Inhibition Mechanism of Caffeine in Tea Pathogenic Fungi *Botryosphaeria Dothidea* and *Colletotrichum Gloeosporioides*

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Research article

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Abstract

Background Caffeine acts as an antifungal agent; however, its underlying inhibition mechanism remains unclear. To investigate variations in caffeine antifungal activity, this study was evaluate the inhibition mechanisms of caffeine on phytopathogens *Botryosphaeria dothidea* and *Colletotrichum gloeosporioides* through transcriptomic analysis, which causes leaf necrosis and anthracnose on tea leaves, respectively.

Results Our *in vitro* results shows that the antifungal activity of caffeine quite different between *B. dothidea* and *C. gloeosporioides*. Furthermore, microscopic observation and bioactivity assay results proved that caffeine destroyed the fungal protective layers (cell wall and cell membrane) and subcellular organelles. The transcriptomes of these two fungi exposed to different caffeine concentrations were analyzed by high-throughput sequencing and the results showed that caffeine repressed gene expression involved in the pathways of mRNA surveillance, ribosome biogenesis in eukaryotes, aminoacyl-tRNA biosynthesis, etc. Particularly, caffeine affected gene expression in melanin biosynthesis in *C. gloeosporioides*, resulting in the disturbances of melanin production and fungal pathogenicity. Manually infected tea leaves showed melanin pigment was important to appressorium development and fungal infection by histopathological observation and fungal biomass quantification. Besides, *C. gloeosporioides* successfully enters into the host tissue even plant could build multilayer defenses against the fungus, this might be due to the variation in pathogen targets and quantities of secondary metabolites by the host cells.

Conclusions Overall, our findings indicate that caffeine in the environment can reduce the fungal growth and physiological activities. However, the genome-wide expression showed different transcriptional profiles between these fungal isolates. Thus, we believe that target of the caffeine varied among isolates and melanin may play a major role in fungus caffeine tolerance. Further in-depth research required to conclude this hypothesis.

Background

Tea (*Camellia sinensis* (L.) O. Kuntze) is an important commercial crop grown worldwide. Tea plants can be severely affected by biotic and abiotic factors [1–3], and the major crop loss in tea by biotic factors (pests and diseases) is around 30 million kg/year, particularly, the economic loss of tea due to diseases is huger than pests [4]. The perennial nature of tea plants makes them ideal hosts for diverse pathogens in an ecological niche. Among them, *Colletotrichum gloeosporioides* ranked 8th in a list of the top 10 fungal pathogens is an important fungal pathogen that causes anthracnose in several agricultural crops including the tea plant [5]. *C. gloeosporioides* also affects the quantity and quality of tea production [2, 6]. *Botryosphaeria dothidea* is one of the most common fungal species that is found on many hosts and causes canker and dieback in trees [7], recently, *B. dothidea* was reported to cause leaf necrosis in tea plants [8].

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid and can be found in more than 80 plant species [9]. Caffeine is a vital secondary metabolite and has a critical function on chemical defense against biotic attackers (insects and fungi) in several plant species including coffee and tea [10]. And caffeine is a toxic compound for some pathogens and herbivores [11, 12]. The physiological role of caffeine in plants is not fully understood, though caffeine is known to be directly or indirectly involved in controlling microbial growth. The direct mechanism is observed when exogenously applied caffeine inhibits the growth and development of several organisms including bacteria, fungi and insects, and the indirect mechanism is to promote plant host's defense against pathogens [12–14]. Tea plant contains many secondary metabolites such as polyphenols and purine alkaloids, among them, catechins and caffeine may be involved in the tea anthracnose resistance mechanism [15]. Phenylpropanoid compounds and phytohormones played a major role against anthracnose fungus in the tea and strawberry plants, respectively [6, 16].

The effect of caffeine varies among microorganisms and ranges from harmful to beneficial. Some bacterial species utilize caffeine for their carbon requirement, whereas other bacterial growths are arrested by caffeine [17]. Different caffeine concentrations strongly inhibited the growth of *Crinipellis perniciosus* and tea plant pathogenic fungi, such as *Colletotrichum fructicola*, *C. camelliae*, *Pestalotiopsis theae* and *Phyllosticta theicola* [15, 18, 19]. Caffeine also acts as a powerful inhibitor of cAMP phosphodiesterase in bacteria, fungi and insects [14]. Sugiyama et al. [9] and Harren et al. [20] reported that fungal growth inhibition by caffeine varied according to the role and quantity of phosphodiesterase. Furthermore, Sugiyama et al. [9] found that caffeine played dual roles by both suppressing the growth of beneficial fungi, such as *Trichoderma* sp., and enhancing the antagonistic activity of the beneficial fungi against fungal pathogens.

Although several studies have focused on the antifungal activity of caffeine, the effect of caffeine on plant pathogenic fungi at the molecular level still requires investigation. The antifungal activity of caffeine against different tea plant pathogenic fungi, such as *Alternaria alternata*, *Pestalotiopsis* sp., *C. gloeosporioides* and *B. dothidea*, has previously been determined by us. Among them, *B. dothidea* and *C. gloeosporioides* are identified to be sensitive and tolerant to caffeine, respectively. Thus, these two pathogenic fungi were selected for further investigation to elucidate their inhibition mechanism under different caffeine dosages by using RNA-seq combined with microscopic observation, enzymatic activity determination, and gene expression analysis. The results of this study could provide novel insights into how the inhibition mechanism of caffeine controls pathogenic fungi in tea plants.

Results

Antifungal activity of caffeine against *B. dothidea* and *C. gloeosporioides*

Caffeine exhibited strong antifungal activity against both *B. dothidea* and *C. gloeosporioides*. *B. dothidea* was a fast-growing fungus, and it covered 9-cm control culture plates within 5 days incubation, its

mycelial growth was measured up to 3 mg/mL of caffeine concentration, above this dosage, fungal growth was completely inhibited. The MIC and EC₈₀ of caffeine against *B. dothidea* were 3 and 1.4 mg/mL, respectively. The mycelial growth rate of *C. gloeosporioides* was slower than *B. dothidea*, taking 7 days to cover control PDA plates, its mycelial growth was observed at the caffeine concentration of up to 7 mg/mL, above this dosage, the growth was arrested. The MIC and EC₈₀ of *C. gloeosporioides* were 7 and 3.2 mg/mL, respectively (Fig. 1A and 1B). On the basis of their MICs, the fungi were classified as caffeine sensitive (*B. dothidea*) and tolerant (*C. gloeosporioides*). Subsequent investigations used the following caffeine concentrations: 1 and 3 mg/mL for *B. dothidea*, 3 and 7 mg/mL for *C. gloeosporioides*.

Effect of caffeine on cell structural changes

SEM results revealed that *B. dothidea* and *C. gloeosporioides* hyphae were thicker and serrated with roughened walls in caffeine treated samples, whereas healthy hyphae, with smooth surfaces and uniform growth were noticed in the control (Fig. 2A and 3A). An ultrathin section of the treated *B. dothidea* and *C. gloeosporioides* revealed that the cell wall and cell membrane were damaged, and some cell organelles were abnormal and degraded, such as the mitochondria, nuclei, and endoplasmic reticulum (Fig. 2B and 3B).

Effect of caffeine on bioactivity

Bioactivity – namely alkaline phosphatase (AKP), superoxide dismutase (SOD), and methanedicarboxylic aldehyde (MDA) – was investigated in both fungal isolates, and changes in bioactivity were observed between the caffeine-treated and control samples. The obtained results indicated that except MDA content was significantly higher in high-dose caffeine treated *C. gloeosporioides*, there were no significant differences in AKP, SOD and MDA activity between the high-dose and low-dose caffeine treated samples (Figure S1).

RNA-seq summary and caffeine response

RNA-seq profiling was performed to investigate the gene regulation of *B. dothidea* and *C. gloeosporioides* in control and caffeine-treated environments. The distribution of clean reads between the control and caffeine-treated RNA sequences exhibited relatively little difference (Table S2). In *B. dothidea* and *C. gloeosporioides*, most DEGs were observed in higher caffeine treated fungal culture (3 and 7 mg/mL caffeine, respectively), followed by lower caffeine treated fungal culture (1 and 3 mg/mL caffeine, respectively). The numbers of up-regulated and down-regulated genes are presented in Fig. 4. The similar results and trends were noticed in the gene ontology (GO) of *B. dothidea* and *C. gloeosporioides* (Table 1).

Table 1
Functional annotation of genes with significant transcript changes

GO terms	No. of genes (<i>B. dothidea</i>)			No. of genes (<i>C. gloeosporioides</i>)		
	1 vs 3 mg/mL	0 vs 1 mg/mL	0 vs 3 mg/mL	3 vs 7 mg/mL	0 vs 3 mg/mL	0 vs 7 mg/mL
Biological regulation	2	-	7	1	10	32
Cellular component organization or biogenesis	1	2	8	1	5	25
Cellular process	25	22	63	22	70	198
Detoxification	1	-	1	-	-	2
Developmental process	1	-	-	-	-	2
Growth	-	-	-	-	-	1
Localization	13	7	22	21	22	92
Metabolic process	43	33	104	51	105	304
Multicellular organismal process	1	1	1	1	1	1
Negative regulation of biological process	1	-	1	-	-	1
Positive regulation of biological process	1	-	3	-	2	5
Regulation of biological process	2	-	6	1	6	22
Reproduction	-	-	2	1	-	3
Reproductive process	-	-	2	1	-	3
Response to stimulus	6	3	11	1	5	3
Signaling	-	-	2	-	1	3
Single-organism process	21	19	61	33	59	188
Cell	16	8	43	8	1	122
Cell part	16	8	43	8	1	122
Extracellular region	-	1	1	1	1	2
Macromolecular complex	1	2	15	1	1	21
Membrane	13	8	31	22	28	108

GO terms	No. of genes (<i>B. dothidea</i>)			No. of genes (<i>C. gloeosporioides</i>)		
	1 vs 3 mg/mL	0 vs 1 mg/mL	0 vs 3 mg/mL	3 vs 7 mg/mL	0 vs 3 mg/mL	0 vs 7 mg/mL
Membrane part	12	6	24	20	26	93
Membrane-enclosed lumen	1	-	4	-	2	5
Organelle	7	2	19	1	18	71
Organelle part	4	-	11	1	6	28
Supramolecular fiber	-	-	-	-	-	2
Virion	-	-	-	-	1	1
Virion part	-	-	-	-	1	1
Antioxidant activity	1	-	2	2	3	7
Binding	24	26	72	33	67	219
Catalytic activity	50	50	117	72	123	344
Electron carrier activity	-	-	-	-	-	1
Molecular function regulator	-	-	-	-	2	4
Nucleic acid binding transcription factor activity	1	-	3	1	5	15
Nutrient reservoir activity	1	1	3	-	1	1
Signal transducer activity	-	-	-	-	-	1
Structural molecule activity	2	1	4	-	-	4
Transporter activity	7	2	15	10	10	39

The KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment pathways differed between the caffeine treatments and control. The number of pathways gradually decreased as the caffeine concentration decreased and number of genes was higher in increased caffeine concentration (Figure S2). The prominent biological functions were cell cycle, oxidative phosphorylation, glycolysis/glycogenesis, fatty acid metabolism and degradation, fatty acid biosynthesis, sphingolipid metabolism, ribosome biogenesis in eukaryotes, DNA replication, protein processing in the endoplasmic reticulum, and aminoacyl-tRNA biosynthesis. All genes were either accelerated or repressed in caffeine-

treated *B. dothidea* and *C. gloeosporioides*. Significantly enriched critical KEGG pathways of both fungi were presented in Table S3 and S4.

A Venn diagram illustrated the shared and unique genes among the different caffeine-treated samples (Fig. 4). There were 13 shared genes in the *B. dothidea* samples and 25 in the *C. gloeosporioides* samples. Heatmaps of the relative expression levels of the genes involved in the caffeine-treated samples were exhibited in Fig. 5. Among them, 10 genes were selected for qRT-PCR analysis, and the relative expression levels and their fragments per kilobase of transcript per million mapped reads (FPKM) values from the RNA transcriptome data were also combined to illustrate the expression tendency (Fig. 5). In addition, 5 genes (both accelerated and repressed) were also chosen for confirmation through qRT-PCR, including genes related to ribosomes, protein processing in the endoplasmic reticulum, fatty acid synthesis, sphingolipid metabolism, and melanin synthetic pathway which were detected during the transcriptome analysis. The relative transcript abundance patterns for the control and caffeine treatments were compared using transcriptome data. The gene expression analysis results were consistent with the RNA-seq data. However, slight quantitative differences were noted in gene expression level (Figure S3).

Important genes in primary metabolic pathways were suppressed by caffeine

We observed that the number of key genes significantly downregulated in various primary metabolic pathways. Among them, ribosome biogenesis in eukaryotes, mRNA surveillance, RNA degradation and transport, ribosomes, and aminoacyl-tRNA biosynthesis were all involved in amino acid and protein synthesis mechanisms in both fungal species. Most of the genes in these pathways, such as integral membrane protein (*IMP-jgi293974*), heat shock protein (*HP-jgi285255*), phospholipase D (*PLD-jgi295869*), G protein subunit alpha transducin family (*GNAT-18734207*, *18745356*), integral membrane protein (*IMP-18734425*), putative stress response protein (*csbB-18743821*), carbohydrate kinase family (*fggy-18735662*), basic leucine zipper (*bZIP-18742633*) and translation initiation factor (*IF-18737650*) were all repressed. Sphingolipid is the important components for fungal cell membrane formation [21]. Arylsulfatase (*ARS-jgi294066*) and sulfatase (*ST-jgi292050*) are involved in galactosylceramide formation in sphingolipid metabolism. Disturbance of these genes indicated that caffeine probably affected *B. dothidea* cell membrane. Fatty acid biosynthesis pathway has been a common target for antimicrobial agents in recent scenario [22]. Fatty acid biosynthesis mechanism was highly disturbed in the caffeine-treated fungi, 67% genes were downregulated in *C. gloeosporioides*. Though, there were no significant changes in *B. dothidea* fatty acid biosynthesis.

In summary, RNA-seq results revealed that caffeine treatment severely affected various physiological and metabolic processes of *B. dothidea* and *C. gloeosporioides*. And *C. gloeosporioides* can produce various secondary metabolites supported to fungal pathogenicity mechanism. Among them, melanin is one of the most important secondary metabolites and plays a major role in the fungus pathogenicity by

influencing appressorium development. Melanin biosynthesis pathway in *Colletotrichum* and other fungi starts from malonyl-CoA/Acetyl-CoA, intermediates are as follow: 1,3,6,8-tetrahydroxynaphthalene, scytalone, 1,3,8-trihydroxynaphthalene, vermeline and 1,8-dihydroxynaphthalene [23, 24]. The short-chain dehydrogenase reductases (*SDR*) family, specifically 1,3,6,8-tetrahydroxynaphthalene reductase (*T4HNR*-18739341), was repressed in the caffeine-treated *C. gloeosporioides* and the result suggested the disturbance of scytalone formation in melanin biosynthesis pathway (Fig. 6). The downregulation of *T4HNR* affected melanin synthesis and led to interruptions of growth development and *C. gloeosporioides* pathogenicity. Based on the caffeine tolerance and importance of anthracnose pathogen (*C. gloeosporioides*) in tea plant, we further extensively investigated the physical characterization of melanin pigment and its inevitable role in fungal growth development and pathogenicity.

Physical characterization of purified melanin pigment

The black colour pigment was noticed in control fungal culture, while it was absent in caffeine-treated fungal culture (Fig. 7A). And the purified black colour melanin powder is shown in Fig. 7B. Figure 7C shows the SEM image of the purified melanin. The appearance suggested that the extracted melanin was round edged and amorphous crystal shapes. Then, the melanin pigment was characterized by UV-visible, FTIR and NMR spectrum. The highest peak absorption was observed at 221 nm (Fig. 7D), which was close to the maximum absorption wavelength of standard melanin. Melanin in *Lachnum* YM226 (LM) and *Rhizoctonia solani* has maximum absorption peaks at 223 and 217 nm, respectively [25, 26]. The spectrum analysis of extracted melanin compared with commercial melanin was shown in Fig. 7E. FTIR showed broad absorption band at 3424 cm^{-1} , which indicated the presence of hydroxyl or amine groups. The broadband ranged from 3420 to 3400 cm^{-1} , which denoted that the presence of phenolics, carboxylic and aromatic amino functions in pyrrolic and indolic systems [27]. Another absorption band was noticed at 2924 cm^{-1} contributed by aliphatic C – H groups. Stretching vibration of aliphatic C – H groups bands at 2922 cm^{-1} and 2852 cm^{-1} were observed in both synthetic and *R. solani* melanin [26]. We observed band at 1636 cm^{-1} , which denoted that the presence of the aromatic ring C = C and C = O in the sample. Olaizola [28] considered that the bands close to 1600 cm^{-1} were attributed to stretching vibrations of C = C bonds of aromatic structures and this band was considered importantly for the identification of melanin. The important features of extracted melanin were almost on par with the procured standard melanin. The ^1H NMR spectrum of *C. gloeosporioides* melanin pigment was presented in Figure S4. The signals at 6.0-8.5 ppm and 0.8–1.5 ppm indicated the presence of aromatic and aliphatic groups, respectively. The signals at 0.8-1.0 ppm and 1.3 ppm can be assigned to methyl and methylene groups of alkyl fragments, respectively. The signals between 7.5 and 6.6 ppm indicated the presence of indole, which is acted as a major precursor of melanin synthesis [29, 30]. Katritzky et al. [31] reported that sequence of four broad signals at 7.60, 7.35, 7.00 and 6.60 ppm in human hair melanin belongs to protons of indole or pyrrole.

Influence of melanin on fungal growth

The experimental results revealed that the mycelial dry weight significantly increased in melanin with caffeine-treated environment. Further, we noticed the dense mycelial structures and number of germinated and non-germinated conidia in 3 mg/mL caffeine with synthetic melanin but it was absent in 7 mg/mL caffeine with synthetic melanin (Fig. 8 and S5). Whereas, there were no conidia development noticed in caffeine treated fungal culture (3 and 7 mg/mL).

Pathogenicity test, histopathological observation and fungal biomass quantification

We performed pathogenicity assay to find symptoms production by the fungus on three different varieties tea leaves. The enlarged necrotic lesions were noticed around 14–16 days interval in all the wounded leaves of susceptible tea cultivar (Longjing 43) but no significant changes were noticed between moderately resistant (Longjing Changye) and resistant (Zhongcha 108) tea cultivars lesion size (Fig. 9a). The histopathological observations indicated that high fungal biomass production, germinated conidia and appressorium formation in susceptible tea cultivar, the fungal mycelium moved freely on infected tea leaves and the number of appressorium was higher. Whereas, the moderately resistant and resistant tea clone leaves, the spreading of fungal biomass, germinated conidia and appressorium development were limited (Fig. 9b). Aside, we performed fungal gDNA quantification in infected tea leaves of three varieties. The obtained results revealed that the amount of the fungal gDNA in susceptible tea leaves was higher than moderately resistant tea leaves, but there was no reasonable amount of gDNA quantified in resistant cultivar tea leaves (Fig. 9c).

Caffeine quantification and gene expression analysis

Caffeine content in chosen tea varieties was analyzed and the concentration marginally increased in the fungal inoculated tea leaves of susceptible and moderately resistant tea varieties compared with control. Though, there was no significant change between the control and resistant variety (Figure S6). The caffeine quantification results are closely associated with relative gene expression of caffeine biosynthesis genes (*TCS1*, *TIDH* and *SAMS*). The gene expression of *SAMS* and *TIDH* was higher in infected leaves of resistant variety, while the expression of *TCS1* was higher in susceptible and moderately resistant varieties. Simultaneously, phenylpropanoid biosynthesis, plant hormone biosynthesis, signaling pathways and plant-pathogen interaction and other stress-responsive genes expression were analyzed to understand resistant response against anthracnose pathogen in tea plants through the molecular mechanism and the data were shown in Figure S7.

Discussion

The divergent antifungal activity of caffeine against *B. dothidea* and *C. gloeosporioides* was analyzed. Zhang et al. [19] noted the strong antifungal activity of caffeine (10 mg/mL) against tea foliar pathogens such as *C. camelliae*, *P. theae* and *P. theicola*. The MIC value of caffeine against *C. fructicola* was 4 mg/mL [15]. The inhibition dosage of caffeine differed between bacteria (62.5 to > 2000 µg/mL) and molds (> 5000 µg/mL) [32]. These results demonstrate that the antifungal activity of caffeine varies among the fungal species. Microscopic observation and bioactivity assay revealed that caffeine strongly disturbed the fungus cell wall, cell membrane and cell organelles. These findings are in agreement with the damage observed in caffeine-treated *C. fructicola* [15]. Several researchers have investigated the action mechanism of caffeine against bacteria and fungi, such as affecting DNA, enzyme, and protein synthesis in bacteria [33], spore germination [34], cAMP signaling pathways [35], sugar synthesis (glucose, fructose and maltose) in mold [36] and cell walls and cell membranes [15]. However, the present study provides the first transcriptomic analysis of the inhibition process of caffeine against the tea plant pathogenic fungi *B. dothidea* and *C. gloeosporioides*.

RNA-seq results revealed the effect of caffeine on *B. dothidea* and *C. gloeosporioides*. Major cell organelles, such as the endoplasmic reticulum, ribosomes, mitochondrion and nucleus, were highly responsive to caffeine, with the results being significantly associated with the physiological observations of *B. dothidea* and *C. gloeosporioides*. The downregulation of genes in ribosome biogenesis in eukaryotes, mRNA surveillance, RNA degradation and transport, ribosome and aminoacyl-tRNA biosynthesis indicated that caffeine disturbed the protein formation of fungal cells. The repression of *IMP* may affect many cellular functions, such as signaling receptors, intracellular trafficking mediation, organelle biogenesis, and molecular transport across cellular membranes [37]. Caffeine treatment has been reported to induce stress response genes and repress ribosomal and other growth-related genes in budding yeast (*S. cerevisiae*) [38]. Caffeine toxicity also targeted many other crucial pathways such as the regulation of mitophagy, MAPK signaling pathway, endocytosis, nucleotide, base excision repair, and purine and pyrimidine metabolism.

Plant pathogenic fungi require melanin for growth development, pathogenicity and surviving in unfavorable climatic conditions. And the melanin biosynthesis is an ideal target for development of targeted specific inhibitor to control the fungal pathogens [39]. Schumacher [40] and Spraker et al. [41] reported that the production of pigments in filamentous fungi was not directly involved in their growth and development, but that it might contribute to survival in unfavorable environmental stress conditions such as UV radiation, heat, and oxidation, bacterial invasion and pathogenesis related mechanisms. Based on this result, we hypothesize that melanin pigment might be the reason for the caffeine tolerance of the fungus. Our findings indicated that down-regulation of the aforementioned gene disturbed the melanin pigment production, biological functions and pathogenicity of *C. gloeosporioides*.

Tarangini and Mishra [42], observed small spheres shape melanin pigment, but our results indicated it round edged and amorphous crystal shape. Melanin pigment was structurally diverse due to the high molecular and oxidative polymerization. The UV-spectra absorption rates decreased from the UV region to visible region (210 to 700 nm). The result indicated the absence of residual protein, nucleic acid and the

presence of complex conjugated structures in the melanin molecule [25, 43]. FTIR spectrum is one of the important analyses for melanin pigment identification and provides precise information [44, 45]. FTIR spectra indicated that the presence of aromatic, hydroxylic, alcoholic, phenolic, amine, alkene and aliphatic groups in the melanin pigment [44, 46]. Melanin with caffeine treatment enhanced fungus vegetative growth, conidial formation and their germination. Schumacher [40] reported that DHN melanin contributed in *Botrytis cinerea* reproductive structure longevity. Besides, melanin playing various roles in pathogenic fungal growth development was reported [24, 47, 48].

Caffeine concentration and *TCS1* expression marginally increased in fungal treated susceptible and moderately resistant tea varieties and the expression of *SAMS* and *TIDH* were higher in fungal inoculated resistant tea variety. Wang et al. [15] observed that number of stress response-related cis-acting elements in *SAMS* and *TCS1* during the fungal infection and speculated that cis-acting elements activated through phytohormones and the respective genes were triggered by the fungus. So, further, we examined the expression of defense related genes involved in phenylpropanoid biosynthesis, plant hormone biosynthesis, signaling pathways and plant-pathogen interaction and other stress responsive. The expression of defense-related genes constantly differed between infected and healthy tea leaves of chosen tea varieties. Plant can produce more than 50,000 secondary metabolites [49], of these, catechins, epigallocatechin-3-gallate and caffeine protect the tea plant from phytopathogenic fungi [15, 50]. The growth inhibition of pathogens by caffeine depends on the dosage, in higher concentration it acts as antifungal agent and at low concentration, it acts as a priming agent [12, 15, 19]. Our gene expressions indicated that the caffeine is inevitable content in tea plant defense mechanism, this may be directly involved in the disease resistance or either via modulation of plant hormones [15] or trigger the salicylic acid in host plant during foreign bodies entry [14].

Conclusions

In present study concluded that the majority of genes involved in many important pathways of the fungal isolates (*B. dothidea* and *C. gloeosporioides*) and the expressions were changed in the caffeine-treated samples. Ribosome biogenesis in eukaryotes, mRNA surveillance, RNA degradation and transport, ribosome, aminoacyl-tRNA synthesis, protein processing endoplasmic reticulum (ER), DNA replication, meiosis and cell cycle, fatty acid biosynthesis, sphingolipid metabolism, OXPHOS and glycolysis/glycogenesis (energy-related pathways) etc., these could be significantly repressed by caffeine exposure. Particularly, higher caffeine concentration strongly inhibits the growth *B. dothidea* hyphae by the downregulation of sphingolipid metabolism gene (*ARS*), it's supporting to fungal vegetative and pathogenicity development. *T4HNR* gene was highly repressed in both caffeine-treated sample, it's indicated that caffeine disturbed conidial germination, appressorium formation and melanin synthesis of *C. gloeosporioides*. The present findings concluded that mode of action of caffeine is differed from one to another fungal isolate due to the target specific. Thus, differentially regulated pathways were provided novel insight of caffeine mode of action to control the plant pathogenic fungi.

Methods

Pathogens isolation and culture conditions

Infected tea leaves were collected from the experimental tea garden of Anhui Agricultural University (Hefei, China). After surface sterilization of leaves [51], fungi were isolated from infected leaves and two of them were identified as *B. dothidea* and *C. gloeosporioides*. The isolated fungal colonies were grown on potato dextrose agar (PDA) and incubated at 28 °C in dark conditions. The fungal cultures were subcultured 3 times in a month for further experimental analysis.

Antifungal bioassay of caffeine

The antifungal activity of caffeine against *B. dothidea* and *C. gloeosporioides* strains was evaluated using the poison food technique in solid media as described by Wang et al. [15] with minor changes. Caffeine (Sigma-Aldrich, the USA) was dissolved in sterile distilled water and then added to sterilized molten PDA at 45–50 °C to obtain final caffeine concentration ranging from 0.5 to 7.0 mg/mL, sterile distilled water served as the control. Caffeine-added PDA culture media and control were poured into 9-cm Petri dishes, and mycelial disks (9 mm diameter) obtained from the edge of 7-day-old fungal cultures were inoculated in the center of each Petri dish. The inoculated plates were incubated at 28 °C, and mycelial growth diameters were measured up to 7 days after inoculation. The inhibition relative to the control was calculated as described previously [52], minimal inhibitory concentration (MIC) and effective concentration (EC₈₀) were both calculated.

Effect of caffeine on the bioactivity of fungal hyphae

The hyphae bioactivity of *B. dothidea* (Control, 1 and 3 mg/mL caffeine) and *C. gloeosporioides* (Control, 3 and 7 mg/mL caffeine) was analyzed as proposed by Shao et al. [53] and Wang et al. [15]. Assay kits (Nanjing Jiancheng Institute of Bioengineering, China) were used to determine the bioactivity of alkaline phosphatase (AKP), superoxide dismutase (SOD) and methane dicarboxylic aldehyde (MDA) according to the manufacturer's instructions.

Fungal RNA extraction and sequencing

The liquid broth cultures of *B. dothidea* and *C. gloeosporioides* were prepared, and growth was initiated by inoculating a 9-mm agar plug taken from the edge of 7-day-old PDA plates into a 250-mL flask containing 50 mL potato dextrose broth (PDB) culture medium. Each isolate grew for 10 days with different caffeine concentrations (based on their rounded value of EC₈₀ and MIC) for *B. dothidea* (1 and 3 mg/mL) and *C. gloeosporioides* (3 and 7 mg/mL). Each isolate was also grown in PDB culture medium without caffeine as a control. Flasks were shaken for 10 days at 200 rpm, 28 °C. The mycelium was

harvested from PDB by using a filter paper and a Buchner funnel after 10 days' incubation and immediately frozen in liquid nitrogen.

Total RNA was extracted from the mycelium by using the standard TRIzol (Sangon Biotech, China) method and further quantified through NanoDrop at 260/230 and 260/280 nm and then qualified by 1.5%-2.0% agarose gel electrophoresis. The extracted total RNA samples were treated with RNase-free DNase I (Biomiga, China) to avoid DNA contamination. A total of 18 cDNA libraries from 6 groups (Three biological replicates/group; $n = 3$) of two fungal species were sequenced using Illumina HiSeq 4000 platform (Beijing Genomics Institute, Shenzhen, China).

Melanin extraction and purification

Extraction and purification of melanin from the mycelia were performed according to Kaverinathan et al. [48] with minor modification. Briefly, the mycelia were ground with liquid nitrogen to attain mycelia powder and then treated with 2 M NaOH (pH 10.5) for 36 h. Thereafter, the mixture was centrifuged at 4000 g for 15 min and the supernatant was acidified with 2 M HCl to pH 2.5, incubated for 2 h at room temperature and centrifuged at 4000 g for 15 min. The obtained precipitate was purified by acid hydrolysis using 6 M HCl at 100 °C for 2 h to remove carbohydrates and proteins. Later, the samples were treated with chloroform to wash away lipids. Purified melanin pigment was lyophilized and stored at -20 °C for further use.

Physical analysis of melanin

The purified melanin powder was dissolved in 0.5 M NaOH solution and then scanned by UV-spectrophotometer (Pultton P200/P200+) under the wavelength ranging from 210 to 700 nm, and 0.5 M NaOH solution was served as blank. FTIR is helpful to find functional groups of an unknown compound. The melanin and KBr (1:100, m/m) were mixed and ground with pestle and mortar to break up the clumped melanin and KBr mixture. The mixture disc was scanned at $4000 - 400 \text{ cm}^{-1}$ in an FTIR spectrophotometer (Thermo Scientific, FTIR-Nicolet is 50). For comparison, synthetic melanin (Sigma-Aldrich, China) was procured and used as a standard. The ^1H NMR spectrum was recorded by Agilent DD2 600 MHz spectrometer in 5-mm NMR tubes at 25 °C. The sample was dissolved in DMSO- d_6 and the operating parameters for the analysis were: frequency 599.796 MHz, field strength 14.09 T (600 MHz), number of scan 64, resolution 0.1-0.001 Hz and acquisition time 1.704 s.

Influence of melanin on fungal growth and development

Caffeine (3 and 7 mg/mL) and synthetic melanin (0.01 mg/mL) were added to the sterilized PDB, while PDB with melanin only was served as control. Then, the *C. gloeosporioides* mycelia were inoculated into

the flasks and kept in a rotatory shaker in 200 rpm at 28 °C. After 10 days, the fungal biomasses were harvested, dried and quantified [54].

Pathogenicity test, histopathological observation and fungal biomass analysis

Pathogenicity test was conducted by Ponmurugan et al. [4] with minor modification, three tea varieties (Longjing 43, Longjing Changye and Zhongcha 108) were procured from local tea garden; identification of the cultivars was done by the State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, Hefei, Anhui Province, China, which were selected based on their pathogenicity tolerance against tea anthracnose [6, 15], and ten two-year-old plants per variety were used. The upper and middle leaves of tea plants were injured on the upper surface by sterile needle (two needle pricks per leaf). And conidial suspension (10^6 conidia mL^{-1}) was spread over the wounds, while sterile water was served as control. After inoculation, each plant was covered by a plastic bag containing moist cotton wool for 10 days at 28 °C to maintain relative high humidity (85–90%). We removed the bags after 10 days and maintained the plants in suitable condition for 5 days. The lesion development was evaluated and the pathogen was re-isolated from the test leaves and confirmed its identity.

For the histopathological observation, the infected leaves were harvested after mature lesion development and stored in ethanol/glacial acetic acid (1:1, v/v) for 3 to 4 days. After that, the incubation leaves were stained with blue cotton in lactophenol as described by Marques [55].

Besides, we performed the fungal biomass quantification in infected tea leaves according to Richa et al. [56] with slight modification. Plant genomic DNA (gDNA) was extracted from healthy and infected tea leaves using Biomiga EZgene™ CP Plant Miniprep Kit (Biomiga, San Diego, CA). The final volume of gDNA was 100 μL and the concentration was assessed by a NANODROP 2000 Spectrophotometer (Thermo Fisher Scientific, USA). We developed standard calibration curves to quantify *C. gloeosporioides* biomass in infected tea leaves of three varieties. Extracted fungal genomic DNA (gDNA) was serially diluted to 0.00025, 0.0025, 0.025, 0.25, 2.5 and 25 ng/mL. The diluted gDNA was mixed with a constant amount of 10 ng gDNA isolated from uninfected tea leaves. Calibration curves were generated for the selected tea varieties by quantitative real time-polymerase chain reaction (qRT-PCR) based on their CT values. For the absolute quantification of fungal DNA in inoculated leaf samples, 20 ng/mL gDNA was used as a template. CT values obtained were then used to calculate the absolute amount of target DNA in a given reaction. The fungal gDNA quantified with three biological replicates and were statistically analyzed. The qRT-PCR protocol for fungal gDNA quantification was as follows: the initial incubation at 95 °C for 30 s, followed by 50 cycles of 95 °C for 5 s and 60 °C for 30 s. The final dissociation curve was obtained between 65 and 95 °C.

Caffeine quantification and RNA extraction in infected half leaf and uninfected half leaf

Two needle pricks per leaf, using a sterile needle and fungus was inoculated to one half of the leaf and another half leaf was treated with sterile water as control. After the infection, we removed the infected area of the infected half leaf and the corresponding pricked area of the uninfected half leaf to get two parts healthy leaves. These two parts leaves were ground to powder in liquid nitrogen respectively and 20 mg freeze-dried powder of each part was transferred to 2 mL tubes and extracted with 1 mL 80% (v/v) methanol. After vortex for 1 min, the samples were sonicated at room temperature for 30 min, and then centrifuged at 12,000 rpm for 10 min. Finally, the supernatants were filtered through 0.22 μm filter membranes to glass vials. Caffeine content was quantified by ultra-performance liquid chromatography (UPLC) system (Waters Corporation, USA) equipped with a reverse-phase C18 column and tunable ultraviolet detector at a flow rate of 0.3 mL min^{-1} , according to the method described by Li et al. [57]. The RNAs were also extracted from those two parts by using Fruit-mate™ for RNA Purification (Takara, Beijing, China) for the gene expression analysis.

Gene expression verification through qRT-PCR

Differentially expressed genes (DEGs) from *B. dothidea* and *C. gloeosporioides* for RNA-seq data validation and tea genes were selected for expression analysis through qRT-PCR with TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Takara, Beijing, China). Total RNA was reverse-transcribed to cDNA in a total volume of 10 μL using PrimeScript™ RT Master Mix (Perfect Real Time) cDNA synthesis kit (Takara, Beijing, China). Primers were designed for selected genes by using the Primer Premier 6 (Premier Biosoft, USA), and listed in additional files (Table S1). Gene expression analysis was performed using a qRT-PCR CFX96 thermocycler (Bio-Rad, USA) with *GAPDH* served as an internal reference. Each well of 96-well optical reaction plate was loaded with 25 μL of the reaction mix which was prepared by the instruction of the reagent. The reactions were performed with an initial incubation at 95°C for 30 s,

followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The final dissociation curve was obtained between 65 and 95 °C. Relative expression values were calculated according to formulae of Livak and Schmittgen [58]. All samples were examined in three biological replicates and three technical replicates.

Microscopic observation

The fungal hyphae morphological changes and melanin structure were analyzed by using S-4800 scanning electron microscopy (SEM) (Hitachi, Japan) and ultra-thin section of fungal hyphae [59] analyzed by using HT-7700 transmission electron microscopy (TEM) (Hitachi, Japan) [44, 60]. For visualization of the fungal development on tea plant leaves, fungal mycelial growth, conidia and appressorium development observations were made on Olympus BX51 microscope and micro-photographed using an Olympus DP25 digital camera.

Statistical analysis

Microsoft Excel and SPSS v.16.0 (SPSS Inc., USA) were employed to conduct statistical analyses. All data were statistically analyzed with appropriate replications and the values were expressed as the means $\pm$ standard deviations (SD). Furthermore, fungus biomass dry weight and bioactivity data were analyzed using one-way ANOVA with a Tukey's correction.

Abbreviations

ANOVA: Analysis of Variance

CT: Cycle Threshold

DMSO-d₆: Dimethyl Sulfoxide-deutero₆

FTIR: Fourier-Transform Infrared Spectroscopy

GO: Gene Ontology

¹H NMR: *Proton Nuclear Magnetic Resonance*

KBr: Potassium Bromide

KEGG: *Kyoto Encyclopedia of Genes and Genomes*

MHz: Megahertz

qRT-PCR: quantitative Real Time-Polymerase Chain Reaction

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding authors on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

K. Thangaraj, C. Deng and L. L. Cheng performed the experiments; K. Thangaraj, C. Deng and W. W. Deng wrote the manuscript; W. W. Deng and Z. Z. Zhang revised the manuscript and guided the entire research process. Finally, all authors read, commented on and approved the manuscript.

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Figures

Figure 1

Figure 1

Antifungal activity analyses of caffeine against *B. dothidea* and *C. gloeosporioides*. (A) Growth of *B. dothidea* and *C. gloeosporioides* on caffeine-amended culture plates. (B) Growth rates of fungal isolates for different caffeine dosages. Error bars indicate SD, n = 4 biological replicates.

Figure 2

Figure 2

(A) SEM images of *B. dothidea* treated with caffeine (3 mg/mL) and control: (a-b) morphology of healthy hyphae; (c-d) hyphae treated with caffeine. (B) TEM images to illustrate effects of caffeine on the subcellular components of *B. dothidea*: (a,c,e) cross and transverse section of healthy hyphae and (b,d,f) cross section of caffeine-treated (3 mg/mL) hyphae. Cell wall (cw), cell membrane (cm), vacuole (v); (1) cell organelle abnormality, (2) cell organelle degradation, (3) cell membrane damage, (4) vacuole abnormality, (5) swollen or budding formation, (6) mitochondrion.

Figure 3

Figure 3

(A) SEM images of *C. gloeosporioides* treated with caffeine (7 mg/mL) and control: (a-b) morphology of healthy hyphae; (c-d) hyphae treated with caffeine. (B) TEM images to illustrate effects of caffeine on the subcellular components of *C. gloeosporioides*: (a,c,e) cross section of healthy hyphae; (b,d,f) cross section of caffeine-treated (7 mg/mL) hyphae; (e) mitochondrion; (f) endoplasmic reticulum. Cell wall (cw), cell membrane (cm), mitochondrion (m), mitochondrion outer membrane (mom), mitochondrion inner membrane (mim), vacuole (v), nucleus (n), vesicle (ve), endoplasmic reticulum (er); (1) swollen hyphae, (2) vacuole abnormality, (3) mitochondrion degradation, (4) mitochondrion cristae, (5) cell membrane damage, (6) cell organelle degradation.

B. dothidea

C. gloeosporioides

Figure 4

Figure 4

DEGs of *B. dothidea* and *C. gloeosporioides*. Venn diagrams show the numbers of shared and unique genes among DEGs in different comparisons.

Figure 5

Figure 5

Validation of DEGs obtained from RNA-seq using qRT-PCR: (A) *B. dothidea* and (B) *C. gloeosporioides*. A heat map of representation of validated unigenes (intensity of color coding (blue to red) indicates the DEGs level).

Figure 6

Figure 6

Simplified diagram of the fungal melanin biosynthesis pathway. Downregulated gene was highlighted (T4HNR) in the pathway. PKS: Polyketide synthase; T4HNR: Tetrahydroxy naphthalene reductase; T3HNR: Trihydroxy naphthalene reductase; SDR: Short-chain dehydrogenase reductase; SCD: Scytalone dehydratase.

Figure 7

Figure 7

Physical characterization of melanin pigment extracted from *C. gloeosporioides*. (A) Melanin synthesis of the fungus in PDB (B) Lyophilized melanin pigment powder (C) SEM image of lyophilized melanin pigment (D-E) UV-visible absorbance spectrum and FTIR spectroscopic analysis of standard melanin and melanin pigment extracted from *C. gloeosporioides*.

Figure 8

Figure 8

Fungal biomass production in melanin with caffeine amended environment (M-Melanin and Caf-Caffeine). Significant differences are labeled with asterisks (** $p < 0.01$ and *** $p < 0.001$); ns means non-significant; error bars indicate SD, $n = 4$ biological replicates. Data were analyzed using one-way ANOVA with a Tukey's correction.

Figure 9

Figure 9

(A) (a) artificially infected three tea varieties (b) front and (c) back surface of the infected leaves (R-Resistant, MR-Moderately Resistant and S-Susceptible). (B) histopathological observations of infected tea leaves. I-appressorium; II-fungal hypha; III-infected region; IV-conidium; V-seta. Scale bars represent 20 μ m. (C) Fungal gDNA quantification in infected tea leaves of three varieties. (a) standard calibration curve (b) Fungal gDNA in infected tea leaves by qPCR targeting *C. gloeosporioides* GAPDH and normalized against tea GAPDH. Error bars indicate SD, n = 3 biological replicates.

Supplementary Files

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