Pharmacological Characteristics and Efficacy of Fluazinam Against *Corynespora cassiicola*, Causing Cucumber Target Spot in Greenhouses

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Abstract

Cucumber target spot, caused by *Corynespora cassiicola*, is a devastating fungal disease in greenhouses in China. Lack of resistant cultivars and unscientific use of fungicides aggravated the difficulty to manage this disease. In recent years, resistance of *C. cassiicola* to benzimidazoles, quinone outside inhibitors, and succinate dehydrogenase inhibitors has occurred in China. Here, we tested the fluazinam sensitivity distribution of 79 *C. cassiicola* isolates from different provinces in China based on mycelial growth inhibition. The EC₅₀ values of fluazinam ranged from 0.1002 to 0.3129 µg/ml with a mean of 0.2136 ± 0.0495 µg/ml, and the sensitivity frequency was normally distributed ($P = 0.2083$, Shapiro–Wilk test). Meanwhile, the EC₅₀ values for spore germination inhibition ranged from 0.0992 to 0.2278 µg/ml with a mean of 0.1499 ± 0.0504 µg/ml. This indicated that fluazinam exhibited an excellent in vitro fungicidal activity on both mycelial growth and spore germination. In addition, fluazinam

also exhibited a good in planta control efficacy on detached cucumber leaves in the protective and curative assays. Moreover, the biological and physiological characteristics of *C. cassiicola* as affected by fluazinam were determined. Fluazinam not only significantly inhibited respiration and adenosine triphosphate production but also caused the increase of cell membrane permeability and the dysfunctions of cellular homeostasis. Interestingly, we found that fluazinam especially damaged vacuole structures, causing the redistribution of vacuole substances. Taken together, our findings provide not only essential references for resistance management of *C. cassiicola* but also interesting insights for further revealing the action mode of fluazinam against plant pathogens.

Keywords: cucumber target spot, *Corynespora cassiicola*, fluazinam, action mode, sensitivity distribution, resistance

Corynespora cassiicola is an important plant pathogenic fungus of the phylum of ascomycetes that causes fungal disease of various plants in the world. In the last decades, *C. cassiicola* had been reported infectious to various plants, including rubber plantation, soybean, pawpaw, and cucumber (Koenning et al. 2006; Oluma and Amuta 1999; Raffel et al. 1999; Silva et al. 1998). In China, cucumber target spot caused by *C. cassiicola* has been reported in some provinces and has been becoming a serious threat to the production of cucumber in commercial greenhouses (Duan et al. 2019). Owing to lack of resistant varieties, programmed application of fungicides has been a primary tool for controlling cucumber target spot caused by *C. cassiicola*. Benzimidazoles, quinone outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs) have been introduced sequentially to the market and provide growers with excellent control of cucumber target spot. However, fungicide resistance occurred because of long-term use and the increased dosage (Adkison et al. 2012; Miyamoto et al. 2009). Recently, multiresistance of *C. cassiicola* has been reported in cucumber greenhouses (Duan et al. 2019; Zhu et al. 2019). More seriously, superresistance of *C. cassiicola* to benzimidazole fungicides has also been reported in China (Duan et al. 2019). Therefore, the occurrence of multiresistance

and superresistance resulted in loss of efficacy of many currently extensively used fungicides in controlling cucumber target spot. Thus, it is urgent to develop or screen a candidate to control cucumber target spot caused by *C. cassiicola*.

Fluazinam is a wide-spectrum fungicide and belongs to the aminopyridines compounds according to the Fungicide Resistance Action Committee. Fluazinam exhibits a powerful fungicidal potency and has a high efficacy in inhibiting growth of many important pathogenic fungi (Cohen et al. 1999; Kalamarakis et al. 2000; Liang et al. 2015; Smith et al. 1991). Fluazinam and its derivatives have been reported to exhibit a strong uncoupling activity and are probably metabolically transferred in mitochondria by a glutathione conjugation mechanism (Brandt et al. 1992). Fluazinam inhibited glucose oxidation in a dose-dependent manner and also suppressed the energy production process through direct inhibition of the adenosine triphosphate (ATP) synthetase in fungal cells (Vitoratos 2014). Moreover, fluazinam decreased basal and ATP-linked respiration significantly in zebrafish embryos, resulting in mitochondrial dysfunction and oxidative damage, and it also affected the dopaminergic system of zebrafish (Wang et al. 2018).

In recent years, fluazinam has been registered to control many plant pathogens in China, such as *Botrytis cinerea*, *Phytophthora infestans*, *Phytophthora capsici*, *Plasmidiophora brassicae*, and *Colletotrichum capsici* (<http://www.chinapesticide.org.cn>). To date, in vitro and in planta antifungal activity of fluazinam against *C. cassiicola* has not been reported. In addition, fluazinam resistance of plant pathogens in the laboratory and in the field has rarely been reported. Therefore, our aims of this study are (i) to determine fluazinam sensitivity distribution of *C. cassiicola* populations from different locations, (ii) to investigate biological and physiological characteristics of *C. cassiicola* as affected by fluazinam, and (iii) to evaluate the application potential of fluazinam in controlling *C. cassiicola* in the field. This study not only increases our knowledge about action mode of fluazinam in plant pathogens but also provides a new active ingredient for the management of aggravating cucumber target spot caused by *C. cassiicola*.

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Materials and Methods

Isolation of *C. cassiicola*. *C. cassiicola* isolates were isolated from the diseased cucumber leaves collected from greenhouses in Henan and Jiangsu Provinces in 2017 and 2018 (Supplementary Table S1). Leaves of typical target spot disease were cut into tissue blocks of a diameter of 5 mm from the border of the scabs, sterilized in 3% NaClO for 3 min, and washed with sterile deionized water (SDW) three times. After naturally drying, the tissues were transferred onto potato dextrose agar (PDA) plates and incubated for 3 to 4 days at 25°C in the dark. The suspected *C. cassiicola* isolates were transferred on new PDA plates for a sporulation assay. All the isolates were purified by a single-spore method and stored on PDA slants at 4°C until use.

Fungicide and media. Technical-grade fluazinam (97%, Jiangsu Lanfeng Bio-Chemical Co., China) was dissolved in methanol to 1×10^4 µg/ml and stored at 4°C as stock solution. For the sensitivity test, stock solution was diluted to a series of concentrations and added into PDA plates.

PDA medium contained 200 g of potato, 20 g of glucose, and 20 g of agar powder in 1 liter of deionized water. Water agar (WA) medium contained 15 g of agar powder diluted in 1 liter of deionized water. Yeast extract peptone dextrose (YEPD) medium contained 3 g of yeast extract, 10 g of peptone, and 20 g of glucose in 1 liter of deionized water. All the media were autoclaved.

Fluazinam sensitivity tests based on the mycelial growth inhibition method. Seventy-nine isolates, isolated from different geographical regions in 2017 and 2018 (Supplementary Table S1), were used to test the sensitivity to fluazinam based on the mycelial growth inhibition method. Mycelial plugs (5 mm diameter) were cut from 6-day-old colonies and transferred onto PDA plates amended with 0, 0.125, 0.25, 0.5, 1, and 2 µg/ml of fluazinam. After incubation for 7 days at 25°C, the diameter of colonies was measured, and the inhibition rate of mycelial growth was calculated to evaluate the concentration inhibiting 50% of growth (EC_{50}) of each isolate as previously described (Liu et al. 2013). There were three replicates for each concentration of each isolate.

Fluazinam sensitivity tests based on the spore germination inhibition method. Five randomly selected *C. cassiicola* isolates were used to test fluazinam sensitivity based on the spore germination inhibition method according to a previous study (Li et al. 2018). The aerial mycelia of 7-day-old colonies on PDA plates were scraped with a sterile spatula, and the PDA plates were incubated for 4 days at 25°C under blacklight lamps to promote spore production. The spores produced on PDA plates were washed with 5 ml of SDW, filtrated with four-layer lens papers, and centrifuged for 5 min at 4,000 rpm. The spores were resuspended and adjusted to 1×10^5 spores/ml, and 50 µl of spore suspensions was spread on WA medium amended with 0, 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 µg/ml of fluazinam. The germination rate of spores was observed with an optical microscope (Olympus, Japan) after incubation for 8 h. Three replicates were performed for each concentration of each isolate.

Cell membrane permeability as affected by fluazinam. Mycelial relative conductivity was determined as previously described with minor modification (Duan et al. 2013, 2018). Ten mycelial plugs (5 mm diameter) cut from 6-day-old colonies were transferred into 100 ml of YEPD. After being incubated for 4 days in a shaker with 180 rpm at 25°C, mycelia were collected with single-layer Miracloth (Millipore, Germany). Mycelia (0.3 g) were suspended in 20 ml of SDW, and the conductivity of mycelia was measured at intervals of 20 min with the CON510 conductivity meter (Eutech/Oakton, Singapore). After 200 min, the mycelia were boiled for 5 min, and the final conductivity was measured when it returned to room temperature. The relative conductivity was calculated with the following equation: relative conductivity (%) = real-time conductivity/final conductivity $\times$ 100. The same volume of methanol was added for the controls. Two randomly selected isolates were tested. Each treatment had three replicates, and the experiments were performed three times.

Respiration as affected by fluazinam. The isolates CN01 and JSHA1701 were used to determine oxygen consumption rate

according the previous study (Duan et al. 2012). Five mycelial plugs (5 mm diameter) were cut from 6-day-old colonies and transferred into 100 ml of YEPD. After being incubated for 4 days in a shaker with 180 rpm at 25°C in the dark, mycelia were collected with single-layer Miracloth (Millipore). Mycelia (fresh weight of 0.02 g) were resuspended in 2 ml of oxygen-saturated deionized water amended with the EC_{50} and $10 \times EC_{50}$ values of fluazinam. After incubation for 1, 2, 4, and 8 h, the oxygen consumption rate was measured with the Oxygraph+ oxygen electrode system (Hansatech Instruments, U.K.). The same volumes of methanol were added to oxygen-saturated deionized water for the controls. The final oxygen consumption rate was standardized by dry weight of mycelia, and the experiments were repeated twice.

Mycelial morphology as affected by fluazinam. Mycelial morphology affected by fluazinam was tested with *C. cassiicola* isolate CN01. Mycelial plugs (5 mm diameter) were cut from 6-day-old colonies and transferred on PDA plates amended with EC_{50} and $10 \times EC_{50}$ of fluazinam. After incubation for 2 days at 25°C, the morphology of the marginal hyphae was surveyed and imaged with an inverted microscope (Olympus). There were three replicates for each concentration, and the experiments were repeated twice.

To analyze how fluazinam affected mycelial cell morphology, mycelial plugs (5 mm diameter) were cut from 6-day-old colonies and transferred into 100 ml of YEPD. After incubation for 2 days in a shaker with 180 rpm at 25°C, fluazinam was added to the cultures. After incubation for an additional 12 h, mycelia were collected with single-layer Miracloth (Millipore), washed with SDW three times, and stained with calcofluor white following the manufacturer's instruction (Sigma Aldrich, U.S.A.). The hyphae were observed with an SP8 confocal microscope (Leica, Germany).

Vacuoles as affected by fluazinam. To further understand the effect of fluazinam on cytoplasm, Cell Tracker blue 7-amino-4-chloromethylcoumarin (CMAC) dye (Keygen Biotech, China) (which reacts with thiols, utilizing glutathione-S-transferase mediated reaction, producing fluorescent conjugates in vacuole lumen) was used to detect the cell membrane-impermeant reaction products. Mycelial plugs (5 mm diameter) were cut from 6-day-old colonies of CN01 and transferred into 100 ml of YEPD. After incubation for 2 days in a shaker with 180 rpm at 25°C, fluazinam was added, and incubation continued for 12 h. Mycelia were stained with CMAC for 30 min at 37°C and washed with SDW to remove the stain. Then, the stained mycelia were observed with an SP8 confocal microscope (Leica).

ATP production as affected by fluazinam. ATP production of mycelia was determined with the isolate CN01 as previously described with minor modification (Xu et al. 2019). Five mycelial plugs (5 mm diameter) cut from 6-day-old colonies were transferred into 100 ml of YEPD. After incubation in a shaker with 180 rpm for 4 days at 25°C, mycelia were exposed to fluazinam for 4 h and were collected with single-layer Miracloth (Millipore). Mycelia (0.1 g) were ground with a mortar and pestle on ice. Extracting solution (1 ml) was added to resolve the tissue powder, and ATP production was tested with an ATP assay kit following the manufacturer's instruction (Solarbio, China). The experiments were conducted twice.

In planta control efficacy of fluazinam against *C. cassiicola*. To further evaluate the activity of fluazinam against *C. cassiicola*, in planta control efficacy was performed on detached cucumber leaves. Cucumber (Jinchun No. 4) was grown in a greenhouse until the trefoil stage. The leaves were detached, disinfected in 0.5% NaClO for 1 min, washed with SDW three times, and air-dried naturally. For protective activity tests, the leaves were sprayed with 25 and 100 µg/ml of fluazinam. After incubation for 12 h in a phytotron (85% humidity, 12-h/12-h photoperiod, 25°C), the leaves were stabbed with a sterile needle and inoculated with mycelial plugs (isolate CN01) from 6-day-old colonies on PDA plates. After incubation for an additional 7 days in a phytotron (85% humidity, 12-h/12-h photoperiod, 25°C), the diameters of the infected lesions were measured. The leaves sprayed with water were controls. For curative activity tests, the leaves were inoculated as described above prior to fluazinam spraying. After inoculation for 24 h in a phytotron (85%

humidity, 12-h/12-h photoperiod, 25°C), the leaves were sprayed with 25 and 100 µg/ml of fluazinam as described above. After inoculation for an additional 7 days, the diameters of the infected lesions were measured. The leaves sprayed with water were controls. Control efficacy of each treatment was calculated as follows: control efficacy = (lesion area of control – lesion area of treatment)/lesion of control × 100. There were 20 replicates for each treatment, and the experiments were performed three times.

Data analysis. The EC₅₀ values were calculated with DPS software (version 7.05). Fluazinam sensitivity distribution of *C. cassiicola* populations and multiple comparisons were analyzed with the Shapiro–Wilk test using SPSS software (version 19.0).

Results

Sensitivity of *C. cassiicola* to fluazinam based on mycelial growth inhibition. PDA plates amended with a series of concentrations of fluazinam were used to test the in vitro sensitivity of *C. cassiicola*. The inhibitory effect of fluazinam increased with the increase of dosage. Under the concentration of 0.125 µg/ml, the inhibitory rate of fluazinam against mycelial growth of *C. cassiicola* was approximately close to 50% (Fig. 1). The results showed that fluazinam effectively inhibited mycelial growth of *C. cassiicola*. Additionally, a total of 79 isolates of *C. cassiicola* were tested for fluazinam sensitivity based on the mycelial growth inhibition method. The EC₅₀ values of fluazinam ranged from 0.1002 to 0.3129 µg/ml, and the mean value was 0.2136 ± 0.0495 µg/ml. The fluazinam sensitivity of all the 79 isolates was normally distributed according to the Shapiro–Wilk test ($P = 0.2083$) (Fig. 2). The results suggested that fluazinam had an excellent antifungal activity against mycelial growth of *C. cassiicola* in vitro.

Sensitivity of *C. cassiicola* to fluazinam based on spore germination inhibition. Spore germination is the origin of fungal infection in host plants. Thus, the inhibition of spore germination can block fungal infection in host plants, increasing control efficacy of plant diseases. Therefore, the inhibitory effect of fluazinam on spore germination was determined with the randomly selected five isolates from mycelial growth inhibition-based sensitivity tests. The EC₅₀ values of fluazinam ranged from 0.0992 to 0.2278 µg/ml with a mean of 0.1499 ± 0.0504 µg/ml (Supplementary Table S2) and were similar to those from the mycelial growth inhibition-based sensitivity tests. The results indicated that fluazinam exhibited an excellent fungicidal activity on both mycelial growth and spore germination.

Cell membrane permeability. Two isolates, JSHA1701 and CN01, were subjected to a cell membrane permeability assay. Relative extracellular conductivity for two isolates increased in mycelia treated with fluazinam, and the increase was in dosage-dependent and time-dependent manners (Fig. 3A and 3B). This suggested that fluazinam disrupted the permeability of the cell membrane, resulting in the increased leakage of mycelial cell electrolyte.

Respiration. Mitochondrial complex I is the initiation complex of mitochondrial complexes where electron transfer begins, and it plays a vital role in cell respiration and ATP synthesis. Fluazinam has been reported to target mitochondrial complex I, causing mitochondrial dysfunction and cell apoptosis (Lee et al. 2012). When the mycelia were exposed to fluazinam, oxygen consumption rate significantly decreased over time. In addition, oxygen consumption rate of mycelia with 10 × EC₅₀ value of fluazinam markedly decreased in comparison with that of mycelia with EC₅₀ value of fluazinam (Fig. 4). The results suggested that fluazinam inhibited respiration of *C. cassiicola*.

Mycelial and cytoplasmic morphology. Curved mycelium or swollen mycelial structure generated by exposure to fluazinam has been reported in some filamentous fungi (Chen et al. 2018; Qu et al. 2018). In our study, CN01 was used to test the effect of fluazinam on mycelial morphology. When exposed to the EC₅₀ value of fluazinam, more mycelial branches were observed, but not curved mycelia (Fig. 5A). Severely swollen mycelial structure emerged when exposed to 10 × EC₅₀ value (Fig. 5A), and severe atrophies of cytoplasts were also observed when treated with fluazinam for 12 h (Fig. 5B). To investigate the effect of fluazinam on cell morphology, the cell wall of CN01 was stained with calcofluor white, and septum constrictions were observed when treated with fluazinam for 12 h (Fig. 5C). The results implied that fluazinam caused abnormality of mycelial morphology and cytoplasmic atrophy, thereby blocking the normal growth of mycelia.

Vacuole integrity. The vacuole is the largest organelle within a cell that is empty of cytoplasm. It is lined with a membrane, filled with fluid, and plays important roles in metabolites and maintaining cellular homeostasis (Cowles et al. 1997; Klionsky et al. 1990; Weber 2002). In this study, spherical vacuoles were observed in mycelial cells without fungicide treatments. However, complete spherical vacuoles were not observed in mycelial cells treated with fluazinam (Fig. 6). The damaged vacuole structures were similar among the different concentrations of fluazinam (Fig. 6). Thus, fluazinam can damage the vacuole structures, even if at the low dosage.

ATP production. ATP is an important cellular energy compound and mainly generated by oxidation phosphorylation in the mitochondria respiration chain. Chemical compounds targeting mitochondria complexes were reported to inhibit cellular ATP production (Xu et al. 2019). Extracted ATP from the isolate CN01 with or without fluazinam was subjected to ATP production assays. Fluazinam significantly inhibited ATP production of *C. cassiicola*, and the inhibitory effect increased with the increase of fluazinam dosage (Supplementary Fig. S1).

In planta control efficacy of fluazinam against *C. cassiicola*. As described above, fluazinam exhibits an excellent antifungal activity in both mycelial growth and spore germination of *C. cassiicola*. Thus, to further confirm the effect of fluazinam in controlling cucumber target spot caused by *C. cassiicola*, in planta control efficacy of

Fig. 1. Inhibitory effect of fluazinam on mycelial growth of *Corynespora cassiicola* on PDA plates.

fluazinam was evaluated on detached cucumber leaves. Fluazinam markedly decreased the diseased lesions of inoculated leaves in both protective activity and curative activity tests (Fig. 7A). For protective activity tests, fluazinam showed 81.01 and 97.47% control efficacies

Fig. 2. Sensitivity distribution of 79 *Corynespora cassiicola* isolates to fluazinam.

Fig. 3. Effect of fluazinam on cell membrane permeability of *Corynespora cassiicola*: A, isolate JSHA1701 ($EC_{50} = 0.2300 \mu\text{g/ml}$); and B, isolate CN01 ($EC_{50} = 0.1048 \mu\text{g/ml}$).

for 25 and 100 $\mu\text{g/ml}$, respectively. However, control efficacies of fluazinam for curative activity tests only were 51.90 and 58.23% for 25 and 100 $\mu\text{g/ml}$, respectively. The results showed that fluazinam exhibited both remarkable protective and curative activities and that protective activity was better than curative activity (Fig. 7B).

Discussion

Cucumber is a daily vegetable cultivated widely in China. According to our survey, a fungal disease named target spot is increasingly becoming a devastating disease, posing a severe threat to cucumber production in greenhouses. As we know, plant pathogen populations conferring fungicide resistance will emerge under the selective pressure of fungicides. Recently, our group and other groups have reported that the multiresistance of *C. cassiicola* populations to benzimidazoles, QoIs, and SDHIs emerged in some provinces in China, resulting in the failure of control efficacy and serious economic losses (Duan et al. 2019; Zhu et al. 2019). That superresistance from multiple mutations (E198A and M163I, E198A and F167Y, and E198A and F200S) of β -tubulin caused significantly increased resistance to benzimidazoles in comparison with the single mutation (E198A) was previously reported in *C. cassiicola* (Duan et al. 2019), resulting in increased difficulty in controlling cucumber target spot in greenhouses. Fluazinam is an uncoupler of oxidative phosphorylation belonging to aminopyridine compounds. In this study, we identified the discriminatory dose of fluazinam on *C. cassiicola* and determined fluazinam sensitivity of 79 isolates from Jiangsu and Henan Provinces in China. For mycelial growth inhibition, the EC_{50} values of 79 isolates ranged from 0.1002 to 0.3129 $\mu\text{g/ml}$ with a mean of $0.2136 \pm 0.0495 \mu\text{g/ml}$, and the frequency of the EC_{50} values was

Fig. 4. Effect of fluazinam on respiration: A, isolate JSHA1701 ($EC_{50} = 0.2300 \mu\text{g/ml}$); and B, isolate CN01 ($EC_{50} = 0.1048 \mu\text{g/ml}$).

normally distributed (Supplementary Table S1, Fig. 2). For spore germination inhibition, the EC_{50} values of the five randomly selected isolates ranged from 0.0992 to 0.2278 $\mu\text{g/ml}$ with a mean of 0.1499 ± 0.0504 $\mu\text{g/ml}$. This implied that fluazinam exhibited a strong inhibition activity on either mycelial growth or spore germination. Similar results were also reported in other plant pathogens (Chen et al. 2018; Matheron and Porchas 2004; Qu et al. 2018). Moreover, no cross-resistance was observed between fluazinam and other fungicides including benzimidazoles, QoIs, SDHIs, and dicarboximides (Liu et al. 2019; Mao et al. 2018). This is in accordance with the findings in this study. In other words, fluazinam has no cross-resistance with other fungicides such as benzimidazoles, QoIs, and SDHIs and can be a candidate for effectively controlling cucumber target spot caused by *C. cassiicola* in greenhouses. Fluazinam was reported to exhibit a good efficacy for controlling *Bipolaris maydis*, *Sclerotinia sclerotiorum*, and *Fusarium fujikuroi* (Chen et al. 2018; Matheron and Porchas 2004; Qu et al. 2018). In this study, fluazinam also exhibited excellent protective and curative activities on detached cucumber leaves (Fig. 7). The results of sensitivity distributions and in planta control efficacy indicated that fluazinam has a great potential to control cucumber target spot caused by *C. cassiicola*.

Fig. 5. Effect of fluazinam on mycelial and cytoplasmic morphology of *Corynespora cassiicola*. **A**, Mycelial morphology of CN01 on PDA plates with added fluazinam ($EC_{50} = 0.1048$ $\mu\text{g/ml}$). Arrows show the mycelial enlargement. **B**, Cytoplasts of CN01 treated with fluazinam for 12 h. Arrows show the abnormal distribution of mycelial cytoplasts. **C**, Calcofluor white staining of CN01 after treatment with fluazinam for 12 h. Arrows show the constriction of the mycelial septum as affected by fluazinam. Bar = 20 μm .

Cytomembrane plays an essential role in preventing abnormal efflux of intercellular electrolyte. The vacuole is a membrane-bound organelle important for the degradation of components and the storage of ions and metabolites. Both the cytomembrane and vacuole play important roles in maintaining cellular homeostasis. Our findings showed that fluazinam increased cell membrane permeability (Fig. 3); this may be due to the efflux of intercellular electrolyte. Interestingly, we also found that vacuole structures were destroyed after exposure to fluazinam (Fig. 6). Thus, we thought that fluazinam might damage the structure of membranes, disrupting cellular homeostasis, and consequently result in defects of mycelial growth.

Mitochondrial complexes are the main sites to complete the respiration process and generate ATP in cells. Some fungicides, such as QoIs, quinone inside inhibitors, and SDHIs, targeted the respiration chain and were highly effective to inhibit spore germination and mycelial growth of some important phytopathogens (Ishii et al. 2016; Mitani et al. 2001). Fluazinam was reported to target mitochondrial complex I in human neuroblastoma SH-SY5Y cells and significantly decreased basal and ATP-linked respiration in embryos of zebrafish (Lee et al. 2012; Wang et al. 2018). We also found that fluazinam significantly decreased the respiration rate of mycelia and cellular ATP

Fig. 6. Morphology of vacuole structure in hyphae as affected by fluazinam. Vacuoles were stained with CMAC and observed with a confocal microscope. Bar = 20 μm .

Fig. 7. Control efficacy of fluazinam against *Corynespora cassiicola*. **A**, Protective and curative activity of fluazinam on the detached leaves. **B**, Lesion areas for protective and curative activity of fluazinam. Column represents mean values of three experiments. The different lowercase letters represent significant difference at $P = 0.05$ level according to the LSD test.

production (Fig. 4, Supplementary Fig. S1). Therefore, we concluded that fluazinam might act on complex I in the respiration chain, resulting in the decrease of respiration, thereby causing the reduction of ATP production.

In summary, fluazinam not only exhibited an excellent in vitro antifungal activity on either mycelial growth or spore germination but also showed a great in planta control efficacy in either a protective assay or a curative assay on detached cucumber leaves. The results indicated that fluazinam was an effective candidate for controlling cucumber target spot caused by *C. cassiicola*, especially *C. cassiicola* populations conferring fungicide multiresistance and superresistance. Additionally, the biological and physiological characteristics of *C. cassiicola* as affected by fluazinam were determined in this study. Fluazinam not only significantly inhibited respiration and ATP production but also caused the increase of cell membrane permeability and dysfunctions of cellular homeostasis. This may contribute to the defects of mycelial growth. Interestingly, we found that fluazinam especially damaged vacuole structures, causing the redistribution of vacuole substances. This is the first report for chemical fungicides disrupting vacuole structures in pathogenic filamentous fungi. These findings will provide new insights for further revealing the action mode of fluazinam against plant pathogens.

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