Time Course of Carbendazim Stimulation on Pathogenicity of *Sclerotinia sclerotiorum* Indicates a Direct Stimulation Mechanism

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Abstract

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Previous studies have demonstrated that subtoxic doses of carbendazim have a stimulatory effect on pathogenicity of *Sclerotinia sclerotiorum* on rapeseed plants. The present study focused on the time-course profile of the stimulatory effect and its relevance to stimulation mechanisms. At 12 h postinoculation (HPI), initial necrotic lesions were visible only for rapeseed leaves treated with carbendazim at 0.2 and 1 $\mu\text{g/ml}$, whereas no disease symptoms were observed for the nontreated control. At 18 HPI, carbendazim stimulation on pathogenicity was more obvious than at 12 HPI. Study with scanning electron microscopy demonstrated that no discernable differences in the development of disease symptoms could be detected at 8 HPI. However, at 12 HPI, necrotic symptoms of the epidermal cells were apparent only for leaves sprayed with carbendazim. These results indicated that stimulations on pathogenicity occurred in the first 12 h, implying that direct stimulation rather than overcompensation to the disruption of homeostasis was likely to be the underlying

mechanism for pathogenicity stimulation. Greenhouse experiments showed that spraying carbendazim at 400 $\mu\text{g/ml}$ on potted rapeseed plants had statistically significant ($P < 0.05$) stimulations on pathogenicity for inoculations at 1, 3, 5, and 7 days after application (DAA). The stimulation action eventually disappeared for inoculations at 14 DAA. Mycelia grown on potato dextrose agar (PDA) supplemented with carbendazim at 400 $\mu\text{g/ml}$ were more pathogenic than the nontreated control. However, after additional growth of the mycelia on fungicide-free PDA for 2 days, the stimulatory effect disappeared completely, indicating that carbendazim was indispensable for pathogenicity stimulations. Studies on biochemical mechanisms indicated that cell-wall-degrading enzymes such as cellulase, pectinase, and polygalacturonase were not involved in pathogenicity stimulations. These results will advance our understanding of the nature and mechanisms of fungicide stimulation on fungal pathogenicity and, thus, are valuable for judicious applications of fungicides.

The filamentous ascomycete fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is a devastating necrotrophic plant pathogen. This pathogen is notorious for its extremely wide host range and can infect more than 400 species of dicotyledonous plants, including many economically important crops (Boland and Hall 1994; Bolton et al. 2006). Diseases caused by *S. sclerotiorum* have been difficult to control because resistant cultivars have not yet been adequately developed and sclerotia are capable of surviving many years in the soil, thus making crop rotations problematic (Adams and Ayres 1979). Application of fungicides is still the mainstay of management measures, and extensive applications have inevitably led to fungicide resistance (Wang et al. 2014). Despite of high levels of carbendazim resistance in eastern China and sporadic occurrences of dimethachlon resistance in major rapeseed-growing regions, carbendazim and dimethachlon remain the two most widely used fungicides for control of *S. sclerotiorum* in China (Wang et al. 2014; Zhang et al. 2003; Zhou et al. 2014b). However, our previous studies have demonstrated that subtoxic doses of carbendazim and dimethachlon have significant stimulatory effects on pathogenicity of *S. sclerotiorum* on rapeseed plants (Di et al. 2015; Zhou et al. 2014a).

Stimulatory effects of subtoxic doses of fungicides on mycelial growth and pathogenicity of plant pathogens are closely relevant to plant disease management because a small number of pathogen isolates will inevitably be exposed to subtoxic doses of fungicides under field conditions (Flores and Garzón 2013). Stimulatory actions of subtoxic doses of fungicides have been reported in a number of plant-pathogenic fungi and oomycetes, such as increased germination of thiabendazole-resistant isolates of *Penicillium expansum* caused by

low concentrations of thiabendazole (Baraldi et al. 2003); increased mycelial growth of *Lyophyllum palustre* (Peck) Singer by propamocarb (Landry et al. 2011) and *Pythium aphanidermatum*, *P. irregulare*, and *P. ultimum* by propamocarb (Moorman and Kim 2004); and increased mycelial growth and pathogenicity of metalaxyl-resistant *Phytophthora infestans* by metalaxyl (Zhang et al. 1997) and *Pythium aphanidermatum* by mefenoxam (Garzón et al. 2011). These stimulations of subtoxic doses of fungicides seem to be a general phenomenon commonly called hormesis, and chemical hormesis has been reported across a tremendously broad range of biological systems and a wide variety of chemical agents (Calabrese 2013; Calabrese and Blain 2011).

Hormetic mechanisms involving cellular receptors and cell signaling pathways have been extensively documented and reviewed (Calabrese 2013; Calabrese and Blain 2011). The possible mechanisms for hormesis include direct stimulation, overcompensation to a disruption of homeostasis, and superimposition of two monotonic dose responses, with one taking effect at lower doses and the other taking effect at higher doses (Calabrese 2001). It is highly likely that multiple metabolic processes are involved in hormetic responses, some acting during the stimulation phase and others acting during the inhibition phase (Calabrese 2008; Garzón and Flores 2013). Up to the present, little has been known about mechanisms of fungicide hormesis. In the case of carbendazim stimulations on virulence of *S. sclerotiorum*, our previous studies have indicated that pathogenicity determinant oxalic acid and sensitivity to reactive oxygen species were not involved, whereas dimethachlon stimulations on pathogenicity were correlated with higher tolerances to oxidative stress that would confer an advantage to *S. sclerotiorum* in infecting host plants (Di et al. 2015; Heller and Tudzynski 2011; Zhou et al. 2014a). A time-course profile of stimulations may give hints about the underlying hormetic mechanisms. Up to now, there has been no detailed report on the time course of fungicide hormesis, perhaps mainly due to difficulties in attaining reproducible and reliable experimental results, especially when very low concentrations of fungicides are used, as is often the case for hormesis studies. The objects of the

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present study were to (i) determine the time course of carbendazim stimulations on pathogenicity of *S. sclerotiorum* and (ii) explore potential mechanisms for carbendazim hormesis.

Materials and Methods

Isolates of *S. sclerotiorum*. Two field isolates (LJ-86 and AH-17) of *S. sclerotiorum* were collected from diseased rapeseed plants with typical symptoms of Sclerotinia stem rot in Anhui Province of China in 2012. Mature sclerotia were stored at 4°C in a refrigerator before being cultured on potato dextrose agar (PDA) media. The 50% effective concentration (EC₅₀) values for carbendazim to the two isolates were higher than 1,000 µg/ml (Di et al. 2015).

Fungicide. Technical-grade carbendazim (98.1% active ingredient; Tian Jin Jin Bei Chemical Co. Ltd.) was dissolved in hydrochloric acid (HCl, 0.1 mol/liter) to produce a stock solution of 8,000 µg/ml. The stock solution was stored at 4°C in a refrigerator for no longer than 2 weeks before being serially diluted for experiments.

Time course of pathogenicity stimulation of subtoxic doses of carbendazim on *S. sclerotiorum* on detached leaves of rapeseed plants. Determination of pathogenicity stimulation of carbendazim on detached leaves of rapeseed plants was performed according to Di et al. (2015), with slight modifications. Briefly, leaves of 8 to 11 cm in diameter were detached from 4- to 5-week-old rapeseed plants growing in the greenhouse at about 25°C. The detached leaves were rinsed with sterile water, air dried on a clean bench, and transferred to 15-cm-diameter petri dishes lined with wetted filter paper to maintain high humidity. Carbendazim solutions were prepared by diluting carbendazim stock solution with sterilized 0.1% surfactant Triton-100 in water to final concentrations of 0.2 and 1 µg/ml. HCl at the same final concentration as in the diluted

carbendazim solutions was used as the nontreated control. Detached leaves were sprayed with carbendazim or HCl dilutions by a hand-held sprayer (800 ml in volume; Xinmeir Co. Ltd.). The treated leaves were air dried for 1 h, then inoculated on the adaxial surface with inverted 5-mm-diameter mycelial plugs (one plug per leaf). The inoculated leaves in petri dishes were incubated at 25°C in a growth chamber, with relative humidity maintained over 85%. Disease symptoms were recorded and photographed with a digital camera at 8, 12, 18, 36, and 48 h postinoculation (HPI). The experiment was performed in triplicate and repeated independently three times.

Microscopic observations of infection process with scanning electron microscopy. Detached leaves inoculated with mycelial plugs were sampled at 4, 8, and 12 HPI. Observations with scanning electron microscopy (SEM) were according to Zhang et al. (2010), with minor modifications. Briefly, the inoculated segments of leaves were cut into small pieces (3 by 3 mm). The samples were fixed in 2.5% (wt/vol) glutaraldehyde solution in sodium phosphate buffer (0.05 M, pH 7.0) at 4°C for 24 h, washed three times in 0.05 M sodium phosphate buffer for 10 min each time, then dehydrated in a graded ethanol series. After critical point drying and gold-coating in a sputter coater (JFC-1600; NTC, Japan), the leaf specimens were examined for infection process with SEM (JSM-6390/LV; NTC, Japan).

Pathogenicity stimulation duration for inoculations on potted rapeseed plants. Determination of pathogenicity stimulation duration of spraying carbendazim on potted rapeseed plants was conducted with a similar procedure as described above. Potted rapeseed plants (about 4 weeks old) grown in 20-cm-diameter pots containing a composite mixture of peat and soil (weight ratio = 1:1) were sprayed with carbendazim at 400 µg/ml until runoff, with rapeseed plants treated

Fig. 1. Time course of infection process of *Sclerotinia sclerotiorum* isolates AH-17 and LJ-86 on leaves of rapeseed plants. Leaves treated with 0.1% Triton-100 in water were used as the nontreated control (the first and third columns). Concentrations of carbendazim for isolate AH-17 (the second column) and for LJ-86 (the last column) were 0.2 and 1 µg/ml, respectively. Arrows indicate first sign of infection at 12 h postinoculation (HPI) for leaves treated with carbendazim.

with HCl at 0.004 mol/liter in 0.1% Triton-100 as the nontreated control. The treated rapeseed plants were incubated as described above. In all, 0, 1, 3, 5, 7, 10, and 14 days after application (DAA), each of the three plants treated with carbendazim at 400 $\mu\text{g/ml}$ and three plants treated with HCl as the control were inoculated on the adaxial surface of three leaves (with one mycelial plug per leaf). The inoculated plants were incubated as described above. Lesion diameters were measured twice at right angles after 48 h of incubation. The experiment was repeated independently six times.

Stimulatory effects of carbendazim-amended PDA on pathogenicity of *S. sclerotiorum*. Determination of the stimulatory effects of growth on PDA supplemented with carbendazim on pathogenicity was conducted according to Zhou et al. (2014a). Leaves of 8 to 11 cm in diameter were detached from 4- to 5-week-old oilseed rape plants grown in the greenhouse at 25°C. The detached leaves were rinsed with sterile water, air dried on a clean bench, and transferred to 15-cm-diameter petri dishes with water-soaked sterilized filter paper to maintain high humidity. Inverted mycelial plugs (5 mm in diameter) cut from fresh margins of 2-day-old colonies grown on PDA amended with carbendazim at a final concentration of 400 $\mu\text{g/ml}$ were inoculated on the adaxial surface of the detached leaves. Leaves inoculated

with mycelial plugs from colonies grown on fungicide-free PDA amended with HCl were used as the nontreated control. The inoculated leaves were incubated as described above. Lesion diameters were measured twice at right angles after 48 h of incubation. In order to study the lasting stimulatory effects of carbendazim, mycelial plugs from PDA supplemented with carbendazim at 400 $\mu\text{g/ml}$ were transferred to PDA free of fungicide and incubated for an additional 2 days; then, mycelial plugs from the PDA without carbendazim were inoculated to detached leaves of rapeseed plants as described above. Lesion diameters were measured as described above. The experiment was conducted in triplicate and repeated independently three times.

Effect of subtoxic doses of carbendazim on activities of cell-wall-degrading enzymes. Mycelia were grown on cellophane-overlaid PDA amended with carbendazim at 1 and 5 $\mu\text{g/ml}$ for 3 days. PDA supplemented with HCl at 0.001 mol/liter was used as the nontreated control. Mycelia were harvested by gentle scraping with a scoop. About 0.5 g of fresh mycelia was weighed, lyophilized, and ground into fine powder with a mortar. Cold sodium acetate-acetic acid buffer (4°C, 1.5 ml, 50 mmol/liter, pH 5.0) was added to suspend the powder. After centrifugation at 10,000 $\times g$ and 4°C for 10 min,

Fig. 2. Scanning electron micrographs showing infection processes of *Sclerotinia sclerotiorum* isolates AH-17 and LJ-86 on rapeseed leaves sprayed with 0.1% Triton-100 in water as the nontreated control (the first column) and sprayed with carbendazim at 1 $\mu\text{g/ml}$. At 12 h postinoculation (HPI), disease symptoms of necrotic epidermis were obvious for leaves treated with carbendazim.

the supernatant was used to assay activities of pectinase and polygalacturonase. For determination of cellulase activity, citric acid at 50 mmol/liter—sodium citrate buffer (pH 4.4) instead of sodium acetate—acetic acid buffer was used to suspend the powder.

Cellulase activity was measured spectrophotometrically using dinitsrosalicylic acid (DNS) as the dyeing agent, according to Bai and Wang (2012). DNS (3 g; Shanghai Chemical Reagent Co. Ltd.) was dissolved in 500 ml of distilled water at 45°C in a 1,000-ml brown volumetric flask. The following reagents were added sequentially into the volumetric flask: 10.4 g of NaOH, 90.1 g of potassium sodium tartrate, 2.5 g of phenol, and 2.5 g of anhydrous sodium sulfite. Distilled water was added to the final volume of 1,000 ml. Sodium carboxymethyl cellulose (CMC; 0.5%) dissolved in citric acid-sodium citrate buffer at 50 mmol/liter was used as the substrate. The activity of cellulase was determined by the conversion of CMC to glucose. The reaction mixtures initially contained 1 ml of supernatant enzyme and 3 ml of 0.5% CMC and were maintained at 50°C for 30 min. The

reaction was stopped by heating in boiling water for 10 min; then, 3 ml of DNS solution was added for the color reaction. Absorbance was measured at 530 nm with a UV-1800 spectrophotometer (Shimadzu Corporation). Reaction mixture with heat-inactivated enzyme was used as the control. A standard curve was developed using a series of glucose solutions. Reaction unit was defined as the production of glucose at 1 mg/min at 50°C.

Pectinase activity was assayed by the method of DNS according to Moyo et al. (2003). The reaction mixture was composed of 2 ml of 0.5% pectin in acetate buffer at 0.05 mol/liter, 7.5 ml of acetate buffer (pH 5.0), and 0.5 ml of supernatant enzyme. The mixture was incubated at 45°C for 30 min in a water bath incubator shaker. Reaction mixture (1 ml) was pipetted to a centrifuge tube and 1 ml each of DNS and 40% potassium sodium tartrate were added. After being vortexed and maintained in boiling water for 10 min, absorbance was measured at 575 nm with a UV-1800 spectrophotometer. One unit of pectinase activity was defined as the amount of the enzyme that catalyzed the formation of galacturonic acid at 1 μ mol/min at 45°C. Polygalacturonase activity was determined by a similar procedure, with 0.5% galacturonic acid instead of pectin as the substrate. The experiment was performed in triplicate and repeated independently three times.

Data analysis. Percentage stimulations were calculated by the following formula, with the diameter of mycelial plugs being subtracted from the measured lesion diameters: percentage stimulation (%) = [(lesion diameter of the treated – lesion diameter of the control)/lesion diameter of the control] $\times$ 100%.

Percentage stimulations were arcsine square-root transformed before statistical analysis. One-way analysis of variance and Dunnett's test ($P = 0.05$) in SPSS (Statistical Product and Service Solutions, ver. 19.0; SPSS Inc.) were employed to evaluate whether the percentage stimulations were significantly higher than the control. In pot experiments, the experimental unit was defined as each rapeseed plant grown in one pot, with the arithmetic mean of lesion diameters as the response. Figures were created with GraphPad Prism (ver. 5.01; GraphPad Software, Inc.) and Microsoft Excel (ver. 2010; Microsoft Corporation).

Results

Pathogenicity stimulations on detached leaves. The study on infection process of detached leaves demonstrated that, at 8 HPI, no necrotic lesions could be observed for all the treatments. At 12 HPI, initial necrotic lesions were visible only for leaves treated with sub-toxic doses of carbendazim, whereas no necrotic lesions were detected for the nontreated control (Fig. 1). From 18 to 48 HPI, pathogenicity stimulations of carbendazim at 0.2 and 1 μ g/ml were obvious for both isolates compared with the control.

Infection process shown by SEM. Observations with SEM gave similar results and, at 4 HPI, no infection structure such as infection cushions could be detected on leaves. At 8 HPI, infection cushions were visible under SEM but no obvious differences could be detected between the nontreated control and those treated with carbendazim

Fig. 3. Pathogenicity stimulation for inoculations of two *Sclerotinia sclerotiorum* isolates, A, AH-17 and B, LJ-86, at different time points after applying carbendazim at 400 μ g/ml on potted rapeseed plants. Error bars denote standard error of the mean for six replicates. Asterisks denote significant ($\alpha = 0.05$) difference from the control.

Fig. 4. Effects of carbendazim on activities of cell-wall-degrading enzymes A, cellulase; B, pectinase; and C, polygalacturonase in *Sclerotinia sclerotiorum* isolate AH-17. Error bars denote standard deviation of three replicates.

(Fig. 2). At 12 HPI, necrotic symptoms of the epidermal cells were more apparent for leaves treated with subtoxic doses of carbendazim than for the nontreated control.

Pathogenicity stimulation duration for inoculations on potted rapeseed plants. For the two carbendazim-resistant isolates, carbendazim at 400 µg/ml had an inhibitory effect only for inoculations immediately after its application. Significant ($P < 0.05$) stimulations on pathogenicity were detected for inoculations from 1 to 7 DAA (Fig. 3). Stimulation magnitude decreased for inoculations at 10 DAA. For inoculations at 14 DAA, pathogenicity stimulations disappeared completely.

Effects of carbendazim-amended PDA on pathogenicity. With a percent inhibition of -1.82%, mycelial growth rate of isolate AH-17 on PDA supplemented with carbendazim at 400 µg/ml was similar to that of the nontreated control. Inoculations to detached leaves with mycelial plugs from carbendazim-amended PDA displayed a pathogenicity stimulation of 19.7%. However, after transferring the mycelia onto PDA without carbendazim and growing for additional 2 days, the stimulatory effect on pathogenicity disappeared completely.

Effect of subtoxic doses of carbendazim on activities of cell-wall-degrading enzymes. Compared with the nontreated control, mycelia grown on PDA supplemented with carbendazim at 1 and 5 µg/ml did not have increased but, instead, slightly decreased activities for the three cell-wall-degrading enzymes (Fig. 4). Statistically, carbendazim at 1 and 5 µg/ml had no significant effects on activities of cellulase ($P = 0.332$), pectinase ($P = 0.961$), and polygalacturonase ($P = 0.142$).

Discussion

The present study showed that the earliest evidence of carbendazim stimulation on *S. sclerotiorum* pathogenicity could be detected with SEM at 12 HPI and with the naked eye at 18 HPI. At 12 HPI, the necrotic lesions on the epidermis of rapeseed leaves were more obvious on leaves treated with carbendazim than the nontreated control. At 8 HPI, infection cushions could be observed with SEM on inoculated leaves of rapeseed but no apparent differences could be detected between the nontreated control and the leaves sprayed with carbendazim at 1 µg/ml. At 4 HPI, no infection cushions were detected with SEM in the present study. Studies by Xiao et al. (2014) demonstrated that infection cushions of *S. sclerotiorum* could be observed with SEM on intact *Arabidopsis thaliana* leaves at 4 HPI. Host plant species and humidity may influence the infection progress. The fact that stimulation on lesion development on the epidermis of rapeseed leaves could be observed as early as 12 HPI indicated that a direct stimulation mechanism rather than overcompensation to disruption of homeostasis may account for the pathogenicity stimulations of carbendazim on *S. sclerotiorum*. Nevertheless, overcompensation to disruption of homeostasis has been inferred as a potential hormetic mechanism from the phenomena of early inhibition and subsequent stimulation. For example, it has been reported that plant growth could be inhibited by a chemical stressor at 24 h but would be stimulated at 72 h (Calabrese 2015b; Townsend 1897).

The direct stimulation mechanism for chemical hormesis has been reported for growth stimulation of herbicide phosphinothricin on the flowering plant *Lotus corniculatus* L. Growth stimulation at lower phosphinothricin concentrations was caused by activation of chloroplastic isoform GS2, while growth inhibition at higher phosphinothricin concentrations was a result of inhibition of chloroplastic isoform GS1 and GS2 (Dragičević et al. 2013). The direct stimulation mechanism may also account for many low-dose stimulatory responses in pharmacology literature (Calabrese 2015a). Studies with pharmacological receptor models demonstrated that the direct stimulations by low doses of chemicals may be caused by two distinct receptor subtypes. One subtype with stimulatory effects has fewer receptors but greater affinity for the agonist, whereas the other subtype with inhibitory effects has more receptors but lower affinity for the agonist. Superimposition of the effects of the two receptor subtypes will lead to stimulation at lower doses and inhibition at higher doses (Calabrese 2013, 2015b).

It should be noted that hormetic dose responses are independent of biological models, endpoints measured, chemical classes, and mechanisms involved. Hormesis (i.e., low-dose stimulation and high-dose inhibition or vice versa) is a common and broadly generalizable phenomenon, and may represent phenotypic plasticity and adaptation of an organism to environment (Calabrese and Mattson 2011). Although quantitative features of hormesis are independent of hormetic mechanisms, studies on time-course profile of hormetic dose response can give hints about the underlying mechanisms for hormesis. Of course, studies on biochemical and molecular mechanisms of hormesis are needed to advance our understanding of the nature of hormesis and, in the case of carbendazim stimulation on *S. sclerotiorum* pathogenicity, insight into the stimulatory mechanisms will be conducive to judicious application of this fungicide for control of *S. sclerotiorum*.

Carbendazim is commonly applied at around 400 µg/ml on rapeseed plants to control Sclerotinia stem rot in China and can provide excellent control on carbendazim-sensitive isolates of *S. sclerotiorum*. However, the present study demonstrated that, for carbendazim-resistant isolates, carbendazim at 400 µg/ml had significant pathogenicity stimulations for inoculations 1 to 7 DAA (Fig. 3). Given the development and prevalence of carbendazim resistance in plant pathogens, especially in *S. sclerotiorum* in eastern China (Wang et al. 2014; Zhu et al. 2016), this has significant implications for applications of carbendazim. This may also explain why Sclerotinia stem rot might become aggravated after application of carbendazim, as occasionally noticed by rapeseed growers (Di et al. 2015).

The first barrier to fungal infection is the plant cell wall, which is mainly composed of cellulose, xyloglucan, pectin, and protein. Plant pathogens release a battery of degrading enzymes, most notably cellulase, pectinase, and polygalacturonase, to weaken the plant cell wall and facilitate their infections (Bashi et al. 2012). However, the present study demonstrated that pathogenicity stimulation by carbendazim was not caused by any of the three cell-wall-degrading enzymes. Biochemical and molecular mechanisms for carbendazim stimulation remain to be elucidated in the future.

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