Stimulatory Effects of Boscalid on Virulence of *Sclerotinia sclerotiorum* Indicate Hormesis May Be Masked by Inhibitions

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Abstract

Hormetic effects of fungicides on phytopathogens are of great importance for proper application of fungicides. The aim of the present study was to investigate the stimulatory effects of the fungicide boscalid on mycelial growth and virulence of the devastating plant pathogen *Sclerotinia sclerotiorum*. Boscalid in potato dextrose agar (PDA) at a dosage range from 0.0005 to 0.002 µg/ml exerted statistically significant ($P \leq 0.015$) stimulations on mycelial growth of *S. sclerotiorum*, and the maximum stimulation magnitudes were $5.55 \pm 0.73\%$ (mean $\pm$ SD) for the four isolates tested. Boscalid in PDA at 0.02 µg/ml inhibited mycelial growth of isolates HLJ3H and HLJ4H by 15.0 and 8.9%, respectively. However, after the growth-inhibited mycelia were inoculated on rapeseed leaves, isolates HLJ3H and HLJ4H exhibited virulence stimulations of 8.7 and 17.8%, respectively, indicating that hormesis may be masked by inhibitions. Boscalid sprayed at 0.0001 to 0.1 µg/ml on detached rapeseed

leaves had significant ($P \leq 0.041$) stimulations on virulence of *S. sclerotiorum*, and the maximum stimulation magnitudes were $17.90 \pm 5.94\%$ (mean $\pm$ SD) for the four isolates tested. Experiments on 12 isolates with different levels of virulence showed there was a negative correlation ($R = -0.663$, $P = 0.019$) between the maximum virulence stimulation magnitude and virulence of *S. sclerotiorum* in the absence of fungicide. Boscalid at stimulatory concentrations had no significant effect on the expression levels of three virulence-associated genes that encode cutinase (*SsCut*), polygalacturonase (*SsPGI*), and oxaloacetate acetylhydrolase (*SsOah1*). The molecular mechanisms for hormetic effects of boscalid on *S. sclerotiorum* remain to be studied in the future.

Keywords: fungi, field crops, oilseeds and legumes, disease management, chemical

Sclerotinia sclerotiorum (Lib.) de Bary is a devastating ascomycete fungus and can infect over 600 plant species, many of which are economically important crops such as rapeseed (*Brassica napus*) and soybean (*Glycine max*) (Bolton et al. 2006; Liang and Rollins 2018). The disease caused by *S. sclerotiorum* on rapeseed plants is widely known as white mold or Sclerotinia stem rot (SSR). SSR is difficult to control due to the long-term and tenacious viability of sclerotia and limited genetic resources for resistance to *S. sclerotiorum*, and thus fungicides remain the mainstay for control of SSR (Derbyshire and Denton-Giles 2016). In the early 1980s, the benzimidazole fungicide carbendazim was registered in China for control of SSR, and in the subsequent two decades, carbendazim was extensively applied to control SSR. Extensive and repetitive applications inevitably led to carbendazim resistance in *S. sclerotiorum* (Shi et al. 2000; Zhang et al. 2003). Therefore, around the year 2000, the dicarboximide fungicide dimethachlone was recommended in China for SSR control. However, in 2009, insensitivity to dimethachlone was reported in Jiangsu Province of East China (Ma et al. 2009), and subsequently, different levels of dimethachlone resistance were detected in northwestern, northeastern, and central China (Zhou et al. 2014b, c). With the development of resistance to carbendazim and dimethachlone, fungicides with other modes of action such as demethylation inhibitors (DMIs) triadimefon, tebuconazole, propiconazole, and prochloraz were registered in China to control SSR (Zhang et al. 2018). Boscalid, a succinate dehydrogenase inhibitor (SDHI) fungicide, was first registered in

2006 in China for SSR control (<http://www.chinapesticide.org.cn/hysj/index.jhtml>).

In the field, there is a great possibility that plant pathogens are in fact under the condition of sublethal doses of fungicides (Garzón and Flores 2013). Sublethal doses of a fungicide may stimulate rather than inhibit mycelial growth and virulence of pathogens (Flores and Garzón 2013). The phenomenon of low-dose stimulation and high-dose inhibition reflects a common biological concept called hormesis. Hormetic model is biphasic, usually represented by either an inverted U-shaped or a J-shaped dose-response curve (Calabrese 2015a). Hormesis is generalizable and widespread across a broad range of biological models, including microbes, plants, invertebrates, and vertebrates. Many cases of hormesis have been reported across a variety of chemical classes (Calabrese 2013, 2015a, b, 2016a, b). Hormesis provides a generalized quantitative estimate of biological plasticity (Calabrese and Mattson 2011). Fungicide hormesis has profound implications for the application of fungicides. There have been numerous reports on fungicide hormesis, such as stimulatory effects of metalaxyl, mefenoxam, propamocarb, and cyazofamid on plant-pathogenic oomycetes *Pythium aphanidermatum* and *Phytophthora infestans* (Garzón et al. 2011; Zhang et al. 1997), and thiabendazole and thiophanate-methyl on fungal pathogens *Penicillium expansum* (Baraldi et al. 2003) and *Sclerotinia homoeocarpa* (Pradhan et al. 2019). In addition, our laboratory reported hormetic effects of carbendazim (Di et al. 2015b), dimethachlone (Zhou et al. 2014a), flusilazole (Lu et al. 2018a, b), prochloraz (Zhang et al. 2019), and trifloxystrobin (Di et al. 2016a) on the pathogenicity of *S. sclerotiorum*.

Owing to its unique mode of action, boscalid is highly efficacious for control of a variety of plant diseases such as powdery mildew, gray mold, leaf spot, and stem rot. Baseline sensitivity and control efficacy of boscalid against *S. sclerotiorum* have been reported recently (Hu et al. 2018; Liu et al. 2018). In order to judiciously apply boscalid for control of SSR, the stimulatory effects of this fungicide on mycelial growth and virulence of *S. sclerotiorum* need to be investigated. The objects of the present study were to (i) assay the stimulatory effects of boscalid on mycelial growth and virulence of *S. sclerotiorum*, (ii) evaluate effects of preconditioning with (pre-exposed to) boscalid on mycelial growth and

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virulence, and (iii) explore molecular mechanisms for hormetic effects of boscalid on *S. sclerotiorum*.

Materials and Methods

Isolates of *S. sclerotiorum*. *S. sclerotiorum* isolates with different levels of virulence were used in the present study to determine the correlation between virulence and the maximum stimulation magnitude. The isolate HLJMG1 was sampled in 2011 from a soybean plant with typical symptoms of Sclerotinia stem rot in Heilongjiang Province, and the isolates JD391 and LJ36 were sampled in 2011 from diseased rapeseed plants in Anhui Province. Three dimethachlone-resistant isolates (HLJ3H, HLJ4H, and HLJ6H) had relatively low virulence to rapeseed plants, and all were sampled from diseased soybean fields of Heilongjiang Province in 2011 (Zhou et al. 2014a, b). Previous studies showed that the expression levels of the group III two-component histidine kinase (HK) gene *Sshk* have an important influence on the virulence of *S. sclerotiorum* (Li et al. 2017); therefore, three *Sshk*-silenced transformants of isolate HLJ4H with increased virulence and three *Sshk*-overexpressed transformants of isolate HLJMG1 with reduced virulence were also used in the present study. The characteristics of these transformants and the transformation procedures were described in our previous paper (Li et al. 2019).

Fungicide. Technical grade boscalid (97.0% active ingredient) was bought from Hubei Kang Bao Tai Fine Chemical Co. Ltd. and dissolved in pure methanol to produce a 1,000 µg/ml stock solution. The stock solution was stored at 4°C and diluted within the next 2 weeks for the following experiments.

Stimulatory effects of boscalid in PDA on the mycelial growth of *S. sclerotiorum*. Two dimethachlone-sensitive isolates (HLJMG1 and JD391) and two dimethachlone-resistant isolates (HLJ3 and HLJ4) with reduced virulence were assessed for the stimulatory effects of boscalid on mycelial growth. Preliminary experiments with a wide range of boscalid concentrations showed that boscalid in potato dextrose agar (PDA) at 0.0001 to 0.02 µg/ml produced typical hormetic dose-response curves with respect to stimulations on mycelial growth of *S. sclerotiorum*. The final concentrations of boscalid in PDA were set for each isolate to produce a typical hormetic profile. PDA amended with methanol at 0.1% (vol/vol) was used as the nontreated control. PDA plates amended with different concentrations of boscalid or methanol alone were inoculated with 5-mm-diameter PDA plugs cut from fresh margins of a 2-day-old colony of each isolate. Three replicate PDA plates were used for each treatment. After being incubated at 23°C in the dark for 48 h, the diameter of each colony was measured twice at right angles. This experiment was independently conducted three times.

Effects of boscalid in PDA on the second generation mycelial growth and virulence. Two-day-old mycelial colonies grown on PDA amended with different concentrations of boscalid as described in the last section “Stimulatory effects of boscalid in PDA on the mycelial growth of *S. sclerotiorum*” were subcultured on fresh PDA plates. PDA plugs were cut from the periphery of each 2-day-old colony with a 5-mm-diameter cork borer and inoculated with the mycelial side down on the center of 9-cm PDA plates. After being incubated at 23°C in the dark for 48 h, the diameter of each colony was measured twice at right angles. This experiment was conducted in triplicate and independently performed three times. For the effects on virulence, leaves of about 9 to 12 cm in diameter were detached from 4-week-old rapeseed plants (cultivar: Zhongshuang 10) growing in the greenhouse at 25°C. The detached leaves were rinsed with tap water, air-dried for 1 h, and then transferred to 15-cm-diameter petri dishes lined with wetted filter paper to maintain high humidity. Two PDA plugs cut from the colonies growing on PDA supplemented with different concentrations of boscalid as described in the last section were inoculated on the adaxial surface of each leaf. The inoculated leaves were incubated at 25°C for 48 h in a growth chamber with relative humidity maintained at approximately 85%. The diameter of each lesion was measured twice at right angles. For each concentration, three detached leaves were inoculated, and the experiments were conducted four times.

Stimulatory effects of boscalid sprayed on rapeseed leaves on the virulence of *S. sclerotiorum*. The fungicide spraying and inoculation procedures were according to the method described by Cong et al. (2018). The stock solution of boscalid was serially diluted with sterile water containing 0.1% (vol/vol) Triton X-100 (Sigma Chemical Co., St Louis, MO). Preliminary experiments showed that boscalid sprayed at the concentration range from 0.00002 to 2 µg/ml produced parabolic stimulation curves. Six serially diluted, evenly spaced concentrations of boscalid were used for each isolate, and the specific concentrations might vary a little depending on their effects on virulence. Leaves of about 9 to 12 cm in diameter were detached from 4-week-old rapeseed plants (cultivar: Zhongshuang 10), rinsed with tap water, and air-dried for 1 h. Boscalid dilutions were sprayed on the detached leaves until runoff by a hand-held sprayer (800 ml in volume, Xinmeir Co. Ltd.), and water containing 0.1% Triton X-100 was used as the nontreated control. After being air-dried, leaves were transferred to 15-cm-diameter petri dishes lined with wetted filter paper to maintain high humidity. Leaves were then inoculated on the adaxial surface with fresh mycelial plugs 5 mm in diameter cut from the margins of actively growing colonies on PDA without any fungicide. The inoculated leaves were incubated at 25°C for 48 h in a growth chamber with relative humidity maintained at approximately 85%. The diameter of each lesion was measured twice at right angles. The experiment was conducted in triplicate and performed independently four times.

Correlation of the maximum magnitude of virulence stimulation with the virulence of *S. sclerotiorum* in the absence of fungicide. Virulence of *S. sclerotiorum* to the detached leaves of 4-week-old rapeseed plants was detected as described in the last section. The virulence of each isolate in the absence of any fungicide was expressed as the diameter in millimeter of disease lesions 48 h after inoculations. Virulence stimulations by boscalid were assayed by the procedure as described in the last section with a total of 12 isolates including three wild-type isolates, three dimethachlone-resistant isolates, three *Sshk*-silenced transformants of isolate HLJ4, and three *Sshk*-overexpressed transformants of isolate HLJMG1. Curve modeling for virulence stimulation was performed according to the method of Deng et al. (2001) with the second order polynomial (quadratic) procedure in the software SPSS (Statistical Product and Service Solutions, ver. 21.0, SPSS Inc., Chicago, IL), and the maximum stimulation magnitude was calculated by the formula $(4ac - b^2)/4a$. The correlation between virulence in the absence of fungicide and the maximum virulence stimulation magnitude was analyzed with the Pearson correlation procedure in the software SPSS.

Effects of boscalid on tolerance of *S. sclerotiorum* to hydrogen peroxide (H_2O_2). Increased tolerance of *S. sclerotiorum* to H_2O_2 is conducive to its infections of host plants (Williams et al. 2011; Zhang et al. 2019). Therefore, effects of boscalid on tolerance of *S. sclerotiorum* to H_2O_2 were assayed in the present study to explore the underlying stimulation mechanisms. The procedure was according to the method described by Cong et al. (2018). PDA was amended with boscalid at the final concentrations of 0.01 and 0.05 µg/ml. PDA supplemented with only methanol at the final concentration of 0.1% (vol/vol) was used as the nontreated control. Mycelial colonies of isolates HLJ3 and HLJ4 were grown at 23°C on PDA plates amended with boscalid and methanol alone for 48 h in the dark, and mycelial plugs 5 mm in diameter were cut with a cork borer from actively growing margins of the mycelial colonies. The fresh mycelial plugs were inoculated on PDA amended with H_2O_2 at 0, 100, and 200 µg/ml. After the PDA plates had been incubated at 23°C for 48 h in the dark, the diameter of each mycelial colony was measured twice at right angles. The experiment was conducted in triplicate and performed independently three times.

Effects of sublethal doses of boscalid on expression levels of virulence-associated genes. The expression levels of three virulence-associated genes were determined by the methods described by Yu et al. (2016) and Li et al. (2017). A mycelial colony of isolate HLJMG1 was subcultured on cellophane-overlaid PDA amended with boscalid at 0.001 µg/ml and incubated at 23°C in

the dark for 48 h. Mycelia subcultured on PDA supplemented with 0.1% (vol/vol) methanol was used as the nontreated control. The actively growing margins of the 2-day-old mycelial colonies were cut with a sterile scalpel into PDA plugs (1 × 1 cm). The PDA plugs were inoculated on leaves of 4-week-old rapeseed plants and incubated at 25°C for 6 h with relative humidity maintained at approximately 85%. After the PDA plugs were dislodged from the inoculated leaves, mycelia were scraped off from the PDA plugs, collected into a mortar and frozen immediately in liquid nitrogen. Total RNA was extracted from the harvest mycelia using the RNAiso Plus kit (TaKaRa Bio Inc.) according to the manufacturer's instructions. RNA purity was determined spectrophotometrically. Qualified

RNA was reverse-transcribed into cDNA with the Hifair II first Strand cDNA Synthesis SuperMix kit (Shanghai Yeasen Biotech Co., Ltd., China) according to the manufacturer's manual, and the obtained cDNA was stored at -80°C for subsequent experiments. The β -tubulin gene was used as the reference gene. Primer sequences for quantitative real-time PCR are listed in Table 1. The primer sequences for the β -tubulin gene were according to the publication of Li et al. (2019). The primer sequences for the cutinase gene *SsCut* were designed online by NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>) based on genomic DNA sequence retrieved from the GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) by the accession number SS1G_07661 (Seifbarghi

Table 1. Nucleotide sequence of the primers used in the study

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
<i>β-tubulin</i>	TTGGATTGCTCCTTTGACCAG	AGCGGCCATCATGTCTTAGG
<i>SsCut</i>	CTTCGGCGATCCAGATAA	ATCCATTCCGTATGTTAGATG
<i>SsPG1</i>	GTGGTCACGGTCTCTCCATC	TGTCCTTGTAGGTAACGCCG
<i>SsOah1</i>	GAAGTTGTCCCTGAAGAAGAG	GTGCGTGCAATCAACACAATA

Fig. 1. Stimulatory effects of boscalid in PDA on the mycelial growth of *Sclerotinia sclerotiorum*. Error bar denotes the standard error of the mean of 3 repeated experiments.

et al. 2017). Primer sequences for the endopolygalacturonase gene *SsPGI* and oxaloacetate acetylhydrolase gene *SsOah1* were according to Wang et al. (2018) and Liang et al. (2015), respectively. Quantitative PCR was performed in a MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). A total volume of 20 μ l reaction mixture contained 1 μ l of cDNA, 1 μ l of each of the forward and reverse primers each at 10 μ M (Table 1), 10 μ l of 2 $\times$ SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, U.S.A.), and 7 μ l of nuclease-free water. The thermal cycling conditions were 95°C initial denaturation for 30 s, 40 cycles of denaturation at 95°C for 5 s, primer annealing and elongation at 60°C for 10 s. Quantitative PCR assay specificity was verified by analyzing a melt curve generated after completion of the amplification reaction by increasing the temperature from 55 to 95°C in a step of 0.5°C every 10 s and monitoring the fluorescence signal at each step. The PCR data for each gene were normalized using the housekeeping gene β -tubulin. The relative levels of gene expression were calculated with the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). Three biological replicates were assayed.

Data processing and analysis. Percent virulence stimulations were calculated by the following formula: Percent stimulation (%) = [(Lesion diameter of the treated – Lesion diameter of the control)/ Lesion diameter of the control] $\times$ 100%. Stimulation curve modeling and test for significance of regression curves were performed with the quadratic regression procedure in the software SPSS (Statistical Product and Service Solutions, ver. 21.0; SPSS Inc.) as described by Deng et al. (2001). The data for mycelial growth, percent stimulations, and relative expression levels of genes were analyzed by the general linear model (GLM) procedure in the software SPSS (ver. 21.0) with Tukey procedure as the Post Hoc multiple comparison method. Percentage data were arcsine square-root transformed for a statistical significance test. Figures were constructed with the software Microsoft Excel (ver. 2013, Microsoft Corporation, Redmond, WA).

Results

Stimulatory effects of boscalid on the mycelial growth of *S. sclerotiorum*. Boscalid in PDA at low dosages of 0.0005, 0.001, and 0.002 μ g/ml had slightly but statistically significant ($P \leq 0.015$) stimulations on the mycelial growth of *S. sclerotiorum* on PDA (Fig. 1). The maximum stimulation magnitude was 5.1, 5.9, 6.4, and 4.8% for the four isolates HLJ3H, HLJ4H, HLJMG1, and JD391, respectively. The effective concentration for 50% inhibition (EC_{50}) values of boscalid for the two dimethachlone-resistant isolates HLJ3H and HLJ4H were at least threefold higher

than those for the two sensitive isolates HLJMG1 and JD391 (data not shown). Consequently, boscalid dosages with the maximum growth stimulation for the two isolates HLJ3H and HLJ4H were also higher than those for the two isolates HLJMG1 and JD391.

Effects of boscalid in PDA on the second generation mycelial growth and virulence. Boscalid at the relatively low dosage range of 0.0005 to 0.002 μ g/ml had stimulatory effects on the first generation mycelial growth of the two isolates HLJ3H and HLJ4H. After the stimulated mycelia were inoculated on fresh PDA plates and incubated for 48 h, mycelia growth stimulations were observed, indicating that the feature of hormesis can last at least several days and can pass down to the next generation of mycelia (Table 2). Boscalid in PDA at the relatively high-dosage range of 0.01 to 0.05 μ g/ml inhibited the first generation mycelial growth by 3.0–28.8%. However, after the growth-inhibited mycelia were inoculated on fresh PDA plates and incubated for 48 h, mycelial growths were stimulated by 3.9–19.8%. After the growth-inhibited mycelia were inoculated on rapeseed leaves, significant stimulations on virulence were obtained. Boscalid in PDA at 0.02 μ g/ml inhibited mycelial growth of isolate HLJ3H by 15.0%, and the inhibited mycelia exhibited virulence stimulation of 8.7% after being inoculated on rapeseed leaves. Similarly, boscalid in PDA at 0.05 μ g/ml inhibited mycelial growth of isolate HLJ4H by 19.6%, and the inhibited mycelia showed virulence stimulation of 13.6%. These results indicate that inhibition may encompass stimulation.

Effects of boscalid sprayed on the virulence of *S. sclerotiorum* to rapeseed leaves. Boscalid sprayed on rapeseed leaves displayed typical bell-shaped stimulatory dose-virulence curves (Fig. 2). Compared with mycelial growth stimulation profiles, the stimulatory doses for virulence were higher, and the maximum stimulation magnitudes were also greater. For isolate HLJ4, boscalid sprayed at the concentration range of 0.00005 to 0.2 μ g/ml showed significant ($P = 0.007$) stimulations on the virulence, and the maximum virulence stimulation magnitude was 21.5%. Similarly, statistically significant ($P = 0.003, 0.006, \text{ and } 0.041$) hormetic dose-response curves were obtained for the other three isolates tested.

Correlation between maximum virulence stimulation magnitude and virulence in the absence of fungicide. Correlation analysis based on data of 12 isolates showed that there was a significantly negative correlation ($R = -0.663, P = 0.019$) between maximum virulence stimulation magnitude and the virulence in the absence of fungicide (Fig. 3). However, no significant correlation ($P = 0.232$) was obtained between the maximum virulence stimulation magnitude and mycelial growth rate on PDA (data not shown).

Table 2. Percent stimulations of boscalid in PDA for the first and second generation mycelial growths and virulence of *Sclerotinia sclerotiorum*

Stimulations	Boscalid concentration in PDA (μg/ml)								
	0.0005	0.001	0.002	0.005	0.01	0.02	0.05	0.1	0.2
Isolate HLJ3H									
First generation mycelial growth	2.34 ^a (1.58) ^b	5.10 (2.31)	3.45 (1.61)	−2.60 (2.14)	−8.06 (2.85)	−14.96 (2.73)	−28.76 (2.51)	−41.58 (2.34)	−49.69 (1.33)
Second generation mycelial growth	3.79 (0.71)	3.32 (1.45)	7.06 (1.60)	7.24 (1.21)	8.07 (2.27)	8.04 (1.95)	7.44 (0.80)	−0.02 (2.26)	NA ^c
Curve modeling ^d	$y = -4.63x^2 - 20.04x - 13.37, R^2 = 0.708$								
Virulence	0.38 (0.80)	7.95 (4.99)	9.94 (2.45)	11.29 (0.85)	17.79 (2.93)	8.73 (0.26)	3.90 (2.96)	−17.93 (2.24)	NA
Curve modeling	$y = -16.98x^2 - 78.37x - 74.98, R^2 = 0.880$								
Isolate HLJ4H									
First generation mycelial growth	4.82 (1.84)	5.92 (3.23)	3.01 (0.75)	2.58 (0.44)	−3.04 (0.78)	−8.85 (1.11)	−19.57 (1.95)	−28.69 (2.56)	−36.97 (1.96)
Second generation mycelial growth	1.05 (4.00)	6.55 (0.68)	5.81 (1.89)	6.87 (0.54)	5.34 (1.01)	4.22 (0.49)	4.85 (2.61)	3.08 (2.64)	−4.76 (1.39)
Curve modeling	$y = -4.17x^2 - 18.57x - 13.78, R^2 = 0.793$								
Virulence	4.87 (4.73)	19.28 (7.76)	24.24 (6.99)	22.57 (8.63)	19.81 (7.72)	17.75 (5.80)	13.59 (3.59)	−0.10 (9.53)	−14.42 (9.39)
Curve modeling	$y = -15.31x^2 - 69.40x - 54.47, R^2 = 0.955$								

^a Data are percent stimulations compared with the nontreated control.

^b Data in parentheses denote the standard error of the mean of 4 repeated experiments.

^c NA denotes the experiments were not conducted due to the high mycelial growth inhibitions on PDA amended with boscalid at 0.2 μ g/ml.

^d The quadratic regression was performed with the logarithmic transformed concentrations and percent stimulations.

Effects of boscalid on tolerance of *S. sclerotiorum* to H₂O₂. Boscalid in PDA at virulence stimulating doses of 0.01 and 0.05 µg/ml had no significant ($P > 0.05$) effects on tolerance of *S. sclerotiorum* to H₂O₂ (Fig. 4).

Effects of boscalid on the expression levels of virulence-associated genes. Boscalid at 0.001 µg/ml in PDA increased the virulence of isolate HLJMG1 by 10.8% but had no significant ($P > 0.05$) effects on the transcription levels of any of the three virulence-associated genes, including cutinase gene *SsCut*, polygalacturonase gene *SsPG1*, and oxaloacetate acetylhydrolase gene *SsOah1* of *S. sclerotiorum* (Fig. 5).

Discussion

The present study demonstrated that boscalid had stimulatory effects on mycelial growth and virulence of *S. sclerotiorum*. The stimulatory curves of boscalid to mycelial growth and virulence of *S. sclerotiorum* conformed well to the typical hormetic dose-response model. Notably, the stimulation magnitude for mycelial growth was smaller than that for virulence to rapeseed leaves. The relatively smaller stimulation magnitude for growth stimulation may be the reason why there are fewer reports on mycelial growth stimulations than

virulence hormesis. Given the unavoidable experimental random errors, smaller stimulation magnitude implies it is more difficult to obtain statistically significant stimulations. Previously, we have reported considerable stimulatory effects of dimethachlone on mycelial growth of dimethachlone-resistant isolates of *S. sclerotiorum* (Zhou et al. 2014a). The greater mycelial growth stimulation magnitude of dimethachlone-resistant isolates may be related to the reduced mycelial growth rates compared with sensitive isolates (Zhou et al. 2014b). However, the present study showed similar stimulation magnitude for boscalid between dimethachlone-resistant and -sensitive isolates. It seemed that in addition to the general low-magnitude hormesis which is independent of biological models and chemical agents as reviewed by Calabrese (2015a, b), there was a more specific stimulation which may be high in magnitude but occurs only in specific biological models and for specific inducing chemical or physical stressors.

After the stimulated mycelia were inoculated on fresh PDA plates and detached rapeseed leaves, significant stimulations of both mycelial growth and virulence were observed, indicating the phenomenon of hormesis can pass on to the next growth and development stage. It might seem surprising that growth-inhibited mycelia also showed

Fig. 2. Stimulatory effects of boscalid sprayed on detached rapeseed leaves on the virulence of *Sclerotinia sclerotiorum*. Error bar denotes the standard error of the mean of 4 repeated experiments.

increased mycelial growth and virulence after being inoculated on PDA plates and rapeseed leaves. These results demonstrate that the occurrences of hormesis may be far more common and frequent than we previously recognized or realized. Therefore, when we determine the occurrence of hormesis in the future, it should be kept in mind that one endpoint measured at a specific time point may not represent the real occurrence of hormesis. Our previous studies also demonstrated that mycelia of *S. sclerotiorum* slightly inhibited by flusilazole displayed increased virulence after being inoculated on rapeseed leaves (Lu et al. 2018b). Mycelia of *B. cinerea* inhibited by carbendazim by about 30% in terms of colony growth also exhibited increased virulence on cucumber leaves and produced higher numbers of sclerotia (Cong et al. 2019). These results indicate definitely that inhibitions may encompass hormesis and hormesis may be hidden among inhibitions. Under

appropriate conditions, hormesis may emerge in inhibited biological organisms. These phenomena have profound implications for understanding the concept of hormesis and also highlighted the prevalence and concealment of hormesis.

Hormesis is a generalizable biological concept, and the quantitative features of hormesis are consistent across a broad range of biological models and independent of chemical stressors, with the maximum stimulation magnitude being about 30–60% compared with the control (Calabrese 2015a). However, our previous study showed that the virulence stimulation magnitude of *S. sclerotiorum* by dimethachlone varied considerably and it seemed that larger stimulation magnitudes were obtained for dimethachlone-resistant isolates which usually have reduced virulence (Zhang et al. 2019; Zhou et al. 2014a). Therefore, the present study employed a group of *S. sclerotiorum* isolates with different levels of virulence to determine the correlation of the maximum stimulation magnitudes with virulence, and a significant negative correlation with $P = 0.019$ and $R^2 = 0.439$ was obtained. For those isolates with reduced virulence on rapeseed leaves, relatively larger stimulation magnitudes were achieved. However, no significant correlation was obtained between the maximum virulence stimulation magnitude and mycelial growth rate (data not shown). These results indicate that the stimulation magnitude may be affected by or correlated with a specific feature of the organism tested. This phenomenon is also consistent with the generalizable concept of hormesis, and the greater stimulation magnitude may result from the removal of a possible constraint on virulence. In other words, the so-called stimulation may only make an organism reach its potential limits.

The present study demonstrated that boscalid at the stimulatory concentrations did not have any significant effect on tolerance of *S. sclerotiorum* to H_2O_2 or expression levels of cutinase gene *SsCut*, polygalacturonase gene *SsPG1*, or oxaloacetate acetylhydrolase gene *SsOah1*. It has long been recognized that host plants generate reactive oxygen species (ROS) such as H_2O_2 as the early defense responses to resist pathogens (Amselem et al. 2011). Previous studies showed that dimethachlone and prochloraz at the concentrations eliciting virulence stimulations promote tolerance of *S. sclerotiorum* to H_2O_2 (Zhang et al. 2019; Zhou et al. 2014a), but carbendazim and flusilazole at subtoxic concentrations have no significant effects on tolerance to H_2O_2 (Di et al. 2015; Lu et al. 2018b). The result of the present study showed that tolerance to H_2O_2 could not be a universal hormesis mechanism. Oxalic acid plays a crucial role in the pathogenicity of *S. sclerotiorum*, and

Fig. 3. Correlation of the maximum virulence stimulation magnitude of boscalid to *Sclerotinia sclerotiorum* with virulence in the absence of a fungicide. Virulence was represented by the diameter of disease lesions on rapeseed leaves 2 days after inoculation.

Fig. 4. Effects of boscalid on tolerance of *Sclerotinia sclerotiorum* isolates HLJ3H and HLJ4H to H_2O_2 . Error bar denotes the standard error of the mean of 3 repeated experiments. CK denotes the nontreated control.

Fig. 5. Effects of sublethal doses of boscalid on relative expression levels of cutinase gene *SsCut*, polygalacturonase gene *SsPG1*, and oxaloacetate acetylhydrolase gene *SsOah1* of *Sclerotinia sclerotiorum* isolate HLJMG. Error bar denotes the standard error of the mean of 3 repeated experiments.

one major pathway responsible for oxalic acid biogenesis is oxaloacetate acetylhydrolase (OAH, EC 3.7.1.1) which catalyzes the conversion of oxaloacetate to oxalic acid and carbon dioxide (Liang et al. 2015). Sublethal doses of boscalid had no significant effect on the expression of OAH encoding gene *SsOah1*, indicating the hormetic effect on virulence may not be associated with oxalic acid production. Similarly, the present study also demonstrated that boscalid at virulence stimulating doses had no significant effect on expression levels of cutinase gene *SsCut* or polygalacturonase gene *SsPG1*. Therefore, the underlying mechanisms for virulence hormesis of boscalid remain to be studied in the future.

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