Reproduction response of *Colletotrichum* fungi under the fungicide stress reveals new aspects of chemical control of fungal diseases

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Summary

Systemic fungicides and antifungals are used as frontline treatments for fungal diseases in plants and humans. It is generally accepted that fungicides will bring significant negative side-effects to the environment and result in fungicide resistance in the pathogenic fungi. Although previous research has focused on fungicide application rates and fungicide resistance for a long time, little attention has been paid to fungicide residues after treatment, especially their potential role in fungal growth and sporulation. Here we investigated the effect of fungicides at sublethal concentrations on fungal sporulation. The results showed that two kinds of 14α-demethylase inhibitors (DMIs) fungicides increased the number of isolates of *Colletotrichum* spp. to sporulate on PDA. Both on PDA medium and plant tissue, low concentration of DMI fungicides could promote spore production of *Colletotrichum* spp., whereas pyraclostrobin, a quinone outside inhibitor (QoIs), had no significant effects on sporulation of *Colletotrichum* spp. Transcriptomic analysis suggested that the DMIs fungicide stress signal may be transmitted to the central regulatory pathway through the FluG-mediated signalling pathway, and further confirmed the morphological effect of DMI fungicide on promoting sporulation of *Colletotrichum*. To our knowledge, this is the first study to provide insights into the reproductive response of fungi in response to fungicide stress. Our findings indicate that fungicides have two-way effects on the growth and reproduction of pathogenic fungi and provide a new basis for the scientific and rational use of fungicides.

Introduction

Crop-destroying fungi account for ~20% yield losses worldwide annually, with a further 10% loss postharvest (Fisher et al., 2018). Fungal effects on human health are currently spiralling, and the global mortality rate for fungal diseases now exceeds that for malaria or breast cancer and is comparable to those for tuberculosis and HIV (Thammahong et al., 2017). Chemical control is one of the most important measures to control fungal pathogens that cause diseases in crops and animals. However, with the wide use of fungicides, many problems have emerged, the most important of which are fungal resistance and fungicide residues (Fisher et al., 2018). For plant pathogens, at present, the resistance frequency of powdery mildew, eyespot, *Fusarium* ear blight and Septoria tritici blotch to common fungicides in many areas has led to the decline or failure of chemical control (Lucas et al., 2015). In humans, long periods of prophylactic treatment in at-risk patients can similarly lead to the emergence of antifungal drug resistance (Robbins et al., 2017). In order to improve the control effect, farmers blindly increased the application rates or used a variety of off-label pesticides indiscriminately, which is the main source of pesticide residues and environmental pollution in China. Regarding post-application fungicide effects, previous studies mainly focused on the harmful effects of pesticide residues on fruits and vegetables (Chiu et al., 2015; Chen et al., 2016; Concha-meyer et al., 2019); little attention has been paid to fungicide residues after treatment, especially their potential roles in fungal growth and sporulation of the target fungi.
Anthracnose caused by *Colletotrichum* spp. is one of the most serious plant diseases, causing devastating damage to a wide variety of economic crops, particularly to tropical and subtropical cereals and fruit trees (Dean et al., 2012). Sporulation is one of the most important reproductive strategies in *Colletotrichum* fungi and is responsible for disease epidemics (Kirk et al., 2008). The conidia serve as inoculum in infecting fruits, leaves, stems and other plant parts, causing dark brown lesions. After initial infection, conidia form in large quantities on the infected tissues and spread through rainwater splash and wind, providing sources for secondary infection and field disease epidemics (Tedford et al., 2018; Rigling and Prospero, 2018). Conidia arising from lesions spread the disease during the growing season.

Fungicides, especially sterol C-14 α-demethylase inhibitors (DMIs) and quinone outside inhibitors (QoIs), are widely used in anthracnose disease control. DMI fungicides bind to the haem iron of the cytochrome P450 sterol 14α-demethylase (CYP51) and thus interfere with the biosynthesis of ergosterol, the primary sterol in fungal membranes. The QoI fungicides act at the Quinone ‘outer’ binding site of the cytochrome bc1 complex, inhibit the binding of hydroquinone to Qo site and prevent the transfer of electron from cytochrome b to cytochrome c1 in complex III, thus inhibiting the production of ATP. Adverse environments can promote the sporulation of many fungi (Berman and Hadany, 2012; Huang and Hull, 2017), and fungicides on plant tissues present an adverse environment for pathogens. The reproductive response of fungi under fungicide stress remains unknown, and there is a lack of research on the effect of different concentrations of fungicide residues on fungi, especially on the ability of fungi to sporulate. The aim of this study was to take *Colletotrichum* fungi and DMIs as a model to better understand the role of low-dose fungicide residues on fungal sporulation and to develop strategies for optimal fungicidal control both in terms of dosage and timing. Here, we performed a morphological and transcriptomic analysis of *Colletotrichum* fungi under low-dose fungicide stress. The results provided new insights into chemical control of plant diseases.

### Results

**Morphological response of *Colletotrichum* fungal to fungicide stress**

Conidia production under fungicide stress of 108 strains of five *Colletotrichum* spp. isolated from different regions in China was assessed on potato dextrose agar (PDA) amended with different concentrations of fungicides by monitoring changes in colony colour. The change to pink in colony colour was previously shown due to production of conidia (Weir et al., 2012). During the 5-day incubation on PDA without any fungicide at 28°C in the dark, all the strains showed no visible change in colony colour except two *C. nymphaeae* isolates. The two *C. nymphaeae* isolates produced clear pink colonies visible to naked eye on PDA after 5 days incubation (Table 1).

### Table 1. Number of the *Colletotrichum* isolates with formation of pink-coloured conidia masses on PDA amended with different concentrations of three fungicides.

| Fungicides       | Species (Number of isolates) | C₀ᵃ | C₁ | C₂ | C₃ | C₄ | C₅ | C₆ |
|------------------|------------------------------|-----|----|----|----|----|----|----|
| **Difenoconazole** | *C. siamense* (89)           | 0   | 1  | 4  | 6  | 70 | 71 | 75 |
|                  | *C. aenigma* (4)             | 0   | 1  | 1  | 1  | 2  | 2  | 2  |
|                  | *C. fructicola* (7)          | 0   | 0  | 1  | 1  | 1  | 2  | 2  |
|                  | *C. gloeosporioides* (5)     | 0   | 2  | 2  | 2  | 5  | 5  | 5  |
|                  | *C. nymphaeae* (3)           | 2   | 2  | 3  | 3  | 3  | 3  | 3  |
| Total (108)      |                              | 2   | 6  | 11 | 13 | 81 | 82 | 85 |
| **Prochloraz**   | *C. siamense* (89)           | 0   | 1  | 1  | 1  | 4  | 52 | 73 |
|                  | *C. aenigma* (4)             | 0   | 0  | 0  | 0  | 1  | 1  | 1  |
|                  | *C. fructicola* (7)          | 0   | 0  | 1  | 1  | 1  | 1  | 1  |
|                  | *C. gloeosporioides* (5)     | 0   | 1  | 1  | 1  | 2  | 3  | 4  |
|                  | *C. nymphaeae* (3)           | 2   | 2  | 2  | 2  | 2  | 3  | 3  |
| Total (108)      |                              | 2   | 4  | 5  | 6  | 8  | 60 | 83 |
| **Pyraclostrobin** | *C. siamense* (89)           | 0   | 0  | 0  | 0  | 0  | 0  | ND |
|                  | *C. aenigma* (4)             | 0   | 0  | 0  | 0  | 0  | 0  | ND |
|                  | *C. fructicola* (7)          | 0   | 0  | 0  | 0  | 0  | 0  | 0  |
|                  | *C. gloeosporioides* (5)     | 0   | 0  | 0  | 0  | 0  | 0  | 0  |
|                  | *C. nymphaeae* (3)           | 2   | 2  | 2  | 2  | ND | ND | ND |
| Total (108)      |                              | 2   | 2  | 2  | 2  | 0  | 0  | 0  |

ND, not determined due to negligible vegetative growth.
Isolates were inoculated on PDA plates amended with fungicides and incubated at 28°C for 4 days.

ᵃ C₀ to C₆. Concentrations of difenoconazole at 0, 0.0319, 0.0797, 0.1593, 0.7967, 1.5933, 7.9667 µg ml⁻¹; prochloraz at 0, 0.0016, 0.0081, 0.0162, 0.0808, 0.1620, 0.8080 µg ml⁻¹; and pyraclostrobin at 0, 0.0033, 0.0163, 0.0327, 0.1630, 0.3270 and 3.2700 µg ml⁻¹.
The asexual reproduction of Colletotrichum isolates was promoted by difenoconazole (Fig. 1) and prochloraz. With the increasing concentrations of difenoconazole or prochloraz, more isolates showed change in colony colour, indicating more conidia being produced. On PDA with 7.9667 µg ml⁻¹ difenoconazole or 0.8080 µg ml⁻¹ prochloraz, there were 85 and 83 Colletotrichum strains that produced pink colonies (Fig. 1), respectively, indicating increased conidial production (Table 1). However, pyraclostrobin had no significant effects on sporulation of Colletotrichum on PDA (Table 1).

Conidiation of Colletotrichum spp. on PDA amended with different concentrations of three fungicides

Based on the morphological response in colony colour, 20 isolates in five Colletotrichum species were selected to test their conidiation on PDA under fungicide stress. Results showed that conidiation of the five species was promoted by addition of difenoconazole or prochloraz in PDA. The conidial yields of the tested 20 Colletotrichum strains on PDA with 1.5933 µg ml⁻¹ difenoconazole increased 28 to 524 times compared to the yield on PDA. Similarly, the conidial yields increased 3–1215 times with the addition of prochloraz at 0.1620 µg ml⁻¹ in PDA. The conidial production of the five Colletotrichum species increased with the increase in concentrations of difenoconazole and prochloraz (Table 2). A slight increase of conidia was observed with pyraclostrobin at 0.0033 l gm l⁻¹, which was 1.65 times of that without pyraclostrobin. On the other hand, there were no significant changes in the conidial production with increasing concentrations of pyraclostrobin (Table 2).

Effects of difenoconazole on sporulation on plant

Strawberry leaves were treated with difenoconazole at different concentrations and then inoculated with C. fructicola. The conidial yield of C. fructicola on strawberry leaves increased at the first 12 or 16 days after inoculation (dai) and then decreased (Table 3). At the early stage of inoculation (4 days and 8 days), difenoconazole inhibited the growth of C. fructicola, resulting in lower conidial production than the untreated control. The conidial yields of treatment with 7.9667 µg ml⁻¹
was also significant lower than that of the control. However, at 12 dai and 16 dai, the yields of conidia on leaves treated with difenoconazole were significantly higher than that of the untreated control (Table 3). Overall, the highest conidial yields on the difenoconazole-treated leaves at 0.0319 μg ml⁻¹ and 7.9667 μg ml⁻¹ were 1.29 times and 1.46 times of the highest conidial yield in the untreated control, respectively.

Transcriptomic response of *C. fructicola* to DMIs fungicide stress

After 4 days of incubation at 28°C in the dark, masses of conidia of *C. fructicola* isolate Gwha-1 were produced on PDA amended with difenoconazole, while no conidia were observed in the control (Fig. S1). RNA-Seq was performed to compare the gene expression profiles of control and fungicide-treated *C. fructicola*. To develop an accurate transcriptome, the analysis was performed three times and had excellent reproducibility as confirmed by a Pearson’s correlation coefficient greater than 0.95 (Fig. S2). Of the total reads, over 69.98% of the reads from both samples were uniquely mapped to the reference genome (Liang et al., 2018). Sequences with at least one read matched approximately 73.31% of the 18 496 coding genes in the *C. fructicola* genome (Table S1), indicating that the majority of *C. fructicola* genes were expressed under difenoconazole stress. In the present study, genes with an adjusted P-value < 0.05 found by DESeq and an absolute value of the log2 (ratio) > 1 were considered differentially expressed. Based on this

| Table 2. Conidial production of *Colletotrichum* species under fungicide stress. |
| --- |
| **Fungicides** | **Species (Numbers of isolates)** | **Conidia yield per unit area (×10⁴ conidia cm⁻²)/ Total conidia production (×10⁴ conidia per plate)** |
| **Fungicides** | **Species (Numbers of isolates)** | **C₀⁻¹** | **C₁** | **C₂** | **C₃** | **C₄** | **C₅** | **C₆** |
| **Difenoconazole** | *C. siamense* (5) | 0.9/21.4 | 10.8/164.2 | 23/260.8 | 33.7/271.0 | 33.7/271.0 | 51.8/296.6 | 73.7/333.4 | 170.8/336.6 |
| | *C. aenigma* (4) | 0.1/3.7 | 0/15.4 | 4.2/86.8 | 4.1/51.5 | 15.7/169.7 | 57.6/434.7 | 45.7/242.6 |
| | *C. fructicola* (4) | 0.2/6.4 | 5.0/94.3 | 4.4/61.4 | 4.5/48.4 | 20/124.4 | 24.5/122.7 | 52.6/133.0 |
| | *C. gloeosporioides* (4) | 1/0.2 | 1/1.7 | 1.1/15.2 | 2/8.9/95.3 | 1.1/15.2 | 1/1.7 | 1/1.7 |
| | *C. nymphaeae* (3) | 40/0 | 0.2/11 | 1/15.2 | 2/8.9/95.3 | 1.1/15.2 | 1/1.7 | 1/1.7 |
| **Prochloraz** | *C. siamense* (5) | 1/0.2 | 7.9/194.6 | 18.3/345.1 | 15.8/198.5 | 24.4/196.2 | 44.4/254.2 | 115.2/261.5 |
| | *C. aenigma* (4) | 0.1/2.2 | 0.1/1.7 | 0.2/2.6 | 1.5/14.4 | 120.5/909.5 | 133.7/463.1 | 158.2/210.0 |
| | *C. fructicola* (4) | 0.2/5.8 | 4.4/120.3 | 12.9/214.4 | 4.9/64.7 | 12.2/75.1 | 8.9/64.7 | 12.2/75.1 |
| | *C. gloeosporioides* (4) | 1/0.2 | 13.7/362 | 5.6/118.9 | 12.6/209.4 | 19.6/120.7 | 9.3/26.4 | ND |
| | *C. nymphaeae* (3) | 40/0 | 267.4/2721.8 | 291.8/2346.8 | 342.5/2754.5 | 137.7/476.9 | 135.4/239.3 | ND |
| **Pyraclostrobin** | *C. siamense* (5) | 1/0.2 | 22.7 | 3.0/25.7 | 3.0/17.2 | 2.7/12.2 | 4.0/9.1 | ND |
| | *C. aenigma* (4) | 0.1/3.5 | 0.1/1.7 | 0.0/0.0 | 0/0.0 | 0/0.0 | 0/0.0 | ND |
| | *C. fructicola* (4) | 0/0.0 | 0.0/0.0 | 0/0.0 | 0/0.0 | 0/0.0 | 0/0.0 | ND |
| | *C. gloeosporioides* (4) | 1/0.2 | 5.8/72.9 | 9.7/73.2 | 5.1/23.1 | 1.3/3.7 | 1.2/3.8 | 0/0.0 |
| | *C. nymphaeae* (3) | 40/0 | 65.8/216.6 | 11.1/130.7 | 21.4/174.1 | ND | ND/ND | ND |

ND, not determined due to negligible vegetative growth.

Isolates were inoculated on PDA plates amended with fungicides and incubated in 28°C for 4 days.

| Concentrations of difenoconazole (µg ml⁻¹) | 0 days | 4 days | 8 days | 12 days | 16 days | 20 days |
| --- | --- | --- | --- | --- | --- | --- |
| 0.0000 | 3.30⁵ | 0.0319 | 3.30⁵ | 3.30⁵ | 3.30⁵ | 3.30⁵ |
| 0.0319 | 3.30⁵ | 1.63 | 3.30⁵ | 1.63 | 3.30⁵ | 1.63 |
| 7.9667 | 3.30⁵ | 1.63 | 3.30⁵ | 1.63 | 3.30⁵ | 1.63 |
| LSD | 1.63 | 1.63 | 2.63 | 2.63 | 2.63 | 2.63 |

Strawberry leaves were treated with difenoconazole at 0.000, 0.0319 and 7.9667 μg ml⁻¹, air-dried and then inoculated with conidial of *C. fructicola*, followed by incubation in 28°C. The conidial productions of *C. fructicola* on leaves were monitored every 4 days after inoculation. Treatment means in the same column followed by different letters indicated significant difference (P = 0.05).
genes were significantly upregulated under fungicide stress, while 1517 genes were significantly downregulated. To further confirm the reliability of RNA-seq expression data measured by FPKM fold changes, 22 differentially expressed genes (DEGs) were randomly selected to monitor their expression levels with RT-qPCR. The relative transcript levels determined by RT-qPCR and RNA-seq were positively correlated with a Pearson coefficient $R^2 = 0.7088$ (Fig. S3). These results support the validity of our RNA-seq data and can be used in downstream analysis.

To reveal the potential functions of the 1045 DEGs, the COG, GO and KEGG databases were used to analyze functional classification and pathways. Among the 25 COG categories, except for the cluster for ‘general function prediction only’, the largest group was the cluster for ‘amino acid transport and metabolism’ and ‘carbohydrate transport and metabolism’, followed by the clusters for ‘inorganic ion transport and metabolism’, ‘secondary metabolites biosynthesis, transport and catabolism’, ‘lipid transport and metabolism’ and ‘cell cycle control, cell division, chromosome partitioning’ (Fig. S4).

To further investigate the function of DEGs, we employed function enrichment analysis including GO enrichment and KEGG pathway enrichment for 2725 DEGs. Among them, 1186 DEGs were successfully categorized into GO groups. The most enriched GO group was ‘metabolic process’ in the biological process category, followed by the ‘catalytic activity’ in the molecular function category (Figure S5). For KEGG pathway enrichment, 493 DEGs were mapped to 93 canonical reference pathways (Table S3) and the top 20 enriched pathways were presented in Figure S6. Pathway ‘pentose and glucuronate interconversions’ contained 18 genes, and ‘starch and sucrose metabolism’ with 23 genes included were significantly enriched.

**DEGs involved in the ergosterol biosynthesis pathway**

In fungi, ergosterol is synthesized from acetyl-coenzyme A (acetyl-CoA) through a complex pathway. The ergosterol-biosynthetic steps have been well characterized in *Saccharomyces cerevisiae*, and 21 genes are involved in ergosterol biosynthesis (Espenshade and Hughes, 2007). The amino acid sequences of these genes were downloaded. Local BLAST searches were used to find the homologues of these genes in *C. fructicola*. Among the DEGs under the difenoconazole stress, 21 homologues involved in ergosterol biosynthesis were found in *C. fructicola*. Within the genes involved in ergosterol biosynthesis, the homologues of the *S. cerevisiae* Erg11 protein (the target of DMI fungicides) were found to be upregulated compared to the control. Four predicted genes, which are homologues of the *S. cerevisiae* Erg6, Erg27, Erg3 and Erg5 proteins, were also found to be upregulated compared to the control. The homologues of the *S. cerevisiae* Erg4 protein were found to be repressed compared to the control. The expression levels of the other 15 genes were not change significantly under difenoconazole treatment (Fig. 2).

**DEGs involved asexual sporulation**

The entire processes of the initiation, progression and completion of asexual sporulation are regulated by various positive and negative genetic elements that direct the expression of genes required for conidial formation. A comprehensive model for the regulation of asexual sporulation in *A. nidulans* has been reviewed by Park and Yu (2016), and the amino acid sequences of these genes were downloaded. Local BLAST searches were used to find homologues of these genes and identified 31 homologues in *C. fructicola* related to asexual sporulation. Within these genes, AbaA and WetA are central regulators of conidiation, and the expression of these genes was greatly increased under the stress of difenoconazole. NsdC, NsdD, PpoA, PpoB, OsaA and PpoC play roles in developmental balance. The expression of NsdC, NsdD, PpoA, PpoB and OsaA were not significantly changed in difenoconazole-treated samples. The expression of PpoC significantly increased under difenoconazole stress. The expression of FluG, SfgA, FlbA, FlbC, FlbE and FlbD were involved in the FluG-mediated signalling pathway. The expression levels of FlbA and FlbD significantly increased, while the expression of SfgA significantly decreased under difenoconazole stress. The expression of FluG, FlbA, FlbC and FlbE was not changed. The other 14 genes, which play roles in the light-dependent signalling pathway (*LreA, LreB* and *FphA*), MAP kinase signalling pathway (MpkB, MdkB, SteC and *SteD*), G protein signalling pathway (*FadA* and PkaA), transcription factors (*SlfA, AreA, ZipA, MtfA* and *RlmA*) and Velvet family proteins (*VelIB* and *VosA*), were not significantly altered under difenoconazole treatment (Fig. 3).

**Discussion**

Exposure of cells to suboptimal growth conditions or to any environment that reduces cell viability or fitness can be considered a stress. Fungi use different strategies to overcome environmental stress, such as changes in morphology (Zajc et al., 2013), reinforcement of cell walls, increase growth rate, accumulation of osmolytes such as glycerol and change in genetic
structures (Kralj Kuncic et al., 2010; Duran et al., 2010; Kis-Papo et al., 2014; Hallsworth, 2018). The formation of conidia plays an important role in fungal reproduction, dissemination and resistance to adverse environmental conditions (Cray et al., 2016; Riquelme et al., 2018). A total of 108 Colletotrichum strains isolated from different regions were used in this study for morphological response testing. The effect on sporulation varied among the fungicides. Two DMI fungicides, difenoconazole and prochloraz, could significantly promote the sporulation of Colletotrichum spp., The number of isolates producing conidia increased with increasing concentrations of the DMI fungicides. Pyraclostrobin, which belong to QoIs, had little effect on the sporulation of C. siamense, C. gloeosporioides and C. nymphaeae and had no significant effect on C. aenigma and C. fructicola (Table 1). This result indicated that the promoting effect of fungicides on fungal sporulation was dependent on the types of fungicides. The induction of sporulation by DMIs was not limited to certain specific isolates, but was common to all isolates of the five Colletotrichum spp. tested.

Hormesis is a toxicological concept characterized by high-dose inhibition and low-dose stimulation. The hormetic zone includes a range of subinhibitory doses that are stimulant, with a peak at the maximum stimulation dose, and ends at the no observable adverse level, that typically precedes the inhibitory doses. One of the most common endpoints used in hormesis research is growth, but several others have been studied including cellular functions, longevity, CO₂ production and other metabolic processes (Garzon and Flores, 2013). All three fungicides tested in this study affected mycelial growth under tested concentration. In all treatments, the mycelial growth rate of Colletotrichum isolates were inhibited by fungicides, the higher fungicide concentration, the stronger the inhibition of mycelium growth were observed (Fig. 1). The concentration range of the three fungicides used in this study was higher than that of the no observable adverse level in hormetic phenomenon (Garzon and Flores, 2013). With the increasing of difenoconazole and prochloraz in PDA medium, the mycelial growth was inhibited, but the conidial production was promoted. This is not consistent with the traditional hormesis phenomenon. Maybe we need to update our understanding and interpretation of the hormesis zone. It is clear that the hermetic zone for spore production of Colletotrichum is in higher fungicide concentrations than that for mycelium growth.

Both in PDA medium and in plant tissue, low concentrations of DMI fungicides could promote spore production of Colletotrichum spp. (Table 2). When used on crops, the concentrations of difenoconazole applied to plants are approximately 50 to 100 µg a.i. m⁻³. Such concentrations of fungicide on plants are high enough to inhibit the mycelial growth of Colletotrichum spp.;
however, the effective concentrations of the fungicides decrease over time due to the influence of ultraviolet radiation and rainfall. The half-life of difenoconazole is 4.9-5.8 days in soil and 3.6-5.5 days on wheat straw (Zhang et al., 2015). When the effective fungicide concentration declines on plants after application, it becomes non-effective in inhibiting fungal growth, the DMIs fungicides may actually induce conidiation of Colletotrichum, which may facilitate plant disease epidemics and increase the cost of chemical control in the production season. The results indicate that DMI fungicides can promote spore production of C. fructicola on plant tissues, and further illuminate the risk of DMI fungicide usage in spreading disease in the field.

Ergosterol is an important component of the fungal cell membrane, essential for fungal growth and development, and plays an important role in conidial germination (Liu et al., 2011; Deng et al., 2015). CYP51 catalyses the 14a-sterol removal of 14a-methyl groups to form ergosterol, which is a key enzyme in the ergosterol biosynthesis pathway (Liu et al., 2011). Because DMI compounds all have a nitrogen heterocycle, the nitrogen atoms on the heterocycle can bind to the haem iron active centre of CYP51 by coordination bonds (Siegel, 1981). Among the different expression genes under difenoconazole stress, the homologues of the S. cerevisiae Erg11 protein (CYP51) were found to be induced compared to the control (Fig. 2). Constitutive over-expression of the CYP51 gene has been shown to cause DMI resistance in many plant pathogenic fungi (Wang et al., 2015). Knocking out the CYP51 gene leads to an increase in sensitivity to DMI fungicides and a decrease in conidial production as well (Liu et al., 2011). The FgERG3, FgERG4 and FgERG5 genes in the ergosterol biosynthesis pathway of Fusarium graminearum were also closely related to conidial formation. Knocking out these genes leads to reduced conidial yield and increased sensitivity to DMI fungicides (Liu et al., 2011; Yan et al., 2011). Four predicted genes, which are homologues of the S. cerevisiae Erg3, Erg5, Erg6 and Erg27 genes, were also found to be induced by difenoconazole stress in this study (Fig. 2). The binding of difenoconazole with CYP51 resulted in a decrease in ergosterol production in C. fructicola. As a feedback of ergosterol deficiency,
gene expression in ergosterol synthesis pathway increased.

Sporulation (asexual and sexual) is the most critical stage in fungal reproductive history. Conidia are specialized structures produced in the asexual life cycle of most filamentous fungi, and they are important for fungal dissemination and propagation (Ebbole, 2010). The molecular mechanisms underlying the induction of conidia in fungi have been intensively studied, mainly in model organisms such as A. nidulans and Neurospora crassa. A number of genes have been found to be involved in this process, affecting different steps of the signal transduction pathway (Roncal and Ugalde, 2003; Park and Yu, 2016). The central regulatory pathway, the BrlA pathway, is involved in the regulation of conidiogenesis in A. nidulans and A. fumigatus (Park and Yu, 2012). Almost all conidiation regulatory signals are affected by the central regulatory pathways. Our transcriptome analysis also identified some homologues of regulatory factors, such as the FluG-mediated signalling pathway, that participate in regulating conidiogenesis. Therefore, the genetic control of conidiation in C. fructicola may shares conserved elements known from other model fungi. The expression of AbaA and WetA homologues in the central regulatory pathways significantly increased under difenoconazole stress (Fig. 3). The results of gene expression further confirmed the roles of DMI fungicides on promoting sporulation in Colletotrichum spp.

However, it is not clear how the difenoconazole stress signal is transmitted to the central regulatory pathway. Moreover, some upstream development activators (e.g. FluG, FlbC, FlbD and FlbE) and downstream development regulators (e.g. velvet complex consisting of VosA, VelB, VelC and LaeA) also act in regulating conidiation (Park and Yu, 2012; Alkhayyat et al., 2015). Transcriptome analysis and similarity searches performed thorough BLAST also identified some homologues of these regulatory factors. In the FluG-mediated signalling pathway, the expression of FlbD and FlbA was upregulated, while Sfg4A was downregulated under difenoconazole stress (Fig. 3). We speculate that the stress signal of fungicides may be transmitted to the central regulatory pathway through the FluG-mediated signalling pathway, thus affecting Colletotrichum fungal sporulation. Functional characterization of these genes may contribute to uncovering the mechanism by which DMI fungicide stress promotes sporulation in Colletotrichum spp.

**Experimental procedures**

Fungal isolates and growth conditions

A total of 108 isolates of Colletotrichum spp., including 89 of C. siamense, 7 of C. fructicola, 5 of C. gloeosporioides, 4 of C. einigma and 3 of C. nymphaeae, were used in this study. These isolates were obtained from diseased strawberry and yam plants from 2012 to 2016 in central China (Han et al., 2016). All isolates were stored at 10°C for approximately 7-14 days before use.

Morphological response of Colletotrichum spp. to fungicide stress

Two DMI fungicides, difenoconazole (95.0%, Jiangsu Fengdeng Crop Protection Co., Ltd, Jiangsu, China) and prochloraz (97.0%, Jiangsu Huifeng Agricultural Chemical Co., Ltd, Jiangsu, China), and one QoI fungicide pyraclostrobin (98.3%, Wuhan Dinghui Chemical Co., Ltd., Hubei, China) were dissolved in acetone at 1 mg ml⁻¹ as stock solutions. The fungicide stock solutions were added individually to potato dextrose agar (PDA) after the medium cooled to approximately 50°C. The final concentrations of difenoconazole in PDA were 0.0319, 0.0797, 0.1593, 0.7967, 1.5933 and 7.967 µg ml⁻¹; prochloraz in PDA were 0.0016, 0.0081, 0.0162, 0.0808, 0.1620 and 0.8080 µg ml⁻¹, and pyraclostrobin in PDA were 0.0033, 0.0163, 0.0327, 0.1630, 0.3270 and 3.2700 µg ml⁻¹. PDA plates without fungicide were used as controls. Three replicates of each fungicide concentration were used for each isolate. Mycelial agar discs of the 108 Colletotrichum isolates were removed with a 5-mm cork borer from the leading edge of an actively growing 5-day-old colony and placed mycelium-side-down on the centre of petri dishes (9 cm in diameter) containing fungicide-amended or non-amended PDA. The colony morphology and sporulation status were checked by naked eye after incubation at 28°C for 5 days in the dark.

Sporulation of Colletotrichum spp. on PDA under fungicide stress

A total of 20 isolates, including 5 of C. siamense, 4 of C. gloeosporioides, 4 of C. fructicola, 4 of C. einigma and 3 of C. nymphaeae, were selected to evaluate the effect of fungicide stress on their asexual reproduction. Mycelial inoculation and incubation were performed using the same methods described above. After 5 days, colony diameters were measured and used to calculate the total conidia production in a plate. Nine (colony diameter more than 12 mm) or six (colony diameter less than 12 mm) uniformly distributed mycelial discs of each petri dish were harvested from the colonies. Then, the mycelial discs from each dish were transferred to a tube with 1 ml lactic acid (0.5%). The conidia were washed from the mycelial discs using a variable speed vortex mixer (Vortex Genie 2; Scientific Industries, Bohemia, NY, USA). The conidial concentration in the resulting conidial suspension was measured with a hemocytometer and
was used to calculate the conidia yield per unit area. Three replicates of each fungicide concentration from three separate plates were used for each isolate, and the entire experiments were performed twice.

Effects of difenoconazole on sporulation during plant infection

The effects of difenoconazole on sporulation during plant infection were evaluated by in vitro leaf inoculation. *C. fructicola* isolate Gwha-1 and leaves of strawberry plants (‘benihopp’, susceptible to anthracnose) were used for plant inoculation experiments. The conidia suspension was prepared as described before (Han et al., 2016). Healthy strawberry plants devoid of any exposure to fungicides were used for the plant inoculation experiments. The third to fifth leaves from the inside to the outside of the plants were detached and sterilized with 75% alcohol and then rinsed twice with sterile water. Leaves were divided into three groups and soaked in water with difenoconazole concentration of 0 µg ml⁻¹, 0.0319 µg ml⁻¹ and 7.9667 µg ml⁻¹, respectively, for 5 min. Leaves were dried and then inoculated by spraying conidial suspensions (1 × 10⁶ conidia ml⁻¹) of Gwha-1 until runoff. The leaves were cut to leaf discs with a 15-mm cork borer, and then put in petri dish with a piece of wet filter paper at the bottom. Ten pieces of leaf discs were put in one petri dish. The petri dishes were incubated at 28°C for 20 days under white fluorescent light sources with the regime of 12-h light and 12-h dark. Three petri dishes of each treatment were taken out every 4 days to determine the conidial yield on leaf discs. Leaf discs were put into a centrifuge tube with 20ml of sterile water and then vibrated for 5min with a variable speed vortex mixer (vortex Genie 2; Scientific industries) to take conidia off from the leaf disc. The concentration of conidia was measured by blood cell counting plate to calculate the conidia yield on the leaf disc. Leaf discs without difenoconazole were used as the controls. Three replicates of each difenoconazole concentration were used for each sampling time, and each replicate contains 10 leaf discs. The whole experiment was repeated twice.

RNA extraction, RNA-Seq analysis and RT-qPCR analyses

Isolate Gwha-1 of *C. fructicola* was selected for transcriptomic analysis. To obtain the fungicide stress-induced mycelia, 200 µl of the conidial suspension was inoculated onto an autoclaved cellophane membrane placed on a PDA plate amended with difenoconazole at a concentration of 0.1593 µg ml⁻¹. Inoculation of the conidia on autoclaved cellophane membrane placed on PDA without any fungicides was used as control. The cultures were incubated at 28°C for 3 days in darkness. RNA extraction and RNA-Seq analysis were conducted as described by Mao et al., (2013). Reads from each library were separated based on their bar codes and aligned to *C. fructicola* strain 1104-7 genomes (GenBank accession no. MVNS00000000) (Liang et al., 2018). DEGs were identified using three biological replicates for each treatment. RT-qPCR analyses were conducted as described previously (Livak and Schmittgen, 2001). 22 DEGs were selected to monitor their expression levels with RT-qPCR, primers (Table S4) used for RT-qPCR were designed based on sequences of *C. fructicola* genes. Transcript levels for each gene were calculated by the Advanced Relative Quantification method using GAPDH, Tub2 and Actin as reference genes. Relative expression values were calculated according to the 2⁻ΔΔCt method (Livak and Schmittgen, 2001). Transctipt levels were quantified in three biological replicates, each based on three technical replicates.

Data analysis and sequencing data access

Data from different difenoconazole treatments at the same sampling time were subjected to analysis of variance using the Statistical Analysis Systems (SAS, version 8.0; SAS Institute, Cary, NC) in order to determine the significance of treatment differences. Means for different treatments were separated using the least significance difference (LSD) test at $P = 0.05$ level. The sequencing data from this study have been submitted to the NCBI Short Read Archive under accession number SUB5426027. Raw reads obtained from *C. fructicola* isolate Gwha-1 with and without difenoconazole-induced mycelium were submitted into GenBank under the accession numbers SRR9118233 to SRR9118238.

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Conflict of interests
None declared.

Author contributions
Yong-chao Han and Li Ren designed the research. Yong-chao Han, Xiang-guo Zeng, Qing-hua Zhang, Feng-ying Chen and Li Qin performed the experiments. Yong-chao Han, Li Ren, Cong Guo and Weidong Chen analysed the data and wrote the manuscript.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Comparison of morphological differences of *Colletotrichum fructicola* isolate Gwha-1 after difenoconazole addition. A conidial suspension was inoculated onto an autoclaved cellophane membrane placed on a PDA without fungicide (CK, left) amended with difenoconazole at 0.1593 µg ml⁻¹ (right), after incubation at 28°C for 3 days in the dark.

**Fig. S2.** Expression correlation heat map between two samples of *Colletotrichum fructicola* isolate Gwha-1 after exposure to difenoconazole.

**Fig. S3.** Validation of RNA-seq data by real-time quantitative RT-PCR. Twenty-two differentially expressed genes (DEGs) were selected and real-time quantitative RT-PCR was conducted to verify the quality of expression changes in RNA-seq (log2 fold).

**Fig. S4.** KOG function classification of consensus sequence.

**Fig. S5.** Differential expression genes classify information on the second level structure of GO.

**Fig. S6.** Statistics of pathway enrichment.

**Table S1.** Summary of RNA-Seq data and mapping.

**Table S2.** Genes different expressed between control and fungicide-treated samples.

**Table S3.** KEGG pathway enrichment of DEGs.

**Table S4.** Primer used in this study.