Stimulatory Effects of Flusilazole on Virulence of *Sclerotinia sclerotiorum*

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Abstract

Flusilazole, a member of the demethylation inhibitor fungicides, is highly efficacious for control of *Sclerotinia sclerotiorum*. To achieve judicious applications of flusilazole, its hormetic effects on virulence of *S. sclerotiorum* were investigated. Flusilazole sprayed at concentrations from 0.02 to 0.5 µg/ml caused statistically significant ($P < 0.05$) stimulatory effects on virulence of *S. sclerotiorum* to potted rapeseed plants, and the maximum stimulation magnitudes were 11.0 and 10.7% for isolates GS-7 and HN-24, respectively. Studies on the time course of the infection process showed that a stimulatory effect on virulence could be discerned at 18 h postinoculation, indicating a direct stimulation mechanism rather than an overcompensation for initial inhibitions. In order to determine whether the stimulations were caused mainly by effects of flusilazole on *S. sclerotiorum* or on rapeseed plants, mycelia grown on flusilazole-amended potato dextrose agar (PDA) media were inoculated on leaves

of rapeseed plants without spraying the fungicide. Mycelium radial growth on PDA supplemented with flusilazole at concentrations from 0.005 to 0.16 µg/ml was inhibited by 10.11 to 48.7% for isolate GS-7 and by 4.1 to 24.9% for isolate HN-24. Observations with a scanning electron microscope showed that flusilazole in PDA at 0.04 and 0.08 µg/ml caused slightly deformed mycelia and twisted mycelial tips. Nevertheless, after inoculating on leaves of potted rapeseed plants, virulence of the inhibited mycelia was statistically significantly ($P < 0.05$) greater than that of the nontreated control, and the maximum stimulation magnitudes were 16.2 and 19.8% for isolates GS-7 and HN-24, respectively. Studies on a physiological mechanism for virulence stimulations showed that tolerance to hydrogen peroxide did not increase significantly for mycelia grown on flusilazole-amended PDA, thus excluding the possibility of tolerance to reactive oxygen species as a potential mechanism for virulence stimulations.

The ascomycete fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is a necrotrophic plant pathogen notorious for its broad host range of more than 400 plant species in 75 families, including many economically important crops and vegetables such as rapeseed, soybean, sunflower, and tomato (Boland and Hall 1994; Bolton et al. 2006). Crop diseases caused by *S. sclerotiorum* have always been difficult to control due to limited genetic resources for *S. sclerotiorum* resistance in major crops and long survival ability of sclerotia in the soil (Adams and Ayres 1979). *S. sclerotiorum* may cause heavy yield losses in many crops and it has been reported that rapeseed production losses due to *Sclerotinia* stem rot range from 10 to 80% annually in China (Wang et al. 2014). In the United States, annual economic losses caused by *S. sclerotiorum* in five crops (i.e., soybean, sunflower, dry edible bean, canola, and pulse) are about \$482 million (USDA 2012).

Among the integrated disease management tactics, fungicides remain the mainstay for the control of *S. sclerotiorum*. Sterol demethylation inhibitor (DMI) fungicides have been widely used in agricultural and medical settings against a wide variety of pathogenic fungi in crops, animals, and humans (Becher and Wirsal 2012). The DMI fungicide flusilazole is a broad-spectrum fungicide with highly preventative and curative efficacies against *S. sclerotiorum* (Lu et al. 2015). At present, flusilazole has not been registered in China to control diseases caused by *S. sclerotiorum* (<http://www.chinapesticide.gov.cn/hysj/index.jhtml>). Due to evaporation, drift, or uneven penetration into the crop canopy, fungicide spraying in the field will inevitably lead to exposure to sublethal doses of fungicides, at least for a portion of pathogens or isolates (Flores and Garzón 2013). Sublethal doses of fungicides may increase rather than reduce mycelial radial growth on artificial media and virulence on host plants. Stimulatory effects have been reported for thiabendazole on germination of spores of *Penicillium expansum* (Baraldi et al. 2003), propamocarb and cyazofamid on mycelial growth of *Pythium aphanidermatum* (Flores and Garzón 2013), metalaxyl on mycelial growth of

Phytophthora infestans (Zhang et al. 1997), and mefenoxam on mycelial growth and virulence of *Pythium aphanidermatum* (Garzón et al. 2011; Moorman and Kim 2004). With respect to *S. sclerotiorum*, stimulatory effects on virulence have been reported for dimethachlone, carbendazim, and trifloxystrobin (Di et al. 2015, 2016a; Zhou et al. 2014). Studies with detached rapeseed leaves showed that spraying dimethachlone at 300 mg/liter increased virulence of an isolate of *S. sclerotiorum* resistant in the field (Firoz et al. 2016). Stimulatory effects of low doses of fungicides on virulence of fungal plant pathogens have much relevance to management of fungal diseases. The toxic activity of flusilazole on *S. sclerotiorum* has been reported (Lu et al. 2015). The objectives of the present study were to (i) assess stimulatory effects of sublethal doses of flusilazole on virulence of *S. sclerotiorum* to rapeseed plants, (ii) determine the time course of the stimulations on virulence of *S. sclerotiorum*, and (iii) elucidate possible physiological mechanisms for the stimulatory effects of flusilazole.

Materials and Methods

Collection and preservation of isolates of *S. sclerotiorum*. Two isolates of *S. sclerotiorum*, HN-24 and GS-7, were collected in 2012 from rapeseed fields in Hunan and Gansu Provinces, respectively. The collected mature sclerotia were stored at 4°C in a refrigerator. Both isolates were sensitive to flusilazole and the effective concentrations at which mycelial growth rate on potato dextrose agar (PDA) was inhibited by 50% (EC₅₀ values) for isolates HN-24 and GS-7 were 0.085 and 0.104 µg/ml, respectively (Lu et al. 2015).

Fungicide. A technical-grade formulation of flusilazole (96.4% active ingredient; Hubei Kang Bao Tai Fine-Chemical Co. Ltd.) was stored at 4°C in a refrigerator and a stock solution at 1,000 µg/ml was prepared by dissolving the fungicide in pure acetone.

Stimulatory effects of spraying low doses of flusilazole on virulence of *S. sclerotiorum* to potted rapeseed plants. The procedure for assessing stimulatory effects of spraying flusilazole was according to Di et al. (2016a). Stock solution of flusilazole was serially diluted with 0.1% Triton-100 in water to concentrations of 8, 4, 2, 0.5, 0.2, 0.05, 0.02, 0.002, and 0.0002 µg/ml. Flusilazole solutions were sprayed until runoff on 4-week-old rapeseed plants growing in the greenhouse. Rapeseed plants sprayed with 0.1% Triton-100 in water were used as the nontreated control. After air drying for 1 h, two leaves per plant were inoculated on the adaxial surface with inverted mycelial plugs (5 mm in diameter), with one mycelial plug for each

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leaf. The inoculated plants were incubated at 23°C with photoperiod of 16 and 8 h (light and darkness, respectively) and relative humidity was maintained at about 85%. After 48 h of incubation, lesion diameters were measured twice in two perpendicular directions. All experiments in the study were performed in triplicate and repeated at least twice.

Stimulatory effects of growing on flusilazole-amended PDA on virulence to potted rapeseed plants. The procedure was according to Zhou et al. (2014). Inverted mycelial plugs were cut from 2-day-old cultures growing on PDA supplemented with flusilazole at final concentrations of 0.0025, 0.005, 0.04, 0.1, 0.16, 0.25, or 0.32 µg/ml, and were inoculated, with the mycelium side down, on the adaxial leaf surface of 4-week-old rapeseed plants. Mycelial plugs from a colony on PDA supplemented with acetone at a final concentration of 0.5% (vol/vol) were used as the nontreated control. The inoculated plants were incubated and lesion diameters were measured as described above.

Time course of stimulation on virulence of *S. sclerotiorum*. The procedure for assessing the time course of virulence stimulation was according to Di et al. (2016a,b). Rapeseed plants were grown at 23°C in a greenhouse for about 4 weeks, and three leaves of 8 to 11 cm in diameter were detached from each plant. The detached leaves were rinsed with sterile water, air dried for 1 h in the laboratory, and sprayed with flusilazole dilutions at 0.002 and 0.02 µg/ml by a hand-held sprayer (800 ml in volume; Xinmeir Co. Ltd.). Leaves sprayed with acetone in 0.1% Triton-100 at a final concentration of 0.5% (vol/vol) were employed as the nontreated control. After being air dried for about 1 h, leaves were transferred into Petri dishes (15 cm in diameter) that were embedded with a piece of wetted filter paper to maintain high humidity. The treated leaves were then

inoculated on the adaxial surface with inverted mycelial plugs, as described above. Petri dishes were lidded and the inoculated leaves were incubated at 23°C, with relative humidity maintained at about 85%. Disease symptoms were photographed with a digital camera (Canon Powershot G12) at 12, 18, 24, 36, and 48 h postinoculation (HPI).

Effects of low doses of flusilazole on tolerance capacity of *S. sclerotiorum* to hydrogen peroxide. Two-day-old mycelial colonies grown on PDA were subcultured on flusilazole-supplemented PDA. The final concentrations of flusilazole in PDA were 0.02, 0.04, 0.08, 0.16, and 0.32 µg/ml. Mycelia subcultured on PDA supplemented with 0.5% (vol/vol) acetone were used as the fungicide-free control. After the PDA plates had been incubated at 23°C for 48 h, mycelial plugs cut from actively growing margins of colonies were transferred to PDA media supplemented with hydrogen peroxide (H₂O₂) at final concentrations of 0 (without H₂O₂), 200, 400, or 800 µg/ml. After growing at 23°C for 48 h, mycelial growth rates were determined by measuring each colony diameter twice at right angles.

Electron microscopic observations on effects of flusilazole on mycelial morphology of *S. sclerotiorum*. The procedure was according to Lu et al. (2015). Mycelia were grown for 48 h on flusilazole-amended PDA media overlaid with a piece of cellophane. The final concentrations of flusilazole in PDA were 0.04 and 0.08 µg/ml. Mycelia grown on cellophane-overlaid PDA media supplemented with acetone at 0.5% (vol/vol) were employed as the fungicide-free control. Samples were collected from 2-day-old colonies by cutting growing margins into small pieces of 3 by 3 mm with a scalpel. The samples were fixed at 4°C for 24 h in 2.5% (wt/vol) glutaraldehyde solution in sodium phosphate buffer (0.05 M, pH 7.0). After being rinsed three times (10 min each time) in 0.05 M sodium phosphate buffer, the samples were dehydrated in a graded ethanol series. After critical point drying and gold coating in a sputter coater (JFC-1600; NTC), the leaf specimens were examined with a scanning electron microscope (SEM; JSM-6390/LV; NTC).

Data processing and analysis. Percent stimulations of low doses of flusilazole on virulence were calculated by the formula: percent stimulation (%) = [(lesion diameter of the treated – lesion diameter of the control)/lesion diameter of the control] × 100%. Arcsine square-root transformation was performed on the percent stimulation data before statistical analysis. Replicate data from different repetitions of the same experiment were pooled together because differences among repetitions were not significant. The multiple comparison procedure of Dunnett's test ($\alpha = 0.05$) in SPSS (Statistical Product and Service Solutions, ver. 21.0; SPSS Inc.) was employed to assess statistical significance of percent stimulation of each treatment compared with the nontreated control. Figures were constructed with Microsoft Excel software (ver. 2010; Microsoft Corporation).

Results

Stimulatory effects of spraying low doses of flusilazole on virulence of *S. sclerotiorum*. Spraying flusilazole at 0.02 to 0.5 µg/ml onto potted rapeseed plants elicited statistically significant ($P < 0.05$) stimulations of virulence of isolate GS-7 (Fig. 1A). Compared with the fungicide-free control, the maximum stimulation amplitude was 11%, at which the concentration of flusilazole was 0.2 µg/ml. For isolate HN-24, the stimulatory range of flusilazole was from 0.002 to 2 µg/ml and the stimulation amplitude was from 7.7 to 10.7% (Fig. 1B). For both isolates, the stimulatory curves did not follow the shape of a typical parabola.

Stimulatory effects of growing on PDA supplemented with flusilazole on virulence to potted rapeseed plants. Flusilazole in PDA at concentrations from 0.005 to 0.16 µg/ml inhibited mycelial growth rates by 10.1 to 48.4% for isolate GS-7 and by 4.1 to 24.9% for isolate HN-24. However, after these inhibited mycelia were inoculated on potted rapeseed plants, virulence (i.e., lesion diameter) increased statistically significantly ($P < 0.05$), compared with the nontreated control (Fig. 2). The maximum percent stimulations were 16.2 and 19.5% for isolates GS-7 and HN-24, respectively. Flusilazole in

Fig. 1. Stimulatory effects of spraying low doses of flusilazole on virulence of *Sclerotinia sclerotiorum* **A**, isolate GS-7 and **B**, isolate HN-24 on potted rapeseed plants. Error bar denotes standard error of the mean. Asterisk denotes significant difference from the control at $\alpha = 0.05$. Data are mean values of 12 replicates.

PDA media at 0.32 $\mu\text{g/ml}$ showed inhibitory effects on virulence for both isolates.

Effects of flusilazole on tolerance capacity of *S. sclerotiorum* to H_2O_2 . Compared with mycelial growth rates on PDA without H_2O_2 , growth rates on PDA amended with H_2O_2 at 200, 400, or 800 $\mu\text{g/ml}$ were strongly inhibited (Fig. 3). However, no statistically significant difference ($P > 0.05$) in mycelial growth rate was detected among different concentrations of flusilazole, indicating that flusilazole had no obvious effect on the tolerance capacity of *S. sclerotiorum* to H_2O_2 .

Time course of virulence stimulation. At 12 HPI, neither the flusilazole treatment nor the nontreated control group showed any sign of necrotic lesions (Fig. 4). At 18 HPI, differences in the development of disease symptoms could be discerned with the naked eye. Early signs of infections were visible only on leaves sprayed with flusilazole at 0.002 $\mu\text{g/ml}$, whereas no symptoms of infections could be discerned with the naked eye for the nontreated control, indicating a direct stimulation mechanism. From 24 to 36 HPI, disease symptoms developed obviously faster on leaves sprayed with flusilazole at 0.002 and 0.02 $\mu\text{g/ml}$ than on the nontreated control leaves.

Effects of flusilazole on mycelial morphology of *S. sclerotiorum*. Microscopic observations with an SEM demonstrated that hyphae without flusilazole treatment seemed natural (Fig. 5). The tips of hyphae grown on PDA supplemented with flusilazole at 0.04 $\mu\text{g/ml}$ were contorted, twisted, and more branched. The hyphae grown on PDA supplemented with flusilazole at 0.08 $\mu\text{g/ml}$ shrank and shriveled. It

was apparent that flusilazole at 0.04 and 0.08 $\mu\text{g/ml}$ inhibited the normal growth of mycelia and substantially affected mycelial morphology of *S. sclerotiorum*.

Discussion

Our previous studies showed that flusilazole sprayed at 90 $\mu\text{g/ml}$ provided preventive efficacy of 85% and curative efficacy of 59% in potted experiments (Lu et al. 2015). The present study demonstrated that spraying flusilazole at doses lower than 1 $\mu\text{g/ml}$ had significant stimulatory effect on virulence of *S. sclerotiorum* to rapeseed plants whereas, at concentrations higher than 4 $\mu\text{g/ml}$, flusilazole showed inhibitory effects on virulence. The inverted U-shaped, biphasic responses of *S. sclerotiorum* to flusilazole conformed to the biological concept of hormesis. In toxicology, hormesis represents a kind of dose-response relationship characterized by low-dose stimulation, which is usually beneficial to the organism, and high-dose inhibition (Calabrese 2015a). As a generalizable phenomenon, studies and published papers on hormesis have dramatically increased in recent years. Quantitative characteristics of hormesis such as stimulation range for chemical or physical stresses and stimulation amplitude have been experimentally observed and thoroughly reviewed, especially by Calabrese (2013, 2015a,b, 2016a,b). Low-dose stimulations of mycelial growth and virulence of *S. sclerotiorum* have been reported for the fungicides dimethachlone, carbendazim, and trifloxystrobin (Di et al. 2015, 2016a; Zhou et al. 2014). The hormetic effects of trifloxystrobin were reported in sensitive isolates of *S. sclerotiorum* (Di et al. 2016a), whereas the stimulatory effects of carbendazim were reported in carbendazim-resistant isolates of *S. sclerotiorum* (Di et al. 2015). With respect to dimethachlone, the hormetic effects were observed in both dimethachlone-sensitive and -resistant isolates (Zhou et al. 2014). In the present study, stimulatory effects of flusilazole were assayed in sensitive isolates. For both sensitive and resistant isolates of *S. sclerotiorum*, stimulatory effects of fungicides were observed in a dose range immediately below the inhibitory dose range. This was an important feature of the concept of hormesis. In the study on dimethachlone hormesis, we reported that all three dimethachlone-resistant isolates and 24% of sensitive isolates displayed stimulatory responses to their respective sublethal doses of dimethachlone (Zhou et al. 2014). Our subsequent studies indicated that all isolates might display hormetic effects induced by low doses of a fungicide, as long as appropriate sublethal doses and adequate replicates are employed. Several replicates, as commonly used in ordinary experiments, may not have enough power to detect stimulatory effects, especially for sensitive wild-type

Fig. 2. Stimulatory effect of growing on flusilazole-amended potato dextrose agar (PDA) on virulence of *Sclerotinia sclerotiorum* isolates **A**, GS-7 and **B**, HN-24 on potted rapeseed plants. Error bar denotes standard error of the mean. Asterisk denotes significant differences from the control at $\alpha = 0.05$. Data are mean values of 12 replicates. For isolate GS-7, mycelial growth inhibitions on PDA supplemented with flusilazole at 0.0025, 0.005, 0.02, 0.04, 0.08, 0.16, and 0.32 $\mu\text{g/ml}$ were 3.9, 10.1, 15.7, 25.2, 39.6, 48.4, and 59.7% respectively. For isolate HN-24, mycelial growth inhibitions on PDA supplemented with flusilazole at 0.0025, 0.005, 0.02, 0.04, 0.1, 0.16, and 0.32 were 2.2, 4.1, 3.5, 9.5, 14.4, 24.9, and 41.0%, respectively.

Fig. 3. Effects of growing on flusilazole-amended potato dextrose agar (PDA) on sensitivity of *Sclerotinia sclerotiorum* isolate HN-24 to H_2O_2 . FL denotes flusilazole. Error bar denotes standard error of the mean. Each column represents the mean value of six replicates. Mycelial growth inhibitions on PDA supplemented with flusilazole at 0.02, 0.04, 0.08, 0.16, and 0.32 $\mu\text{g/ml}$ (as the pretreatment) were 5.1, 16.4, 23.9, 32.0, and 47.4%, respectively.

isolates, because of a mild stimulatory amplitude, as shown in the present study, where the maximum stimulations were only a little greater than 10%. Thus, for studies on hormetic effects, a rigorous experiment design is needed (Flores and Garzón 2013). Another characteristic of hormetic dose-response is high fluctuations in stimulatory responses. In preliminary experiments of the present study, we found that stimulatory virulence and mycelial growth responses were not stable (data not shown). The relatively large error bars in Figures 1 and 2 also reflected the instability of stimulatory responses of *S. sclerotiorum* to flusilazole. The feature of high fluctuations in fungicide stimulations has also been reported by Garzón et al. (2011), whose studies showed that, although stimulations were

observed in mycelial growth of *P. aphanidermatum* on corn meal agar supplemented with sublethal doses of mefenoxam in all repetitions, no statistically significant differences were found, mainly due to unstable responses. Whether the stimulation is stable or not, and whether the stimulation magnitude is great or small, our experience with studies on stimulatory effects of several fungicides indicates that fungicide hormesis should be a general and common phenomenon, at least in the ascomycete fungus *S. sclerotiorum*. The hormesis database established by Calabrese and Blain (2011) that contained approximately 9,000 dose responses indicated that hormesis is a generalizable, fundamental concept in biology, and has been reported in a wide range of biological models across a large variety of chemical classes.

Fig. 4. Temporal profile of virulence stimulation of *Sclerotinia sclerotiorum* isolate GS-7 by low doses of flusilazole. Rapeseed leaves treated with 0.1% Triton-100 in water in the first row were used as the nontreated control (CK). HPI = hours postinoculation.

Fig. 5. Scanning electron micrographs showing effects of flusilazole on hyphal morphology of *Sclerotinia sclerotiorum*. In the first column, mycelia grown on potato dextrose agar amended with acetone at 0.5% (vol/vol) were used as the nontreated control (CK).

With respect to hormetic mechanisms, hormetic dose-responses may be caused by direct stimulation or by an overcompensation to a disruption of homeostasis (Calabrese 2001). Numerous examples of direct stimulations and their descriptive and quantitative features have been reviewed and evaluated (Calabrese 2001). Our previous studies on the temporal profile of fungicide hormesis demonstrated that stimulatory virulence responses of *S. sclerotiorum* to low doses of carbendazim and trifloxystrobin were mediated by a direct stimulation mechanism (Di et al. 2016a,b). The present study also showed that virulence stimulations of flusilazole on *S. sclerotiorum* involved a direct stimulation mechanism. Fungal infection of plants is a complex process and plant physiological status may substantially affect progresses of fungal infections. Stimulations of low doses of flusilazole on virulence of *S. sclerotiorum* may be mainly mediated by effects of flusilazole on plant or on fungus. However, mycelia grown on flusilazole-amended PDA also exhibited enhanced virulence to oilseed plants without spraying fungicide, thus indicating that virulence stimulation was induced mainly through effects of the fungicide on mycelia of *S. sclerotiorum*. In the early contact and initial infection by a fungus, the plant will mount a common resistance response called oxidative burst, a rapid production and release of huge amounts of reactive oxygen species, including superoxide radical, hydroxyl radical, and H_2O_2 (Williams et al. 2011; Wojtaszek 1997). A high tolerance ability of the fungus is conducive to virulence. However, the present study demonstrated that growing on flusilazole-supplemented PDA did not increase the tolerance capacity of *S. sclerotiorum* to H_2O_2 , thus ruling out the possibility of tolerance to plant oxidative burst as a physiological mechanism for flusilazole stimulations. Similar results have been reported for the stimulatory effect of carbendazim on virulence of *S. sclerotiorum* (Di et al. 2015). Nevertheless, mycelia of *S. sclerotiorum* grown on PDA supplemented with dimethachlone exhibited higher tolerance capacity to H_2O_2 than the nontreated control (Zhou et al. 2014). These results demonstrated that effects on tolerance capacity of fungi to H_2O_2 varied among different classes of fungicide. It seemed that enhanced tolerance to H_2O_2 was not a common mechanism for hormetic effects of a fungicide on virulence of plant fungal pathogens.

Flusilazole on PDA at concentrations of 0.005 to 0.16 $\mu\text{g/ml}$ inhibited mycelial growth of *S. sclerotiorum*. However, after the mycelia were inoculated on rapeseed leaves, the inhibited mycelia exhibited higher virulence than the fungicide-free control. For isolate GS-7, after mycelia inhibited by 48.4% in terms of colony radial growth on PDA were inoculated on rapeseed plants, virulence stimulation was significantly higher than the control. However, after mycelia inhibited by 59.7% were inoculated on plants, virulence was slightly lower than the control. For isolate HN-24, mycelia inhibited by 14.4% showed significantly greater virulence, whereas mycelia inhibited by 24.9% showed slightly lower virulence than the control. These results indicate that the relationship between mycelia inhibition and subsequent virulence stimulation may vary considerably even among different isolates of the same pathogen species. In the field, a portion of mycelia or spores will survive fungicide applications. Further investigations are warranted to determine whether and to what extent the inhibited mycelia and surviving spores will have stimulated virulence to plants in the field.

Acknowledgments

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