using molecular biology and morphology methods.

RESULTS

Antifungal activities of JHXJZ and its analogs against three fungal pathogens in vitro.

The inhibitory effects of JHXJZ and its analogs on the mycelial growth of strain GZHS-2017-010 were evaluated at doses of 0.00, 1.25, 2.50, 5.00, 10.00, and 20.00 µg/ml (Table 1). JHXJZ had a strong inhibitory effect on mycelial growth (Fig. 1). In addition, compounds 2 to 6 in Table 1 showed antifungal activities but to a lesser extent than JHXJZ and tebuconazole. According to these data, we further evaluated the antifungal activity levels of JHXJZ against strains GZSQ-4 and GZHS-2017-01. The results indicated that JHXJZ inhibits the growth of mycelia of the three strains (Table 1).

Effects of JHXJZ on the morphology of *L. theobromae*.

After *L. theobromae* was treated with JHXJZ at the effective concentration giving 10% inhibition (EC₁₀), 50% inhibition (EC₅₀), and 90% inhibition (EC₉₀), the mycelial morphology

significantly changed. For example, after *L. theobromae* was treated with the JHXJZ EC₁₀ for 16 h, the hyphae were fewer, shorter, more deformed, and slightly protuberant, with darker branches than those in the control (Fig. 2A and B). After *L. theobromae* was treated with the JHXJZ EC₅₀ for 16 h, the hyphal branches further decreased, and the numbers of hyphal protuberances increased (Fig. 2C). After *L. theobromae* was treated with the JHXJZ EC₉₀ for 16 h, the hyphae were severely distorted and

Fig. 2. Effect of jiahuangxianjunzuo (JHXJZ) on morphology of *Lasiodiplodia theobromae*. **A**, Control (CK); **B**, EC₁₀ (0.30 µg/ml), 16 h after exposure (HAE); **C**, EC₅₀ (3.54 µg/ml), 16 HAE; **D**, EC₉₀ (41.64 µg/ml), 16 HAE. All bars = 20 µm.

Table 1. Antifungal activity of jiahuangxianjunzuo and its analogs against three fungal strains

Fungal strains	Compound no.	Regression equation	<i>r</i> ²	EC ₅₀ (µg/ml) ± SD
GZHS-2017-010	1	<i>y</i> = 1.1976 <i>x</i> + 4.342	0.9911	3.54 ± 0.55
GZHS-2017-010	2	<i>y</i> = 2.6101 <i>x</i> - 0.1279	0.9872	92.18 ± 2.66
GZHS-2017-010	3	<i>y</i> = 2.3111 <i>x</i> + 1.2389	0.9574	42.40 ± 3.07
GZHS-2017-010	4	<i>y</i> = 1.3232 <i>x</i> + 2.9626	0.9884	34.65 ± 3.80
GZHS-2017-010	5	<i>y</i> = 2.9366 <i>x</i> + 0.9267	0.9788	24.38 ± 1.99
GZHS-2017-010	6	<i>y</i> = 3.1345 <i>x</i> + 0.5049	0.9905	27.17 ± 2.02
GZHS-2017-010	7	<i>y</i> = 0.7253 <i>x</i> + 5.5923	0.9822	0.15 ± 0.03
GZSQ-4	1	<i>y</i> = 0.6629 <i>x</i> + 4.9108	0.7882	1.36 ± 0.34
GZSQ-4	7	<i>y</i> = 0.5071 <i>x</i> + 5.2104	0.9166	0.38 ± 0.05
GZHS-2017-01	1	<i>y</i> = 1.1464 <i>x</i> + 3.7929	0.9632	11.30 ± 0.80
GZHS-2017-01	7	<i>y</i> = 0.7675 <i>x</i> + 4.6238	0.9896	3.09 ± 0.45

Fig. 1. Inhibition effect of jiahuangxianjunzuo (JHXJZ) on mycelial growth of *Lasiodiplodia theobromae*.

deformed, and the hyphal ends were enlarged. When *L. theobromae* was treated with JHXJZ at the EC₅₀ and EC₉₀, the protoplasts agglutinated in hyphae and the hyphal color deepened (Fig. 2D).

Antifungal activity of JHXJZ against *L. theobromae* in vivo.

Strain GZHS-2017-010 exhibited strong pathogenicity on detached tea leaves, and spots formed rapidly at the inoculation sites (Fig. 3A). The negative medium control did not display spots at the inoculation sites (Fig. 3F). The curative efficacy of JHXJZ at doses of 50.00 and 100.00 µg/ml was 14.55 and 41.78%, respectively (Fig. 3B and C). Additionally, the curative efficacy of tebuconazole was 14.08 and 38.50% at a dose of 50.00 and 100.00 µg/ml, respectively (Fig. 3D and E). The results indicated that JHXJZ exhibited curative activity against tea leaf spots associated with *L. theobromae*.

Transcriptomic analysis.

In total, 53.43 Gb of high-quality sequences, ranging from 8.13 to 9.73 Gb per sample, were obtained from the transcriptome sequencing from two treatment groups (treated and control [CK]) (Supplementary Table S1). The average error rate of the sequences was 0.03% (Supplementary Table S1). The sequencing data were assembled into 34,758 transcripts, with lengths ranging from 201 to 24,864 bases (mean length = 3,395 bases and median length = 2,401 bases), and 31,490 unigenes were obtained (mean length = 3,719 bases and median length = 2,759 bases). The total length of the unigenes was 117.11 Mb (117,113,342 bases).

In total, 28,709 unigenes (91.16% of the total unigenes) were annotated in at least one of the databases used in our study (Supplementary Table S2). The unigenes were primarily annotated with good matches in the National Center for Biotechnology Information (NCBI) nonredundant protein sequences (Nr) database (best hits: median E-value = 6.10E-174 and median similarity = 0.79). According to Gene Ontology (GO) classification, the greatest number of annotations was in biological processes (BP), in which the top three GO terms were cellular, metabolic, and single-organism processes; the second greatest number was in cellular components (CC), in which the top three GO terms were cell, cell part, and organelle; and the least number of annotations was in molecular functions (MF), in which the top three GO terms were binding, catalytic activity, and transporter activity (Supplementary Table S3). For eukaryotic ortholog groups (KOG) classification, the top ten classes were general function prediction only; post-translational modification, protein turnover, chaperones; energy production and conversion; signal transduction mechanisms; lipid transport and metabolism; translation, ribosomal structure and biogenesis; intracellular trafficking, secretion, and vesicular transport; amino acid transport and metabolism; carbohydrate transport and metabolism; and RNA processing and modification (Supplementary Table S4). The unigenes were annotated using

Kyoto Encyclopedia of Genes and Genomes (KEGG) (Fig. 4). Most of the total annotations were related to different metabolic pathways, among which carbohydrate metabolism was the most abundant, followed by amino acid metabolism, the “overview” group, energy metabolism, and lipid metabolism (Supplementary Table S5).

The transcripts spliced with Trinity were used as reference sequences, to which the clean reads of each sample were mapped. The total mapped values for all groups reached 85% (Supplementary Table S6). For the density distributions of gene expression in both treatment groups, the graphs presented a nonstandard normal distribution, with the area and the sum of the probability being 1, respectively (Supplementary Fig. S1A through F). The peak of the density distribution curve represented the numbers of genes at the level of expression.

A graph of the density distribution and a boxplot were used to analyze the overall distribution and dispersion of gene expression between the two treatment groups (Supplementary Fig. S1G and H). Pearson’s correlation analysis against gene expression revealed that the correlation coefficients were close to 0.9 or greater than 0.9 between any three replicates in each treatment group (Supplementary Fig. S1I), thus indicating high similarity between the expression patterns of the two treatment groups.

In total, 899 genes were significantly up-regulated (Supplementary Table S7) and 1,185 genes were down-regulated (Supplementary Table S8) based on the filter conditions of $p_{adj} < 0.05$ and $\log_2^{\text{FoldChange}} > 1$ (Fig. 5A). A Venn diagram indicated that 2,854 and 2,598 genes were individually detected from the treatment and control, respectively, and 18,677 genes were detected in both treatment groups (Fig. 5B; Supplementary Tables S9 through S11). The greatest $\log_{10}^{\text{(FPKM+1)}}$ values clustered together, indicating a relatively high similarity among gene expression patterns in the hierarchical clustering heat map of the differentially expressed genes (DEGs) (Fig. 6). DEG-enriched GO annotations were chosen for quantitative real-time PCR (qRT-PCR) verification, which indicated that these genes were regulated in a manner similar to the expected transcriptional pattern from RNA-seq data (Table 2).

GO analysis with Blast2GO by using DEGs.

DEGs were annotated in the GO database (Fig. 7; Supplementary Table S12). In comparison with the GO annotations for all genes, there were 23,215 background genes and 769 DEGs. DEGs were annotated in BP categories carbohydrate metabolic process, carbohydrate derivative metabolic process, single-organism carbohydrate metabolic process, and carbohydrate derivative biosynthetic process, with the numbers of DEG_item with upregulation trends being 83, 58, 31, and 22, respectively (Supplementary Table S13). DEGs were enriched in BP of carbohydrate metabolic process, carbohydrate derivative

Fig. 3. Antifungal activity of jiahuangxianjunzuo (JHXJZ) and tebuconazole against *Lasiodiplodia theobromae* in vivo. **A**, The pathogenetic control; **B**, JHXJZ treatment at 50 µg/ml; **C**, JHXJZ treatment at 100 µg/ml; **D**, tebuconazole treatment at 50 µg/ml; **E**, tebuconazole treatment at 100 µg/ml; **F**, the healthy control. All bars = 1 cm.

metabolic process, single-organism carbohydrate metabolic process, and carbohydrate derivative biosynthetic process, with the numbers of DEG_item with downregulation trends being 104, 81, 62, and 29, respectively (Supplementary Table S14). DEGs with an upregulation trend were enriched at CC of membrane-bounded organelle, membrane protein complex, membrane-enclosed lumen, endoplasmic reticulum (ER) membrane, integral component of Golgi membrane, late endosome membrane, and ER to Golgi transport vesicle membrane, with the numbers of DEG_item with upregulation trends being 182, 41, 36, 15, 3, 2, and 1, respectively (Supplementary Table S13). DEGs were enriched in CC categories endomembrane system, plasma membrane, ER, late endosome, and Golgi apparatus, with the numbers of DEG_item with downregulation trends being 47, 26, 22, 16, and 6, respectively (Supplementary Table S14). DEGs with an upregulation trend were enriched at MF categories catalytic activity, hydrolase activity, carbohydrate derivative binding, cofactor binding, hydrolase activity, acting on glycosyl bonds, hydrolase activity, hydrolyzing *O*-glycosyl compounds, inorganic cation transmembrane transporter activity, channel regulator activity, calcium channel regulator activity, and carbohydrate transmembrane transporter activity, with the numbers of DEG_item with upregulation trends being 517, 226, 126, 100, 52, 51, 42, 16, 7, and 4, respectively

(Supplementary Table S13). DEGs were enriched in MF categories catalytic activity, ion binding, small molecule binding, carbohydrate derivative binding, ion transmembrane transporter activity, channel activity, steroid dehydrogenase activity, and steroid hormone receptor activity, with the numbers of DEG_item with downregulation trends being 598, 341, 213, 120, 79, 13, 9, and 1, respectively (Supplementary Table S14).

KEGG pathway analysis with Blast2GO using DEGs.

Using annotation according to the KEGG database, the top five enriched KEGG pathways were spliceosome, RNA transport, other glycan degradation, amino sugar and nucleotide sugar metabolism, and steroid biosynthesis, with 15, 14, 12, 11, and 8 genes, respectively, showing an upregulation trend (Supplementary Table S15). The top five enriched KEGG pathways were glycolysis/gluconeogenesis, tyrosine metabolism, fatty acid degradation, pyruvate metabolism, and valine, leucine and isoleucine degradation, with 23, 21, 20, 16, and 14 genes, respectively, showing a downregulation trend (Supplementary Table S16). KEGG enrichment analysis indicated that DEGs were enriched in the pathway of steroid biosynthesis (Table 3, Supplementary Fig. S2). Six genes were up-regulated and two were down-regulated in this pathway, respectively

Fig. 4. Classification of unigenes in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway.

(Supplementary Table S17). The results indicated that JHXJZ influences the expression of genes involved in steroid biosynthesis.

Effects of JHXJZ on the ergosterol content of *L. theobromae*.

The ergosterol content of strain GZHS-2017-010 continuously declined as the dose of JHXJZ increased (Fig. 8), thus suggesting that JHXJZ had a concentration-dependent effect on ergosterol production. In addition, treatment with tebuconazole at the EC₅₀ resulted in lower ergosterol production than that in the control treatment. However, treatment with azoxystrobin at the EC₃₀ did not result in significantly lower ergosterol production than that under the control treatment. Consequently, we hypothesized that the inhibitory effects on ergosterol production of JHXJZ and tebuconazole were equal, and the action mechanism of JHXJZ was similar to that of tebuconazole.

Effects of JHXJZ on the cell-membrane permeability of *L. theobromae*.

JHXJZ enhanced the cell-membrane permeability of strain GZHS-2017-010, and the relative electrical conductivity (REC) was JHXJZ concentration-dependent. When the mycelia were exposed to the EC₉₀, the REC was significantly greater than at EC₅₀ and EC₁₀ (Fig. 9). Thus, JHXJZ changed the structure of the cell membrane, resulting in increased mycelial electrolyte leakage.

Effects of JHXJZ on the cell ultrastructure of *L. theobromae*.

Scanning electron microscopy (SEM) showed that the hyphae of the untreated control exhibited robust, uniform growth, plump cell bodies, and smooth hyphal surfaces (Fig. 10A and B). New hyphae (daughter hyphae) always occurred at some distance from the hyphal tips (data not shown). After treatment with

JHXJZ, the hyphae showed pronounced morphological alterations. The deformed hyphae had a rough hyphal surface after treatment with the JHXJZ EC₁₀ for 16 h (Fig. 10C, white circles). The abnormal extracellular polysaccharide was adhered on the surface of hyphal surfaces after treatment with the JHXJZ EC₃₀ for 16 h (Fig. 10D, yellow arrows). Meanwhile, small protrusions (branching initials) formed on the hyphal tips (Fig. 10E, black arrows), and the generated hyphae were swollen to differing degrees (Fig. 10D and E). Daughter hyphae did not grow normally. When the dosage of JHXJZ was enhanced to EC₅₀, small protrusions were further enhanced on the hyphal surfaces (Fig. 10F, black circles). Meanwhile, the mycelial tube collapsed, because the contents of the mycelium were exuded from the inner parts of cells (Fig. 10F, white circle). When the hyphae were treated with JHXJZ at the EC₉₀, the morphological alterations were extreme, with deformed hyphae and a rough hyphal surface with many protrusions (Fig. 10G to I, black circles) and wrinkles (Fig. 10G to I, arrows). The mycelial tube was further collapsed because the contents of the mycelium were exuded and the organelles were degraded (Fig. 10G and H, white circles).

Transmission electron microscopy (TEM) showed that the hyphae of the untreated control had intact cell walls, plasma membranes (Fig. 11A to C, rectangular outline highlights the cell wall), septa (Fig. 11A, S = septa) and organelles (Fig. 11A and C, M = mitochondria, G = Golgi equivalents). The septum was even and complete (Fig. 11A). The cytoplasm of the hyphal tip showed numerous electron-dense vesicles (Fig. 11B, black arrows). After treatment with JHXJZ, extreme ultrastructural modifications occurred in the hyphae. The density of the hyphal cell walls decreased after exposure to JHXJZ (Fig. 11D to F, rectangular outline highlights the cell wall), and the organelles became disorganized, with morphologies that were not uniform compared with those of hyphae from the untreated control (Fig. 11D and E, black arrows). The septa were also abnormal (Fig. 11D). Vacuoles and lipid bodies increased in the hyphal cytoplasm (Fig. 11E and F,

Fig. 5. The analysis of gene differential expression between treated versus control (CK) groups. **A**, Volcano plot of the analysis of gene differential expression between treated versus CK. **B**, Venn diagram of gene expression between treated and CK. Treat and CK represent mycelia of *Lasiodiplodia theobromae* treated with jiahuangxianjunzuo (JHXJZ) at EC₅₀ or without JHXJZ for 3 h.

Fig. 6. Hierarchical clustering heat map of differentially expressed genes. Mycelia of *Lasiodiplodia theobromae* treated with jiahuangxianjunzuo (JHXJZ) at EC₅₀ (Treat) or without JHXJZ for 3 h (CK). A horizontal line shows the expression of a gene in both treatment groups. The expression of such a gene is significantly different in at least one of the pairwise comparisons between the two treatment groups. Different colors indicate different levels of gene expression. From red to blue, the $\log_{10}^{(\text{FPKM} + 1)}$ value ranges from large to small.

V = vacuoles, L = lipid body). The hyphal cytoplasm was usually necrotic or degenerated for the JHXJZ EC₉₀ treatment, and the organelles became severely disorganized (Fig. 11G to I). The cell walls and septa were not intact (Fig. 11G to I), and the inner wall layer of the original hyphae was retracted (Fig. 11G and I, PM = plasmolysis). Vacuoles further generated in the hyphal cytoplasm (Fig. 11G and I), and a larger number of organelles became degraded (Fig. 11H and I).

Effects of JHXJZ on cell nuclei and septa of *L. theobromae*.

The cell nuclei were distributed in the whole hyphae (Fig. 12A and B, white arrows point to nuclei). After treatment with the JHXJZ EC₁₀, the cell nucleus distribution in strain GZHS-2017-010 was same as that after the control treatment (Fig. 12C). When the dosage of JHXJZ was enhanced to EC₃₀, the number of the hyphal nuclei decreased and the nuclei were dispersed (Fig. 12D). Additionally, fluorescence intensity of the background increased as the dose of JHXJZ increased to the EC₅₀, and fluorescence intensity of the cell nuclei began to decrease (Fig. 12E). When the dose of JHXJZ increased to the EC₉₀, fluorescence associated with cell nuclei nearly disappeared. Meanwhile, the background showed blue fluorescence (Fig. 12F). The results indicated that higher doses of JHXJZ significantly increased the cell membrane permeability, which led to stained hyphae.

When strain GZHS-2017-010 was not treated with JHXJZ, the structures of the hyphal septa remained intact (Fig. 13A and B). Nevertheless, when GZHS-2017-010 was treated with JHXJZ at the EC₁₀ and EC₃₀ for 16 h, the septa became closer as the dose increased (Fig. 13C and D). When the dosage of JHXJZ was enhanced to the EC₅₀, the growth of the hyphae was significantly inhibited, and daughter hyphae did not grow normally. So, the septa did not change (Fig. 13E). Nevertheless, higher doses of JHXJZ significantly increased cell-membrane permeability, the fluorescent substances in the hyphae gradually accumulated as the dose increased to the EC₉₀ (Fig. 13F).

Effects of JHXJZ on related gene expression levels in the ergosterol biosynthetic pathway of *L. theobromae*.

The DEGs of strain GZHS-2017-010 were associated with the sterol synthetic pathway of *L. theobromae*, as determined by the transcriptomic analysis. The genes were further verified with qRT-PCR at the EC₁₀, EC₃₀, and EC₅₀ for 3, 6, 9, and 12 h. Treatment with tebuconazole at the EC₅₀ was used as the positive control. Compared with the control treatment, JHXJZ treatment significantly downregulated *FDFT1* ($P < 0.05$) at the three doses at 3 h as well as at EC₃₀ and EC₅₀ at 9 and 12 h. Nevertheless, the expression trends after JHXJZ treatments contrasted with those after the tebuconazole treatment (Fig. 14A).

For *ERG1*, JHXJZ induced a significant downregulation ($P < 0.05$) at the three doses at 3 h, upregulation at EC₃₀ at 6 h, and downregulation at EC₃₀ and EC₅₀ at 9 h. However, the expression after the tebuconazole treatment was not significantly different from that after control treatment ($P < 0.05$) at 3, 6, and 9 h. In addition, JHXJZ induced an upregulation at EC₁₀ but a downregulation ($P < 0.05$) at EC₅₀ at 12 h. At that timepoint, tebuconazole significantly upregulated gene expression (Fig. 14B).

JHXJZ significantly downregulated the expression of *CYP51* ($P < 0.05$) at EC₃₀ and EC₅₀ at 3 h, but tebuconazole did not result in a significant difference ($P < 0.05$) at that timepoint as compared with the control treatment. JHXJZ upregulated the expression ($P < 0.05$) at EC₃₀ at 6 h and EC₁₀ at 12 h, in agreement with the findings after tebuconazole treatment. However, no treatment at any of the three tested doses at 9 h significantly regulated the gene expression, whereas the tebuconazole treatment induced a significant upregulation ($P < 0.05$) at this timepoint (Fig. 14C).

The JHXJZ treatments did not significantly regulate the expression of *ERG24* ($P < 0.05$) at the three doses at 3 h, whereas tebuconazole induced an upregulation ($P < 0.05$) at that timepoint. JHXJZ downregulated the expression ($P < 0.05$) at the three doses at 6 h and at EC₃₀ at 9 h, in contrast to tebuconazole at those timepoints. However, JHXJZ treatments at EC₁₀ and EC₅₀ for 9 h as

well as at EC₁₀ for 12 h induced an upregulation ($P < 0.05$), similarly to the results after tebuconazole treatment (Fig. 14D).

JHXJZ upregulated the expression of *ERG27* ($P < 0.05$) at EC₃₀ and EC₅₀ at 3 h but downregulated the expression ($P < 0.05$) at EC₁₀ and EC₃₀ at 6 h, findings consistent with those after tebuconazole treatment at the same timepoints. However, JHXJZ

Table 2. Genes selected for the validation of RNA-seq data

Gene ID	Putative function	Gene expression ^a	
		RNA seq (log ₂ foldchange)	qRT-PCR (fold change)
Cluster-4153.9367	Hydrolase activity, hydrolyzing <i>O</i> -glycosyl compounds	3.8234	3.40585902
Cluster-4153.3177	Hydrolase activity, hydrolyzing <i>O</i> -glycosyl compounds	-2.1637	0.178787018
Cluster-4153.13602	Dipeptidase activity	-12.001	0.238427
Cluster-4153.13299	Dipeptidase activity	-3.1187	0.078604366
Cluster-4153.14052	Dipeptidase activity	-3.5899	0.076180344
Cluster-4153.13813	Hydrolase activity, acting on glycosyl bonds	-5.3404	0.062848431
Cluster-4153.21128	Hydrolase activity, acting on glycosyl bonds	-3.0441	0.611272215
Cluster-4153.13551	Tetrapyrrole binding	2.4236	2.203582963
Cluster-4153.9271	Tetrapyrrole binding	2.5064	1.214326216
Cluster-4247.0	Tetrapyrrole binding	-2.0964	0.391321101
Cluster-4153.13382	Iron ion binding	2.6815	1.524332716
Cluster-4153.6705	Iron ion binding	-2.305	0.18279232
Cluster-4153.10997	Iron ion binding	-8.7868	0.852204568
Cluster-4153.22752	Cofactor binding	-1.6284	0.228533894
Cluster-4153.3119	Cofactor binding	-4.1034	0.698885098
Cluster-4153.17571	Catalytic activity	-3.4296	0.309037912

^a qRT-PCR = quantitative real time PCR. Fold-change values represent the fold expression in *Lasiodiplodia theobromae* treated with jiahuangxianjunzuo as compared with that in the control.

Fig. 7. Differentially expressed genes enriched in gene ontology annotation.

Table 3. Steroid biosynthesis of the enriched pathway terms^a

UniGenes	KO ID	KO name	KO description
Cluster 4153.13049	K05917	CYP51	Sterol 14-demethylase
Cluster 4153.14165	K09831	ERG5	C-22 sterol desaturase
Cluster 4153.23948	K00559	E2.1.1.41, SMT1, ERG6	Sterol 24-C-methyltransferase
Cluster 4153.7992	K14674	TGL4	TAG lipase/steryl ester hydrolase/phospholipase
Cluster 4153.6963	K00801	FDFT1	A2/LPA acyltransferase
Cluster 4153.13915	K14674	TGL4	Farnesyl-diphosphate farnesyltransferase
Cluster 4153.7733	K09827	ERG27	TAG lipase/steryl ester hydrolase/phospholipase
Cluster 4153.24848	K00222	TM7SF2, ERG24	A2/LPA acyltransferase
Cluster 4153.8705	K09831	ERG5	3-Keto steroid reductase
Cluster 4153.14572	K00511	SQLE, ERG1	Δ 14-Sterol reductase
Cluster 4153.13551	K05917	CYP51	C-22 sterol desaturase
			Squalene monooxygenase
			Sterol 14-demethylase

^a Data from sample number 11, background 88. The P value is 0.0960937760031 and the corrected P value is 0.516793378915. KO = KEGG orthology.

treatments at EC₁₀ and EC₃₀ for 9 h as well as at EC₃₀ for 12 h downregulated *ERG27* expression ($P < 0.05$), in contrast to the effects of tebuconazole at the same timepoints (Fig. 14E).

The expression of *ERG5* was down-regulated ($P < 0.05$) by JHXJZ at the three doses at 3 h, in contrast to the effects of tebuconazole at the same timepoint. With longer treatment times, JHXJZ upregulated the expression of this gene at EC₃₀ and EC₅₀ at 6 h, but its expression was down-regulated ($P < 0.05$) at the same concentrations at 9 h, a result similar to the effects of tebuconazole at the same timepoints. However, treatments with EC₁₀ for 9 and 12 h upregulated the gene expression ($P < 0.05$) in contrast to findings after tebuconazole treatment (Fig. 14F).

Overall, JHXJZ downregulated the expression of *TGL4* ($P < 0.05$) in a concentration-dependent manner at the three doses at 3 h as well as at EC₁₀ and EC₃₀ at 6, 9, and 12 h, results quite similar to the effects of tebuconazole. In addition, treatment with JHXJZ at EC₅₀ for 12 h upregulated the expression ($P < 0.05$), in contrast to tebuconazole treatment (Fig. 14G).

Effects of JHXJZ on the roughness of the surface of hyphae of *L. theobromae*.

Compared with the control treatment, JHXJZ can enhance the height and roughness of hyphae. The degree of the change for two aspects depended on the dosages of JHXJZ (Fig. 15A to O). In addition, there was a similar result between the JHXJZ EC₅₀ and the tebuconazole EC₅₀ (Fig. 15M to R).

DISCUSSION

In this study, JHXJZ treatments at EC₃₀ and EC₅₀ decreased the ergosterol content from 4.42 to 3.37 and 2.79 $\mu\text{g}/\text{mg}$, respectively, reflecting ergosterol inhibition rates of 23.76 and 36.88%, respectively (Fig. 8). Additionally, two commercial fungicides, tebuconazole and azoxystrobin, which inhibit ergosterol synthesis and block electron transport in the mitochondrial respiratory chain served as controls (Bertelsen et al. 2001; Bowen et al. 1994; Ziogas et al. 1991). Tebuconazole at EC₅₀ decreased the ergosterol

Fig. 8. Effect of jiahuangxianjunzuo (JHXJZ) on ergosterol content of *Lasiodiplodia theobromae* ($P < 0.05$).

Fig. 9. Effect of jiahuangxianjunzuo (JHXJZ) on cell membrane permeability of *Lasiodiplodia theobromae*.

content to 2.83 $\mu\text{g}/\text{mg}$, reflecting an inhibition rate of 35.97%, which was similar to that of JHXJZ at EC_{50} ($P < 0.05$). However, azoxystrobin at EC_{30} decreased ergosterol content to only 4.19 $\mu\text{g}/\text{mg}$ with an inhibition rate of 5.2%, findings significantly different from those for JHXJZ at the same dose (Fig. 8). Treating *Ustilago avenae* spores with 1.5 $\mu\text{g}/\text{ml}$ triadimefon for 3.5 and 6 h has been found to decrease ergosterol content from 1.30 and 1.50 $\mu\text{g}/\text{mg}$ to 0.70 and 0.40 $\mu\text{g}/\text{mg}$, indicating inhibition rates of 46.15 and 73.33%, respectively (Buchenauer 1977). Triazolol and tebuconazole result in concentration-dependent decreases in the ergosterol levels of *Nectria hematococca* var. *cucurbitae* (Ziogas et al. 1991). Independent treatments with triazolol at 2.50 $\mu\text{g}/\text{ml}$ and tebuconazole at 0.50 $\mu\text{g}/\text{ml}$ decreased in the ergosterol content in mycelia from 67.90% to 13.00 and 12.90%, respectively. Thus, JHXJZ, like triazole fungicides, decreases the ergosterol content in hyphae, thereby resulting in their inhibited growth.

Transcriptomic DEGs revealed that JHXJZ can change the abundance of mRNA related to the metabolism of carbohydrate metabolic process at BP as well as the mRNA of membrane protein complex of organelles, related with Golgi equivalents, ER, and cell wall at CC, the mRNA with catalytic activity, hydrolase activity, carbohydrate derivative binding, and transmembrane transporter activity at MF (Supplementary Tables S16 and S17). These changes of DEGs ultimately represent the morphological changes of organelles, the changes of plasma membrane and cell wall (Figs. 10 and 11).

Ergosterol, the major sterol in fungal cell membranes, is involved in cell permeability (Burden et al. 1990; Douglas and Konopka 2014). After ergosterol synthesis was inhibited by JHXJZ, the ergosterol content in the hyphae was significantly decreased (Fig. 8). The structure and integrity of cell membranes was destroyed, thus altering the cell membrane permeability and producing inner cell leakage, as verified by REC experiments (Fig. 9). The REC of the mycelia increased at 11 h after JHXJZ treatments at EC_{10} , EC_{50} , and EC_{90} and the results showed a concentration-dependent trend. Previous studies corroborated our experimental results, in which triazole fungicidal treatments led to changes in the sterol composition of fungal cell membranes and increased cell membrane permeability (Sancholle et al. 1984). Thus, we speculated that the increase in REC was caused by the inhibition of ergosterol biosynthesis, which destroyed the integrity of the cell membrane, thereby affecting cell membrane permeability.

The above conclusion was further verified by the morphological results. After the cells were treated with JHXJZ at EC_{50} and EC_{90} , SEM revealed twisted, collapsed, shrunken, and deformed hyphae (Fig. 10), and TEM showed vacuoles and necrosis in the hyphal cytoplasm (Fig. 11). The optical microscopy coupled with fluorescence staining revealed that the hyphae showed a concentration-dependent distortion, decreased branching, and an accumulation of granular substances after JHXJZ treatments (Figs. 2 and 13). These effects were similar to those of triazole fungicides. For

Fig. 10. Scanning electron microscopy of *Lasiodiplodia theobromae* treated with jiahuangxianjunzuo (JHXJZ). **A** and **B**, Control (CK). **C**, EC_{10} (0.30 $\mu\text{g}/\text{ml}$) **D**, **E**, and **F**, EC_{50} (3.54 $\mu\text{g}/\text{ml}$). **G**, **H**, and **I**, EC_{90} (41.64 $\mu\text{g}/\text{ml}$). All bars = 5.0 μm .

instance, after treatment with tebuconazole at 20.00 $\mu\text{g/ml}$ for 2 days, TEM showed that the plasmalemmas of *Fusarium culmorum* were strongly invaginated, and the septa were abnormally thickened or incompletely formed. Additionally, septation increased markedly, and the septa were very close together. Furthermore, the number of vacuoles has been found to increase in the hyphal cytoplasm, and the organelles become disordered (Kang et al. 2001). Treatment with flusilazole at 0.16 $\mu\text{g/ml}$ results in more vacuoles in the hyphal cytoplasm of *Sclerotinia sclerotiorum* (Lu et al. 2015). In the concentration range of 10^{-8} to 10^{-6} M, 4-h independent treatments with tioconazole and itraconazole have been found to result in shorter *Candida albicans* hyphal lengths, and a 4-h tioconazole treatment has been found to decrease hyphal branching (Odds et al. 1985). The accumulation of fluorescent substances in the hyphae, shorter septal distances, and swollen hyphal tips have been observed after treatment of *Sclerotium rolfii* with cyproconazole at 0.75 $\mu\text{g/ml}$ for 30, 60, and 120 h (Roberson et al. 1989). According to the morphological results, we hypothesized that JHXJZ changed the morphology of hyphae by inhibiting the biosynthesis of ergosterol, thereby resulting in changes in the structures of septa, cell walls, and cell membranes.

In addition, the fluorescence microscopy results did not show significant differences among nuclei between JHXJZ-treatment

at the EC_{10} and the control treatment. However, after cells were treated with JHXJZ at EC_{50} , the nuclei in the hyphae decreased and dispersed and the fluorescence intensity of the background increased. When treated with JHXJZ at EC_{90} , the hyphal nuclei were scarcely visible and the fluorescence intensity of the background was much greater than that of the control. This finding may have resulted from increased cell-membrane permeability after JHXJZ treatments, which, in turn, led to gradual cell death owing to the degradation of hyphal nucleic acids, as demonstrated by the leakage of the fluorescent substance (Fig. 12). Nuclear staining demonstrated that cyproconazole had no apparent effects on the positioning of nuclei (Roberson et al. 1989). These findings indicated that JHXJZ and triazole fungicides cannot directly target nuclei but can alter the synthesis of the major cell wall and the plasma membrane, thereby influencing septal formation.

Many studies have indicated that triazole fungicides regulate the genes involved in ergosterol synthesis. Thus, it is important to determine gene expression levels related to ergosterol synthesis. For instance, the gene transcription profiles of *Fusarium graminearum* have been explored after a 6-h treatment with tebuconazole at 2.50 $\mu\text{g/ml}$ by using the DeepSAGE sequencing technique (Liu et al. 2010). In total, 23 genes detected by DeepSAGE were annotated to sterol biosynthesis, and nine genes, *ERG5*,

Fig. 11. Transmission electron microscopy of *Lasiodiplodia theobromae* treated with jiahuangxianjunzuo (JHXJZ). **A, B, and C,** Control (CK). **D, E, and F,** EC_{50} (3.54 $\mu\text{g/ml}$). **G, H, and I,** EC_{90} (41.64 $\mu\text{g/ml}$). CW = cell walls, Cy = cytoplasm, G = Golgi equivalents, M = mitochondria, N = nucleus, PM = plasma membrane, S = septum, V = vacuoles, L = lipid bodies. All bars = 1.0 μm .

ERG6 (two accessions), *ERG7*, *ERG10*, *ERG11* (*Cyp51*, two accessions), *ERG13* and *ERG25*, showed more than fivefold increases over the control. However, none of the genes were down-regulated by more than fivefold after the tebuconazole treatment. Among the genes upregulated by more than fivefold, *ERG5* was up-regulated by 6.26-fold and the two accessions of *ERG11* were up-regulated by 53.01- and 57.68-fold, respectively (Liu et al. 2010). The expression trends of *ERG5* and *ERG11* were consistent with those observed in our study (Fig. 14C and F). The two accessions of *ERG6* were up-regulated by 77.33- and 272.18-fold. However, this gene was down-regulated in our study. In addition, *ERG7*, *ERG10*, *ERG13*, and *ERG25* were up-regulated by 9.89-, 12.08-, 36.73-, and 12.08-fold, respectively (Liu et al. 2010). However, these genes were not differentially expressed in our study (Supplementary Table S12). Notably, *FDFT1*, *ERG1*, *ERG24*, and *ERG27* were up-regulated and *TGL4* was down-regulated in our study, whereas their differential expression was not reported in the literature (Liu et al. 2010).

The data of the transcriptome indicated that JHXJZ can change the abundance of mRNA involved with the formation of the membrane of organelles and the cell wall, and also change the abundance of mRNA of steroid, carbohydrate transmembrane transporter, channel regulator activity (Supplementary Tables S13 and S14). A KEGG pathway enrichment analysis of DEGs also verified some DEGs to be related with steroid biosynthesis (Supplementary Tables S15 and S16). Little literature reported the transcriptome of triazole fungicides for pathogenic fungus. The actions of JHXJZ and triazole fungicides upregulated *ERG5* and *ERG11* (*Cyp51*), which are key genes in the ergosterol

synthetic pathway, inhibiting ergosterol synthesis, causing damage to the cell-membrane structure and increasing cell-membrane permeability. So, these DEGs may be the intrinsic reason for the specific antifungal targets of JHXJZ and triazole fungicides.

In summary, JHXJZ, a sulfone compound containing 1, 3, 4-oxadiazole, showed excellent antifungal activity both in vitro and in vivo. In this study, its antifungal mechanism against *L. theobromae* was further investigated. JHXJZ appears to upregulate *ERG5* and *CYP51*, which are key genes in the ergosterol biosynthetic pathway that decrease the ergosterol content, disrupt the cell-membrane structure and increase cell-membrane permeability until cell death occurs. Compared with tebuconazole, JHXJZ regulates some different key genes in ergosterol biosynthesis. These findings may provide ideas and a basis for the development of sulfone compounds containing 1, 3, 4-oxadiazole. JHXJZ should be classified as an ergosterol-synthesis inhibitor, and its detailed antifungal targets in the ergosterol biosynthetic pathway deserve further study.

MATERIALS AND METHODS

Preparation of reagents and experimental materials.

JHXJZ and its analogs were synthesized and characterized with nuclear magnetic resonance and elemental and infra-red analyses (Supplementary Table S18) (Li et al. 2013; Xu et al. 2012). Tebuconazole (97% purity) was purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). As pathogens causing tea leaf spot or tea gray blight, strains GZHS-2017-010 of *L. theobromae*, GZSQ-4 of *Phoma segeticola* var.

Fig. 12. Distribution of the nucleus of *Lasiodiplodia theobromae* treated with jiahuangxianjunzuo (JHXJZ) or without JHXJZ. The mycelia were stained with 4',6-diamidino-2-phenylindole at 1 µg/ml and were observed with fluorescence microscope. A and B, Control (CK); C, EC₁₀ (0.30 µg/ml); D, EC₃₀ (1.29 µg/ml); E, EC₅₀ (3.54 µg/ml); F, EC₉₀ (41.64 µg/ml). All bars = 20 µm.

camelliae, and GZHS-2017-01 of *P. camelliae-sinensis*, preserved by our research group, were used (Li et al. 2018, 2019; Zhao et al. 2018a). Three strains have been deposited in China General Microbiological Culture Collection Center (CGMCC), with the preservation numbers of fungi as CGMCC3.20151, CGMCC3.20152, and CGMCC3.20199. Five-year-old potted *C. sinensis* cv. Fuding-dabaicha tea plants were grown in a greenhouse at Guizhou University, Guiyang, China. The tea plants were maintained at 25°C during the day and at 20°C at night, with 14-h light and 10-h dark cycles and a relative humidity of 70 to 80%.

Antifungal activity of JHXJZ and its analogs against three fungal pathogens in vitro.

Antifungal activity was determined according to the mycelial growth rate method, with minor modifications (Duan et al. 2013; Lv et al. 2018). First, JHXJZ and its analogs were dissolved in 200 µl of dimethyl sulfoxide (DMSO) (Solarbio, Beijing) to prepare stock solutions. Then, 10 ml of sterilized double-distilled H₂O (ddH₂O) containing 0.1% Tween 20 (Sangon, Shanghai, China) was mixed with different amounts of the stock solutions, and the solutions were further mixed with 90 ml of potato dextrose agar (PDA) (Solarbio) to obtain final concentrations of 1.25, 2.50, 5.00, 10.00, and 20.00 µg/ml, which were poured into 9-cm Petri dishes. The commercial fungicide tebuconazole was used as a positive control, and sterilized ddH₂O containing 0.1% Tween 20 without any fungicides was used as a negative control. Mycelial plugs (8 mm diameter) were cut from the edges of the colonies with a sterilized punch and were placed at the centers of the PDA plates with or without the antifungal active compound. For each dose, five experimental replications

were conducted. The inhibition rate of each compound against the pathogen was calculated with the formula: $I(\%) = [(C - T) / (C - 0.8)] \times 100$, where I represents the inhibition rate, C represents the fungal colony diameter on a negative control plate, and T represents the fungal colony diameter on the compound-treated plate. The EC₅₀ value of each compound was calculated on the basis of the linear regression of the probability value of the inhibition rate on log-transformed fungicide concentrations and was expressed as mean ± standard deviation. For the compound with the strongest antifungal activity, the EC₁₀, EC₃₀, and EC₉₀ values were also calculated with the same method. The experiment was independently repeated three times.

Antifungal activity of JHXJZ against *L. theobromae* in vivo.

Based on antifungal activity in vitro, the antifungal activity of JHXJZ for *L. theobromae* was further determined in detached leaves according to the method of Duan et al. (2013), with minor modifications. Briefly, healthy tea plants were selected at the one-bud and fifth-leaf stages and were then cleaned with sterilized water. Mycelial plugs (8 mm diameter) were cut from 2-day-old colonies cultured on PDA. The third and fourth leaves were punctured with sterilized syringe needles, were inoculated with mycelial plugs, and were covered with a plastic bag. At 24 h after inoculation, the mycelial plugs were discarded from the tea leaves. Lesions formed on the inoculating sites and reached 12 to 20 mm². JHXJZ and tebuconazole were individually dissolved in DMSO and were then dispersed into ddH₂O containing 0.1% Tween 20. The inoculated leaves were independently sprayed with JHXJZ at 50.00 or 100.00 µg/ml. The antifungal activity tests also incorporated fungicidal, pathogenetic, and healthy controls.

Fig. 13. Distribution of the septum of *Lasiodiplodia theobromae* treated with jiahuangxianjunzuo (JHXJZ) or without JHXJZ. The mycelia were stained with Calcofluor white at 1 µg/ml and were observed with a fluorescence microscope. **A** and **B**, Control (CK); **C**, EC₁₀ (0.30 µg/ml); **D**, EC₃₀ (1.29 µg/ml); **E**, EC₅₀ (3.54 µg/ml); **F**, EC₉₀ (41.64 µg/ml). All bars = 20 µm.

Tebuconazole (50.00 and 100.00 µg/ml) was used as the fungicidal control. PDA plugs with or without mycelia were used as the pathogenetic and healthy control, respectively. For the pathogenetic control, sterilized ddH₂O containing 0.1% Tween 20 was sprayed on the detached leaves inoculated with mycelial plugs. Then, tea twigs were maintained under the same conditions. At 7 days after the spraying treatment, lesion sizes were determined as the means of two diameters at right angles to one another. The antifungal activity of JHXJZ was expressed as the curative activity, calculated as follows: curative activity (%) = (lesion diameter in the pathogenetic control – lesion diameter in the treatment)/lesion diameter in the pathogenetic control × 100. Each treatment included 12 replicates, and the experiment was performed twice.

Changes in mycelial morphology under the JHXJZ treatment.

The observed mycelial morphological changes after treatment with JHXJZ may aid in determining its mechanism of action as a fungicide. The study of mycelial morphology was performed according to the method of Han et al. (2019), with minor modifications. Briefly, four mycelial plugs of *L. theobromae* were transferred to 90 ml of potato dextrose broth (PDB) (Solarbio) in 250-ml flasks, were then incubated in a shaker incubator (BSD-WX2350, Shanghai

Boxun Co., Ltd.) at 33°C and 200 rpm in the dark for 32 h, until the strain entered the logarithmic growth phase. Different amounts of JHXJZ were dissolved in 10 ml of sterilized ddH₂O containing 0.1% Tween 20 to final concentrations of 0.30 µg/ml (EC₁₀), 3.54 µg/ml (EC₅₀), or 41.64 µg/ml (EC₉₀). Sterilized ddH₂O containing 0.1% Tween 20 without JHXJZ was prepared in the same manner as the control. After another 16 h of continuous shaking under the same conditions, mycelia were washed with ddH₂O and were then placed on glass slides. The morphology of the mycelia was observed with a light microscope (Olympus BX43F, Tokyo). There were three replications performed per treatment, and the experiment was repeated three times.

RNA-seq.

Mycelia of *L. theobromae* were treated with JHXJZ at 3.54 µg/ml (EC₅₀) at 33°C and 200 rpm in the dark for 3 h and are noted as the “Treated” group. The negative control was treated with sterilized ddH₂O containing 0.1% Tween 20 and is noted as the “CK” group. After the mycelia had been treated for 3 h, they were ground into a fine powder in liquid nitrogen. Approximately 100 mg of mycelial powder was used for RNA extractions. Total RNA was extracted with an EASYspin Plus plant RNA kit (Aidlab, Beijing). The cDNA libraries were constructed from RNA samples for Illumina paired-end sequencing on the basis

Fig. 14. Effect of jiahuangxianjunzuo (JHXJZ) on related gene expression in ergosterol biosynthesis pathway of *Lasiodiplodia theobromae*. **A**, *FDFT1*; **B**, *ERG1*; **C**, *CYP51*; **D**, *ERG24*; **E**, *ERG27*; **F**, *ERG5*; **G**, *TGL4*. Significant analyses were performed among groups at the same time after treatment with JHXJZ or tebuconazole ($P < 0.05$).

of the Illumina protocol. Paired-end sequencing (2×100 bp) was carried out on the Illumina NovaSeq 6000 platform (Illumina, San Diego CA, U.S.A.) at Novogene Bioinformatics Technology Co., Ltd (Beijing). Each treatment contained four replicates, and the experiment was repeated three times.

To obtain clean reads, sequences with adaptor sequences, reads in which the proportion of ambiguous bases were greater than 10% or those containing sequence of low quality (more than 50% bases with $Q_{phred} \leq 20$) were removed. To generate a transcriptome without a genome as reference, clean reads were assembled to a reference sequence in Trinity (version v2.4.0) with `min_kmer_cov:3` (Grabherr et al. 2011). The longest cluster sequence was obtained with Corset hierarchical clustering for subsequent analyses (Davidson and Oshlack 2014). The longest assembled transcript of a gene was taken as its unigene.

Functional annotations.

Functional annotations of unigenes were based on the following databases: Nr, NCBI nonredundant nucleotide sequences (Nt), Protein family (Pfam) (Finn et al. 2008), KOG/clusters of orthologous groups of proteins (COG) (Koonin et al. 2004), Swiss-Prot (a manually annotated and reviewed protein sequence database) (Boeckmann et al. 2003), KEGG (Kanehisa et al. 2008), and GO (Young et al. 2010). NCBI BLAST 2.2.28+ was used for the alignments of unigenes to Nr, Nt, Swiss-Prot, and KOG. The E-value threshold was set to $1E-5$ in the alignments to Nr,

Nt, and Swiss-Prot. For the alignments to KOG, the E-value threshold was $1E-3$. Hmmscan in HMMER 3.0 was used to search Pfam (Finn et al. 2008). The GO annotations were performed with Blast2GO v2.5 based on the Nr and Pfam annotations (Götz et al. 2008). The KEGG automatic annotation server (KAAS) was used for KEGG annotations (Kanehisa et al. 2008).

Gene expression analysis of the transcriptome.

The transcriptome assembled by Trinity software (v2.4.0) was used as the reference sequence, to which clean reads from each treatment were mapped in RNA-seq by expectation-maximization (RSEM) software (v1.2.15), with the parameter of Bowtie2 having a mismatch of 0 (Li and Dewey 2011).

RSEM software was used to analyze the alignments from Bowtie2, which showed the read counts of each unigene in each sample (Li and Dewey 2011). The read counts were transformed into expected number of fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) for the gene expression analyses (Trapnell et al. 2010). The resulting FPKM values were used to represent the expression levels of unigenes in different samples. The density distribution of FPKM reflects the gene expression pattern of each sample. The peak of the density distribution curve represents the number of genes at that level of expression. To determine the biological duplications of samples, correlations among gene expression levels were tested and analyzed.

Fig. 15. Atomic force microscopy of *Lasiodiplodia theobromae* treated with jiahuangxianjunzuo (JHXJZ) and tebuconazole. **A, B,** and **C,** Control (CK). **D, E,** and **F,** EC_{10} (0.30 $\mu\text{g/ml}$). **G, H,** and **I,** EC_{30} (1.29 $\mu\text{g/ml}$). **J, K,** and **L,** EC_{50} (3.54 $\mu\text{g/ml}$). **M, N,** and **O,** EC_{90} (41.64 $\mu\text{g/ml}$). **P, Q,** and **R,** Tebuconazole at the dosage of EC_{50} (0.15 $\mu\text{g/ml}$). **D** through **O** show results of JHXJZ treatment. **A, D, G, J, M,** and **P** show amplitude error. Bars = 2.0 μm . **B, E, H, K, N,** and **Q** show height sensor. All bars = 2.0 μm . **C, F, I, L, O,** and **R** present the scan for height (y axis) and distance (x axis) values.

Differential gene expression analyses between two treatment groups were performed in DESeq2 software, with the thresholds for significance adjusted to $p_{adj} < 0.05$ and $\log_2^{\text{FoldChange}} > 1$ (Love et al. 2014). A volcano plot, a Venn diagram, and hierarchical clustering were used to illustrate the differential gene expression patterns between the two treatment groups. The $\log_2$ (ratios) of relative gene expression were clustered with heatmap software and H-cluster, K-means, and self-organizing map methods. According to the functional annotations of unigenes, DEGs were selected as candidate genes for the study of the mechanisms of action of JHXJZ.

qRT-PCR analysis.

qRT-PCR was used to validate the DEGs identified by transcriptome sequencing for the two treatment groups. Total RNA was extracted as described previously for the samples used in transcriptome sequencing. With the PrimeScript RT reagent qPCR kit with gDNA eraser kit (TaKaRa, Dalian, China), genomic DNA was removed from total RNA (300 ng RNA per sample), and cDNA was synthesized and diluted 10-fold with ddH₂O. Information regarding the primers is shown in Supplementary Table S19. The PCR mixture contained 10.0 µl abm EvaGreen qPCR MasterMix-No dye (TaKaRa), 7.4 µl ddH₂O, 0.8 µl of each gene-specific primer (10 µM), and 1 µl cDNA template. The qRT-PCR assays were performed in an ABI 7500 system (Applied Biosystems 7500, Foster City, CA, U.S.A.) with the following program: 95°C for 10 min, 40 cycles of 94°C for 15 s and 60°C for 1 min. *β-Tubulin* was used as the reference gene to normalize the expression levels of the target genes (Paolinelli-Alfonso et al. 2016). The relative expression levels of the target genes were calculated with the $2^{-\Delta\Delta C_t}$ method (Adnan et al. 2011; Livak and Schmittgen 2001).

Determination of ergosterol.

Ergosterol extractions were performed according to the methods of Silva et al. (2010) and Kohiyama et al. (2015), with modifications. Briefly, four mycelial plugs of *L. theobromae* were transferred to 100 ml of PDB with JHXJZ at doses of EC₁₀, EC₃₀ and EC₅₀, were then incubated in a shaker incubator at 33°C and 200 rpm for 48 h. Tebuconazole (0.15 µg/ml) and azoxystrobin (50.00 µg/ml), which function by inhibiting ergosterol synthesis and blocking electron transportation in the mitochondrial respiratory chain, respectively, served as control treatments (Bertelsen et al. 2001; Bowen et al. 1994; Ziogas et al. 1991). The negative control was treated with the same solution without fungicides. After 48 h, the mycelia were collected, were washed twice with ddH₂O, and were dried at 60°C. Dry mycelia (0.1 g) from each group were individually transferred to tubes containing 20 ml of methanol, 5 ml of ethanol, and 2 g of potassium hydroxide. Each tube containing the mixture was shaken slightly for 5 min and was incubated in a water bath at 70°C for 40 min. After the mixtures were cooled at room temperature, 5 ml of ddH₂O was added to each tube, and was centrifuged (Anke TGL-20B, Shanghai, China) at 10,000 rpm for 10 min. Supernatants were transferred to a separation funnel, and then, an equal volume of n-hexane was added. The solutions were shaken vigorously for 2 min and were then allowed to sit for 10 min. The n-hexane (upper layer) fraction was collected and placed in a glass flask. The solvent in each flask was evaporated at 40°C and 80 rpm in a rotary evaporator (Yarong RE 2000A, Shanghai, China). The obtained residue was stored at -20°C until analysis.

Ergosterol was determined with high-performance liquid chromatography (HPLC) (Agilent 1100,) equipped with a C₁₈ chromatographic column (5 µm, 4.6 × 150 mm) (Agilent Technologies, Santa Clara, CA, U.S.A.), according to the methods of Silva et al. (2010) and Kohiyama et al. (2015), with minor modifications. The extracts were resuspended in 3 ml of pure ethanol

and were filtered through a 0.22-µm membrane. Then, 20 µl of solution was injected into the HPLC system. HPLC grade methanol (Adamas-beta, Shanghai, China) was used as the mobile phase with a flow rate of 1 ml/min. The retention time was 8.9 min, and the wavelength was 282 nm. The detection and quantification limits were 0.33 and 1 µg/ml, respectively. The standard curve was constructed with 0.50, 1.00, 5.0, 10.00, 50.00, 100.00, 250.00, 500.00, and 1,000.00 µg/ml of pure standard (Sigma-Aldrich, Shanghai, China) dissolved in ethanol. The ergosterol content was quantified by comparison with a standard curve ($Y = 26.568X + 32.547$, $R^2 = 1$). The recovery ratio of ergosterol by HPLC was $95.89 \pm 3.54\%$. The ergosterol content was expressed in micrograms per milligram. Each treatment contained four replicates, and the experiment was repeated three times.

Determination of the leakage of intracellular contents.

Four mycelial plugs of *L. theobromae* were transferred to 100 ml of PDB medium for culturing at 33°C and 200 rpm in the dark for 32 h. The PDB medium was discarded after centrifugation at 3,000 rpm for 2 min. Then, the mycelia were washed with ddH₂O three times and were collected on filter paper. Samples of 1 g (fresh weight) of mycelia were independently exposed to 20 ml of JHXJZ solutions at the EC₁₀, EC₅₀, and EC₉₀. Mycelia were exposed to 20 ml of sterilized ddH₂O containing 0.1% Tween 20. Each treatment included four replicates. To assess the leakage of the cell contents through the cell membranes, the electrical conductivity of each solution was determined 11 times at 1-h intervals with a conductivity meter (Shanghai Precision and Scientific Instrument, Shanghai, China) after treatment. Finally, all samples were boiled for 5 min, and their final conductivity levels were determined after they had cooled to room temperature (Gao et al. 2018). The REC was calculated according to the following formula: REC (%) = conductivity/final conductivity × 100 (Duan et al. 2013).

Effects of JHXJZ on ultrastructure *L. theobromae*.

To observe the effects of JHXJZ on the ultrastructure of *L. theobromae*, we transferred four mycelial plugs of *L. theobromae* to 90 ml of PDB in 250-ml flasks and incubated them in a shaker at 33°C and 200 rpm in the dark for 32 h. The mycelia were treated with JHXJZ at the EC₅₀ and EC₉₀ for 16 h. Solutions without JHXJZ were used as negative controls. The mycelia were treated according to the methods of Yue et al. (2015) and were then observed by SEM (Hitachi TM-1000, Tokyo) and TEM (Hitachi H-7650).

Effects of JHXJZ on cell nuclei and septa of strain GZHS-2017-010.

Four mycelial plugs of *L. theobromae* were transferred to 90 ml of PDB in 250-ml flasks for culturing at 33°C and 200 rpm for 32 h. After the mycelia reached the logarithmic growth phase, 10 ml of sterilized ddH₂O containing 0.1% Tween 20 and different doses of JHXJZ was added to the PDB medium to final JHXJZ concentrations set at EC₁₀, EC₅₀, and EC₉₀. The negative control was treated with sterilized ddH₂O containing 0.1% Tween 20. After another 16 h of culturing under the same conditions, the mycelia were collected and washed twice with 1 ml of PBS. To observe cell nuclei, we dyed the mycelia with 200 µl of 4',6-diamidino-2-phenylindole (BioFroxx, Hessen, Germany) at 1 µg/ml for 30 min in the dark (Chen et al. 2018). To observe septa, we dyed the mycelia with 200 µl Calcofluor White (Sigma-Aldrich) at 1 µg/ml for 30 s in the dark. After being washed three times with 1 ml of PBS, the mycelia were observed through fluorescence microscopy (Olympus DP74 and FVMPE-RS) (Chen et al. 2018).

Effects of JHXJZ on the expression of genes involved in sterol biosynthesis in *L. theobromae*.

Because JHXJZ reduced the ergosterol content of *L. theobromae*, we speculated that JHXJZ might regulate gene expression involved in sterol biosynthesis. Consequently, we analyzed the expression of *L. theobromae* genes located in the sterol biosynthetic pathway with or without JHXJZ treatments at different doses or different treatment times, using qRT-PCR. Four mycelial plugs of *L. theobromae* were transferred to 90 ml of PDB in 250-ml flasks, were then incubated in a shaker at 33°C and 200 rpm in the dark for 32 h. Subsequently, different doses of JHXJZ were dissolved in DMSO and were dispersed into 10 ml of sterilized ddH₂O containing 0.1% Tween 20. The final concentrations of JHXJZ were 0.30 µg/ml (EC₁₀), 1.29 µg/ml (EC₃₀), and 3.54 µg/ml (EC₅₀). Tebuconazole served as the positive control, and sterilized ddH₂O containing 0.1% Tween 20 served as the negative control. After 3, 6, 9, and 12 h of continuous shaking under the same conditions, samples from each treatment were collected and washed twice with PBS. Total RNA extraction, cDNA synthesis, and qRT-PCR analysis were performed as described previously. The primers used in this section are listed in Supplementary Table S20.

Effects of JHXJZ on the roughness of the surface of hyphae of *L. theobromae*.

Four mycelial plugs of *L. theobromae* were transferred to 90 ml of PDB in 250-ml flasks for culturing at 33°C and 200 rpm for 32 h. Once the mycelia reached the logarithmic growth phase, 10 ml of sterilized ddH₂O containing 0.1% Tween 20 and different dosages of JHXJZ were added to the PDB medium, and final JHXJZ concentrations were set at EC₁₀, EC₃₀, EC₅₀, and EC₉₀. The commercial fungicide tebuconazole was used as a positive control at a dosage of 0.15 µg/ml (EC₅₀), and sterilized ddH₂O containing 0.1% Tween 20 without any fungicides was used as a negative control. After another 16 h of culturing under the same conditions, the mycelia were collected and washed three times with 1 ml of sterilized ddH₂O. Then, some of the hyphae were placed on a glass slide and put in a Petri dish, to prevent impurities in the air from flying in; the hyphae were dried in an oven at 37°C and were then observed under an atomic force microscope (Dimension ICON, Bruker, Santa Barbara, CA, U.S.A.) (Dong et al. 2011; Ma et al. 2006; Oberle-Kilic et al. 2013).

Statistical analysis.

The data collected from the qRT-PCR and ergosterol content determinations in this study were analyzed for statistical significance with the least significant difference method in SPSS software (Version 22). A *P* value of 0.05 was used to evaluate significance.

Availability of transcriptome data.

The datasets generated in this study are available in the NCBI Sequence Read Archive (SRA) database with the SRA accession PRJNA540200 (temporary submission ID: SUB5522448).

AUTHOR-RECOMMENDED INTERNET RESOURCES

Bioproject SRA records:

<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA540200>

GO database: <http://geneontology.org>

KAAS: <http://www.genome.jp/kegg/kaas>

KOG/COG database: <http://www.ncbi.nlm.nih.gov/COG>

Pfam database: <http://pfam.xfam.org>

RSEM software: <http://deweylab.github.io/RSEM>

Swiss-Prot database: <http://www.ebi.ac.uk/uniprot>

Trinity software: <https://github.com/trinityrnaseq/trinityrnaseq/wiki>

LITERATURE CITED

- Adnan, M., Morton, G., and Hadi, S. 2011. Analysis of *rpoS* and *bolA* gene expression under various stress-induced environments in planktonic and biofilm phase using 2^(-ΔΔCT) method. *Mol. Cell. Biochem.* 357:275-282.
- Bao, X. T., Dharmasena, D. S. P., Li, D. X., Wang, X., Jiang, S. L., Ren, Y. F., Wang, D. L., Song, B. A., and Chen, Z. 2019. First report of *Epilicium sorghinum* causing leaf spot on tea in China. *Plant Dis.* 103:3282.
- Bertelsen, J. R., Neergaard, E. D., and Smedegaard-Petersen, V. 2001. Fungicidal effects of azoxystrobin and epoxiconazole on phyllosphere fungi, senescence and yield of winter wheat. *Plant Pathol.* 50:190-205.
- Boeckmann, B., Bairoch, A., Apweiler, R., Blatter, M. C., Estreicher, A., Gasteiger, E., Martin, M. J., Michoud, K., O'Donovan, C., Phan, I., Pilbout, S., and Schneider, M. 2003. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. *Nucleic Acids Res.* 31:365-370.
- Bowen, K. L., Hagan, A. K., Olive, J., and Foster, W. 1994. Application rates and spray intervals of ergosterol-biosynthesis inhibitor fungicides for control of *entomosporium* leaf spot of *Photinia*. *Plant Dis.* 78:578-581.
- Buchenauer, H. 1977. Mode of action of triadimefon in *Ustilago avenae*. *Pestic. Biochem. Physiol.* 7:309-320.
- Burden, R. S., Cooke, D. T., and Hargreaves, J. A. 1990. Mechanism of action of herbicidal and fungicidal compounds on cell membranes. *Pest Manag. Sci.* 30:125-140.
- Chen, Y. L., Mao, X. W., Wang, J. X., Wu, L. Y., Zhou, M. G., and Hou, Y. P. 2018. Activity of the dinitroaniline fungicide fluazinam against *Bipolaris maydis*. *Pestic. Biochem. Physiol.* 148:8-15.
- Davidson, N. M., and Oshlack, A. 2014. Corset: Enabling differential gene expression analysis for de novo assembled transcriptomes. *Genome Biol.* 15:410.
- Dong, J., Signo, K. S. L., Vanderlinde, E. M., Yost, C. K., and Dahms, T. E. S. 2011. Atomic force microscopy of a *ctpA* mutant in *Rhizobium leguminosarum* reveals surface defects linking CtpA function to biofilm formation. *Microbiol. Read.* 157:3049-3058.
- Douglas, L. M., and Konopka, J. B. 2014. Fungal membrane organization: The eisosome concept. *Annu. Rev. Microbiol.* 68:377-393.
- Duan, Y., Ge, C., Liu, S., Chen, C., and Zhou, M. 2013. Effect of phenylpyrrole fungicide fludioxonil on morphological and physiological characteristics of *Sclerotinia sclerotiorum*. *Pestic. Biochem. Physiol.* 106:61-67.
- Finn, R. D., Tate, J., Mistry, J., Coghill, P. C., Sammut, S. J., Hotz, H. R., Ceric, G., Forslund, K., Eddy, S. R., Sonhammer, E. L., and Bateman, A. 2008. The Pfam protein families database. *Nucleic Acids Res.* 36 (Database):D281-D288.
- Gao, Y., He, L., Li, X., Lin, J., Mu, W., and Liu, F. 2018. Toxicity and biochemical action of the antibiotic fungicide tetramycin on *Colletotrichum scovillei*. *Pestic. Biochem. Physiol.* 147:51-58.
- Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., Robles, M., Talón, M., Dopazo, J., and Conesa, A. 2008. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* 36:3420-3435.
- Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., and Regev, A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* 29:644-652.
- Han, Y., Zhao, J., Zhang, B., Shen, Q., Shang, Q., and Li, P. 2019. Effect of a novel antifungal peptide P852 on cell morphology and membrane permeability of *Fusarium oxysporum*. *Biochim. Biophys. Acta Biomembr.* 1861:532-539.
- He, L., Li, X., Gao, Y., Li, B., Mu, W., and Liu, F. 2019. Characterization and fungicide sensitivity of *Colletotrichum* spp. from different hosts in Shandong, China. *Plant Dis.* 103:34-43.
- Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima, S., Okuda, S., Tokimatsu, T., and Yamanishi, Y. 2008. KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* 36 (Database):D480-D484.
- Kang, Z., Huang, L., Krieg, U., Mauler-Machnik, A., and Buchenauer, H. 2001. Effects of tebuconazole on morphology, structure, cell wall components and trichothecene production of *Fusarium culmorum* in vitro. *Pest Manag. Sci.* 57:491-500.
- Kohiyama, C. Y., Yamamoto Ribeiro, M. M., Mossini, S. A., Bando, E., Bomfim, N. S., Nerilo, S. B., Rocha, G. H., Grespan, R., Mikcha, J. M., and Machinski, M., Jr. 2015. Antifungal properties and inhibitory

- effects upon aflatoxin production of *Thymus vulgaris* L. by *Aspergillus flavus* Link. Food Chem. 173:1006-1010.
- Koonin, E. V., Fedorova, N. D., Jackson, J. D., Jacobs, A. R., Krylov, D. M., Makarova, K. S., Mazumder, R., Mekhedov, S. L., Nikolskaya, A. N., Rao, B. S., Rogozin, I. B., Smirnov, S., Sorokin, A. V., Sverdlov, A. V., Vasudevan, S., Wolf, Y. I., Yin, J. J., and Natale, D. A. 2004. A comprehensive evolutionary classification of proteins encoded in complete eukaryotic genomes. Genome Biol. 5:R7.
- Li, B., and Dewey, C. N. 2011. RSEM: Accurate transcript quantification from RNA-seq data with or without a reference genome. BMC Bioinformatics 12:323.
- Li, D. X., Bao, X. T., Ren, Y. F., Wang, Y., Song, B. A., Chen, Z., and Wang, Y. 2019. First report of *Lasiodiplodia theobromae* causing leaf spot on tea plant in Guizhou province of China. Plant Dis. 103:374.
- Li, D. X., Zhao, X. Z., Wang, Y., Lian, S. S., Ren, Y. F., and Chen, Z. 2018. Identification of the pathogen causing tea gray blight disease in Huishui county, Guizhou province. (in Chinese) Chin. J. Trop. Crop. 39:1827-1833.
- Li, P., Yin, J., Xu, W., Wu, J., He, M., Hu, D., Yang, S., and Song, B. 2013. Synthesis, antibacterial activities, and 3D-QSAR of sulfone derivatives containing 1, 3, 4-oxadiazole moiety. Chem. Biol. Drug Des. 82:546-556.
- Liu, X., Jiang, J., Shao, J., Yin, Y., and Ma, Z. 2010. Gene transcription profiling of *Fusarium graminearum* treated with an azole fungicide tebuconazole. Appl. Microbiol. Biotechnol. 85:1105-1114.
- Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-(\Delta\Delta C_T)}$ Method. Methods 25:402-408.
- Love, M. I., Huber, W., and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15:550.
- Lu, X. M., Zhu, Z. Q., Di, Y. L., and Zhu, F. X. 2015. Baseline sensitivity and toxic action of flusilazole to *Sclerotinia sclerotiorum*. Crop Prot. 78: 92-98.
- Lv, P., Chen, Y., Zhao, Z., Shi, T., Wu, X., Xue, J., Li, Q. X., and Hua, R. 2018. Design, synthesis and antifungal activities of 3-acyl thiotetronic acid derivatives: New fatty acid synthase inhibitors. J. Agric. Food Chem. 66:1023-1032.
- Ma, H., Snook, L. A., Tian, C., Kaminskyj, S. G. M., and Dahms, T. E. S. 2006. Fungal surface remodelling visualized by atomic force microscopy. Mycol. Res. 110:879-886.
- Oberle-Kilic, J., Dighton, J., and Arbuckle-Keil, G. 2013. Atomic force microscopy and micro-ATR-FT-IR imaging reveals fungal enzyme activity at the hyphal scale of resolution. Mycology 4:44-53.
- Odds, F. C., Cockayne, A., Hayward, J., and Abbott, A. B. 1985. Effects of imidazole- and triazole-derivative antifungal compounds on the growth and morphological development of *Candida albicans* hyphae. J. Gen. Microbiol. 131:2581-2589.
- Paolinelli-Alfonso, M., Galindo-Sánchez, C. E., and Hernandez-Martinez, R. 2016. Quantitative real-time PCR normalization for gene expression studies in the plant pathogenic fungi *Lasiodiplodia theobromae*. J. Microbiol. Methods 127:82-88.
- Paramasivam, M., Deepa, M., Selvi, C., and Chandrasekaran, S. 2017. Dissipation kinetics and safety evaluation of tebuconazole and trifloxystrobin in tea under tropical field conditions. Food Addit. Contam. A. 34:2155-2163.
- Ren, Y. F., Li, D. X., Zhao, X. Z., Wang, Y., Bao, X. T., Wang, X., Wu, X., Wang, D. L., Song, B. A., and Chen, Z. 2019. Whole genome sequences of the tea leaf spot pathogen *Didymella segeticola*. Phytopathology 109:1676-1678.
- Roberson, R. W., Fuller, M. S., and Grabski, C. 1989. Effects of the demethylase inhibitor, cyproconazole, on hyphal tip cells of *Sclerotium rolfsii*: I. A light microscope study. Pestic. Biochem. Physiol. 34:130-142.
- Sancholle, M., Weete, J. D., and Montant, C. 1984. Effects of triazoles on fungi: I. Growth and cellular permeability. Pestic. Biochem. Physiol. 21: 31-44.
- Shi, L., Li, P., Wang, W., Gao, M., Wu, Z., Song, X., and Hu, D. 2015. Antibacterial activity and mechanism of action of sulfone derivatives containing 1, 3, 4-oxadiazole moieties on rice bacterial leaf blight. Molecules 20:11660-11675.
- Silva, R. R., Corso, C. R., and Matheus, D. R. 2010. Effect of culture conditions on the biomass determination by ergosterol of *Leptinocybe crinitus* and *Psilocybe castanella*. World J. Microb. Bio. 26:841-846.
- Sowndhararajan, K., Marimuthu, S., and Manian, S. 2013. Integrated control of blister blight disease in tea using the biocontrol agent *Ochrobactrum anthropi* strain BMO-111 with chemical fungicides. J. Appl. Microbiol. 114:1491-1499.
- Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., Salzberg, S. L., Wold, B. J., and Pachter, L. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28:511-515.
- Wang, Y., Xiong, F., Lu, Q., Hao, X., Zheng, M., Wang, L., Li, N., Ding, C., Wang, X., and Yang, Y. 2019. Diversity of *Pestalotiopsis*-like species causing grey blight disease of tea plants (*Camellia sinensis*) in China, including two novel *Pestalotiopsis* species, and analysis of their pathogenicity. Plant Dis. 103:2548-2558.
- Wang, Y. C., Hao, X. Y., Wang, L., Bin Xiao, Wang, X. C., and Yang, Y. J. 2016. Diverse *Colletotrichum* species cause anthracnose of tea plants (*Camellia sinensis* (L.) O. Kuntze) in China. Sci. Rep. 6:35287.
- Xu, W. M., He, J., He, M., Han, F. F., Chen, X. H., Pan, Z. X., Wang, J., and Tong, M. G. 2011. Synthesis and antifungal activity of novel sulfone derivatives containing 1,3,4-oxadiazole moieties. Molecules 16:9129-9141.
- Xu, W. M., Han, F. F., He, M., Hu, D. Y., He, J., Yang, S., and Song, B. A. 2012. Inhibition of tobacco bacterial wilt with sulfone derivatives containing an 1,3,4-oxadiazole moiety. J. Agric. Food Chem. 60:1036-1041.
- Young, M. D., Wakefield, M. J., Smyth, G. K., and Oshlack, A. 2010. Gene ontology analysis for RNA-seq: Accounting for selection bias. Genome Biol. 11:R14.
- Yue, C., Cao, H. L., Wang, L., Zhou, Y. H., Huang, Y. T., Hao, X. Y., Wang, Y. C., Wang, B., Yang, Y. J., and Wang, X. C. 2015. Effects of cold acclimation on sugar metabolism and sugar-related gene expression in tea plant during the winter season. Plant Mol. Biol. 88:591-608.
- Zhao, X. Z., Chen, Z., Yu, L., Hu, D. Y., and Song, B. A. 2018a. Investigating the antifungal activity and mechanism of a microbial pesticide Shenqinmycin against *Phoma* sp. Pestic. Biochem. Physiol. 147:46-50.
- Zhao, X. Z., Wang, Y., Li, D. X., Ren, Y. F., and Chen, Z. 2018b. Morphological characterization and phylogenetic analysis of the pathogen *Phoma segeticola* var. *camelliae* causing a new tea disease. Acta Phytopathol. Sin. 48:556-559.
- Ziogas, B. N., Oesterheld, G., Masner, P., Steel, C. C., and Furter, R. 1991. Fenpropimorph: A three site inhibitor of ergosterol biosynthesis in *Nectria haematococca* var. *cucurbitae*. Pestic. Biochem. Physiol. 39: 74-83.