Control of Wheat Fusarium Head Blight by Heat-Stable Antifungal Factor (HSAF) from *Lysobacter enzymogenes*

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Abstract

Heat-stable antifungal factor (HSAF), which belongs to the polycyclic tetramate macrolactam family, was isolated from *Lysobacter enzymogenes* fermentations and exhibited inhibitory activities against a wide range of fungal pathogens. In this study, the antifungal activity of HSAF against *Fusarium graminearum* in vitro and in vivo was investigated. A total of 50% of mycelial growth of *F. graminearum* was suppressed with 4.1 µg/ml of HSAF (EC₅₀ value). HSAF treatment resulted in abnormal morphology of the hyphae, such as curling, apical swelling, and

depolarized growth. Furthermore, HSAF adequately inhibited conidial germination and conidiation of *F. graminearum* with an inhibition rate of 100% when 1 and 6 µg/ml of HSAF were applied, respectively. HSAF caused ultrastructural changes of *F. graminearum*, including cell wall thickening and plasmolysis. Moreover, the application of HSAF significantly controlled Fusarium head blight in wheat caused by *F. graminearum* in the field. Overall, these results indicate that HSAF has potential for development as a fungicide against *F. graminearum*.

Fusarium head blight (FHB), primarily caused by *Fusarium graminearum*, is a devastating fungal disease observed worldwide that results in yield and quality losses of wheat and other small grains (Dean et al. 2012). In addition to the yield and quality reduction, the notable problem is mycotoxin contamination of grains, primarily with deoxynivalenol (DON), which is toxic to animals and humans (EFSA Panel on Contaminants in the Food Chain et al. 2017; Katika et al. 2014; Pestka 2010; Sobrova et al. 2010). Different strategies have been applied to control FHB infection, including agronomic practices, resistant cultivars, chemical fungicides, and biological control (Dweba et al. 2017; Wegulo et al. 2015). Of these strategies, chemical fungicide control is the most widely used, particularly the demethylation inhibitors (DMIs, e.g., tebuconazole, prothioconazole, and metconazole) and benzimidazole fungicides (MBC, e.g., carbendazim) (Chen and Zhou 2009; McMullen et al. 2012). However, since the discovery of carbendazim-resistant isolates in China (Chen and Zhou 2009) and tebuconazole-resistant isolates in the United States (Spolti et al. 2014), the need to develop fungicides with new mechanisms of action is urgent.

In the search for new fungicide leads, secondary metabolite heat-stable antifungal factor (HSAF, dihydromaltophilin) belonging to the polycyclic tetramate macrolactams (Fig. 1) was isolated from *Lysobacter enzymogenes* C3 and OH11 fermentations (Lou et al. 2011; Yu et al. 2007). *L. enzymogenes* belongs to the *Xanthomonadaceae* family and is emerging as an effective bacterial biocontrol agent

against fungal plant diseases (Folman et al. 2003, 2004; Jochum et al. 2006; Qian et al. 2009; Zhang and Yuen 1999). *L. enzymogenes* strain OH11 was isolated from the rhizosphere of green pepper and shared 99% genetic similarity with strain C3 according to phylogenetic analysis (Qian et al. 2009). The unusual hybrid polyketide synthase/nonribosomal peptide synthetase biosynthetic mechanism and regulatory pathways of HSAF have been extensively studied in *L. enzymogenes* strain OH11 (Lou et al. 2011; Qian et al. 2013; Su et al. 2017; Xu et al. 2018). HSAF exhibits potent inhibitory activities against a wide range of filamentous fungal pathogens by disrupting sphingolipid biosynthesis, which induces cell wall thickening and depolarizes hyphal growth of *Aspergillus nidulans* (Li et al. 2006, 2009). Furthermore, HSAF is also efficient in killing the nonfilamentous fungus of *Candida albicans* by inducing apoptosis through reactive oxygen species accumulation (Ding et al. 2016). Collectively, the unique characteristics described above make HSAF particularly attractive as a potential chemical pesticide, particularly for controlling fungal pathogens that are resistant to currently applied pesticides. However, there was no specific report about the mode action of HSAF on *F. graminearum* and its control effect in wheat FHB.

In this study, we investigated the inhibitory activity of HSAF against *F. graminearum* in vitro and assessed the potential of HSAF for controlling FHB in the field. The antifungal mechanism of HSAF against *F. graminearum* was also explored.

Materials and Methods

Strains and growth conditions. *L. enzymogenes* strain OH11 (CGMCC no. 1978) was isolated from the rhizosphere of green pepper in Jiangsu Academy of Agricultural Sciences of China (Qian et al. 2009). OH11 was grown in Luria broth (LB) medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl) or 10% tryptic soy broth (TSB) at 28°C. A wild-type strain of *F. graminearum*, PH-1 (NRRL 31084), originally isolated from corn in Michigan, U.S.A., was grown at 25°C on potato dextrose agar (PDA) medium (200 g of potato dextrose, 20 g of glucose, and 15 g of agar in 1 liter of distilled water) for 3 days or yeast extract–peptone–dextrose (YEPD) medium (10 g of peptone, 3 g of yeast extract, and 20 g of

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glucose in 1 liter of distilled water) for 1 day. For conidiation assays, *F. graminearum* PH-1 was inoculated in carboxymethylcellulose (CMC) medium (15 g of sodium carboxymethylcellulose, 1 g of yeast extract, 1 g of NH_4NO_3 , 1 g of KH_2PO_4 , and 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 liter of distilled water) at 28°C for 4 days.

HSAF preparation. We extracted HSAF using methods previously described by Yu et al. (2007) and Zhao et al. (2017). *L. enzymogenes* strain OH11 was inoculated into LB medium and incubated at 28°C with shaking at 200 rpm until the OD_{600} reached 1.0. The OH11 cultures were then transferred into 10% TSB (1:100; vol/vol) and were incubated at 28°C in a rotary shaker at 220 rpm for 24 h. After incubation, bacterial cultures were centrifuged ($10,000 \times g$ at 4°C for 20 min), and the supernatant was collected. To extract HSAF, an equal volume of ethyl acetate was added to the supernatant, which was acidified with 37% hydrochloric acid (0.4% [v/v]). The mixture was fully shaken in a rotary shaker at 180 rpm for 1 h, and the ethyl acetate was evaporated in a rotary evaporator. Finally, the remaining residues containing HSAF were fully dissolved in 100% methanol and purified by high-performance liquid chromatography to obtain HSAF of over 90% purity.

In vitro effects of HSAF on mycelial growth and morphology. To evaluate the antifungal activity, the effects of different concentrations of HSAF against *F. graminearum* were determined. HSAF was diluted into gradient solutions with methanol, and 50 μl of each solution was added into 10 ml of PDA medium to yield final concentrations of 2, 4, 6, 8, and 10 $\mu\text{g}/\text{ml}$. The same volume of methanol was added as a control. A 4-mm mycelial plug was cut from the edge of a fresh *F. graminearum* colony and placed in the center of the HSAF-amended plates. The plates were incubated at 28°C for 3 days. There were three replicates in the experiment, and the experiment was repeated three times. The formula for calculating the percent inhibition was as follows:

$$\text{inhibition}(\%) = (1 - T_d/C_d) \times 100$$

where T_d represents the diameter of the growth zone in the HSAF treatment plate (mm) and C_d was the diameter of mycelium in the control plate (mm). The EC_{50} values (the concentration of inhibiting 50% of mycelial growth) were calculated using probit-log analysis.

To investigate the effect of HSAF on mycelial morphology, *F. graminearum* was incubated on YEPD medium with 0, 2, 4, 6, 8, and 10 $\mu\text{g}/\text{ml}$ concentrations of HSAF at 28°C on a shaker plate at 220 rpm for 24 h, and the mycelium was subsequently transferred onto a glass slide. The morphology of mycelia was examined using the DIC mode of an OLYMPUS-IX71 microscope (Tokyo, Japan) at 40 $\times$ magnification.

Conidial germination and conidiation assay. The inhibitory activity of HSAF on conidial germination and germling elongation of *F. graminearum* was evaluated by microscopic observation. HSAF was dissolved in methanol and diluted into serial concentrations. Different concentrations of HSAF solutions prepared at a volume of 5 μl were added into conidial suspension (1×10^5 conidia/ml) of *F. graminearum*. Next, 500 μl of conidial suspension containing 0.2 to 2 $\mu\text{g}/\text{ml}$ of HSAF was incubated at 28°C for 2, 4, 6, 8, and 10 h, and a conidial suspension containing a corresponding volume of methanol used as the control was incubated 2, 4, 6, 8, and 10 h. The experiments were repeated three times. Germination and conidial morphology of each treatment were examined by microscopy using the methods described above.

For the conidiation assay, five mycelial plugs (4 mm in diameter) from the edge of the 3-day-old colony were inoculated into CMC medium containing 0.2 to 10 $\mu\text{g}/\text{ml}$ of HSAF. Three replicates of CMC medium containing each concentration of HSAF were performed. After incubation at 28°C in a rotary shaker at 180 rpm for 4 days, the number of conidia in each treatment group was determined using a hemocytometer and microscope (OLYMPUS-IX71) at 40 $\times$ magnification.

Transmission electron microscopy (TEM) observation. Five 4-mm mycelial plugs from the edge of a 3-day-old colony were inoculated on YEPD medium with or without 5 $\mu\text{g}/\text{ml}$ of HSAF and incubated at 28°C and 180 rpm for 24 h. Mycelial samples for each

treatment were collected and fixed with 2% glutaraldehyde in 0.1 M, pH 7.0 sodium phosphate buffer (SPB) overnight at 4°C. After fixation, the samples were thoroughly rinsed with SPB (0.1 M, pH 7.0) three times and then transferred into 1% (m/v) osmium tetroxide in SPB for 2 h. Subsequently, the mycelia were rinsed with SPB three additional times. After that step, the mycelial samples were dehydrated in serial ethanol solutions (30, 50, 70, 80, 90, and 95%) and polymerized overnight with Lowicryl K4M hypothermy embedding medium. Blocks were cut into 70-nm slices with an ultramicrotome (LEICA EM UC7) and stained for 5 min with lead citrate and uranyl acetate saturated solution. Finally, the samples were observed with a Hitachi H-7650 TEM.

Field trials. The field trials were conducted in Nanjing, Jiangsu province, China. The experiments were performed as a randomized complete block design with 1-m separation between plots. In each experiment, there were four replicated plots (4 $\times$ 6 m) per treatment. The Chinese common wheat cultivar Yangmai 158, which is *F. graminearum* susceptible, was planted to test the efficacy of HSAF. Carbendazim, which is a widely used fungicide in China to control wheat FHB, was used as the positive control, and the application of water was the negative control. HSAF was dissolved in methanol, and the solution was added to 0.1% Tween 80 aqueous solution to obtain final concentrations of 10, 20, and 40 $\mu\text{g}/\text{ml}$. At anthesis (Feekes stage 10.5.1), wheat spikes were sprayed with different concentrations of HSAF (1,000 ml/hm²), carbendazim (50% wettable powder; Jiangsu Lanfeng Biochemical Co., 638 g of active ingredient/hm²), or sterile distilled water. The treatment sprays were applied with a heavy-duty Jacto HD400 backpack sprayer (Agrolex, Singapore) with a flow rate of 710 ml/min. After 7 days, all treatments were performed for the second time. The experimental site was naturally infected. After 2 weeks of the final treatments, wheat heads were collected from five sampling locations in each field plot, and 100 heads of wheat were collected at each sampling location. Finally, the disease incidence (percentage of diseased heads), disease index (DI), and control efficacy were calculated (Hu et al. 2014). DI in wheat heads was assessed with five evaluation classes according to the percentage of wheat head surface showing FHB symptoms (0, 0%; 1, 1 to 25%; 2, 26 to 50%; 3, 51 to 75%; and 4, >75%). DI of each plot was calculated using the following formula: $[(\sum \text{number of wheat heads in each class} \times \text{class number}) / (\text{total number of wheat heads} \times 4)] \times 100$. The control efficacy of each treatment against FHB in the field was determined by applying the following formula: $[(\text{DI of the water control} - \text{DI of the treatment}) / \text{DI of the water control}] \times 100\%$. The data of disease incidence, DI, and control efficacy were analyzed with variance analysis by Tukey's test at $\alpha = 0.05$ to determine whether the fungicides had a significant effect on controlling FHB.

Statistical analysis. Data were expressed as the mean $\pm$ standard error from three or four replicates per treatment. Differences among different treatments were determined by one-way analysis of variance with Tukey's test at the 0.05 level using SPSS version 25.0.

Results

In vitro effect on mycelial growth, conidial germination, and conidiation of *F. graminearum*. The antifungal activity against *F. graminearum* of different concentrations of HSAF was investigated.

Fig. 1. Chemical structure of heat-stable antifungal factor.

Fig. 2. Inhibitory effect of heat-stable antifungal factor (HSAF) on mycelial growth of *Fusarium graminearum*. **A**, Dose-dependent inhibition of HSAF on mycelial growth. **B**, Diameter of the hyphae in the presence of HSAF, corresponding to **A**. Different letters indicate statistically significant differences according to Tukey's test ($\alpha = 0.05$).

Fig. 3. Inhibitory activity of heat-stable antifungal factor (HSAF) on conidial germination and germling elongation of *Fusarium graminearum*. **A**, Effect of HSAF on the percentage of conidial germination. **B**, Abnormal morphology of germlings after HSAF treatment (from left to right: control, 0.2, 0.4, 0.6, 0.8, 1, and 2 µg/ml of HSAF).

The results showed that the colony diameter of *F. graminearum* decreased significantly with HSAF treatment compared with the negative control. HSAF possessed dose-dependent activity against *F. graminearum* mycelial growth with an EC_{50} value of $4.1 \mu\text{g/ml}$ ($Y = 1.7079X + 3.9597$, $R = 0.9917$) (Fig. 2).

HSAF inhibited conidial germination of *F. graminearum*. Conidia did not start to germinate when incubated at 28°C for 2 h in the assay of the control and HSAF treatment. The control conidia germinated completely after incubation at 28°C for 6 h. Germination was

inhibited and delayed in the presence of 0.2, 0.4, 0.6, or $0.8 \mu\text{g/ml}$ of HSAF, and germination of 100% of conidia happened after 8 h. Moreover, application of 1 or $2 \mu\text{g/ml}$ of HSAF completely prevented conidial germination (Fig. 3A). HSAF inhibited the elongation of germings and resulted in more germination spots and germ tube branching (Fig. 3B).

Conidiation was inhibited in the HSAF treatment, which showed that the number of conidia was significantly decreased in the presence of $0.2 \mu\text{g/ml}$ and higher concentrations of HSAF. When

Fig. 4. Conidial production on carboxymethylcellulose medium with different concentrations of heat-stable antifungal factor (HSAF). Each value represents the means of three independent experiments $\pm$ standard deviation. Different letters indicate statistically significant differences according to Tukey's test ($\alpha = 0.05$).

Fig. 5. Changes in the hyphal morphology of *Fusarium graminearum* with heat-stable antifungal factor (HSAF): **A**, control; **B**, $0.2 \mu\text{g/ml}$ of HSAF; **C**, $0.4 \mu\text{g/ml}$ of HSAF; **D**, $0.6 \mu\text{g/ml}$ of HSAF; **E**, $0.8 \mu\text{g/ml}$ of HSAF; and **F**, $1.0 \mu\text{g/ml}$ of HSAF.

6 $\mu\text{g/ml}$ of HSAF was applied, the production of conidia was completely inhibited (Fig. 4).

Changes in hyphal morphology and ultrastructure of *F. graminearum*. *F. graminearum* mycelia without HSAF treatment (control) displayed normal morphology with linear, regular, and smooth hyphae (Fig. 5A). In the presence of 2 and 4 $\mu\text{g/ml}$ of HSAF, the hyphae were distorted, and 6 $\mu\text{g/ml}$ of HSAF treatment resulted in apex branching (Fig. 5B, C, and D). With 8 and 10 $\mu\text{g/ml}$ of HSAF treatment, the apices of hypha showed multiple ramifications and swelling (Fig. 5E and F).

TEM observation demonstrated that the cell wall and plasma membranes of untreated hyphae were normal and had a uniform organization (Fig. 6A and B). By contrast, treatment with HSAF (5 $\mu\text{g/ml}$) resulted in depolarized hyphal growth and plasmolysis, as well as cell wall thickening (Fig. 6C and D).

Efficacy of HSAF for reducing FHB in wheat. After treatment with different concentrations of HSAF and two control agents, plots

were rated for the disease incidence, DI, and control efficacy. All HSAF treatments significantly reduced the disease incidence and DI compared with the water treatment, especially at higher HSAF concentration. The application of 20 and 40 $\mu\text{g/ml}$ of HSAF was equal to the 50% carbendazim treatment in decreasing disease incidence and DI. However, 10 $\mu\text{g/ml}$ of HSAF was not as effective as 50% carbendazim in reducing disease incidence and DI. The control efficacy of 10 $\mu\text{g/ml}$ of HSAF, 20 $\mu\text{g/ml}$ of HSAF, 40 $\mu\text{g/ml}$ of HSAF, and 50% carbendazim were 63.32, 74.58, 80.32, and 74.02%, respectively. The results indicated that 40 $\mu\text{g/ml}$ of HSAF significantly improved control efficacy against FHB compared with the 50% carbendazim. There was no significant difference in control effect between 20 $\mu\text{g/ml}$ of HSAF and 50% carbendazim, but the effectiveness of 10 $\mu\text{g/ml}$ of HSAF was lower than that of 50% carbendazim (Table 1).

Fig. 6. Transmission electron micrographs for the hyphae of *Fusarium graminearum* with or without heat-stable antifungal factor (HSAF). **A and B**, Control; **C and D**, 5 $\mu\text{g/ml}$ of HSAF. **A** and **C** show a longitudinal section through the hyphae, and **B** and **D** show a tangential section through the hyphae. CM = cell membrane, and CW = cell wall. The arrow at the bottom right in **D** indicates plasmolysis.

Table 1. Evaluation of heat-stable antifungal factor (HSAF) for control of *Fusarium* head blight (FHB) in field experiments in Nanjing on *Fusarium graminearum*-susceptible wheat cultivar Yangmai 158^w

Treatment	Disease incidence (%) ^x	Disease index ^y	Control efficacy (%) ^z
Water	23.30 a	12.64 a	—
50% carbendazim	8.90 c	3.29 c	74.02 b
10 µg/ml of HSAF	12.20 b	4.65 b	63.23 c
20 µg/ml of HSAF	8.90 c	3.21 c	74.58 b
40 µg/ml of HSAF	6.70 c	2.49 c	80.32 a

^w Within columns, means followed by different letters differ significantly (Tukey's test, $\alpha = 0.05$).

^x Disease incidence (%) represents the percentage of diseased heads.

^y Disease index was assessed with five evaluation classes according to the percentage of wheat head surface showing FHB symptoms.

^z Control efficacy (%) was calculated from disease index.

Discussion

FHB is a highly destructive disease of wheat and barley, causing major yield and quality reductions across the world. Contamination of food and feeds with the mycotoxin DON causes a health risk to both humans and animals. Fungicide application is one of the strategies available for management of FHB and DON. However, because DMI and MBC fungicides have been used extensively and widely, resistant *Fusarium* isolates have been discovered in several countries (Becher et al. 2010; Liu et al. 2010; Spolti et al. 2014; Talas and McDonald 2015; Yin et al. 2009). For instance, JS399-19 (2-cyano-3-amino-3-phenylacrylic acetate, phenamacril) was a novel and promising cyanoacrylate fungicide developed in China to manage FHB. JS399-19 exhibited specific activity against fungal plant pathogens of the genus *Fusarium* by strongly inhibiting mycelial growth (Li et al. 2008). The fungicide exhibited excellent efficacy in controlling FHB in field trials by targeting myosin I of *F. graminearum* (Zhang et al. 2015). However, a major gene controlled the resistance to JS399-19 in *F. graminearum*, which makes resistance appear quicker under high selection pressure of the fungicide (Chen et al. 2009). *F. graminearum* isolates resistant to JS399-19 were quickly found because this fungicide was extensively used in China (Chen and Zhou 2009). The discovery of resistant strains indicates a high possibility for fungicide resistance development and brings a new challenge in the management of FHB and DON using chemical methods. Clearly, these situations underscore the need for developing novel chemicals to combat FHB, but with modes of action different from that of presently used fungicides.

Our results suggested that HSAF strongly suppressed mycelial growth, conidiation, and conidial germination of *F. graminearum*. Ascospores, macroconidia, chlamydospores, and mycelium fragments of *F. graminearum* all can be inoculum for FHB infection. *F. graminearum* survives in soil or crop debris in the form of asexual macroconidia and starts new infection cycles in the spring (Bai and Shaner 2004). The study indicated that HSAF not only could affect infection on wheat heads by inhibiting mycelium growth and conidial germination but also might translate to reducing production of macroconidia in crop debris. In addition to the inhibitory activity, HSAF also led to the malformation of mycelia for *F. graminearum*, such as mycelial curving, swelling, and branching. We discovered plasmolysis in the hyphal cell, which was considered to be the result of intracellular osmotic pressure changes. The result indicated that HSAF affected the permeability of the cytomembrane. We observed that HSAF caused depolarized hyphal growth and cell wall thickening of *F. graminearum*. A previous study suggested that HSAF-induced depolarized hyphal growth and cell wall thickening are related to sphingolipid synthesis in *A. nidulans* (Li et al. 2006, 2009). However, the molecular and cellular mechanisms of HSAF against fungi are still not well understood. DMI fungicides disrupt the cell membrane by obstructing the biosynthesis of sterol (Odds et al. 2003). MBC fungicides damage microtubules by interacting

with β -tubulin, of which β 1-tubulin is preferred to β 2-tubulin (Zhou et al. 2016). The mode of action of HSAF is speculated to be different from the existing fungicides for FHB but needs further study.

The in vivo activity of HSAF against *F. graminearum* has never been reported. In this study, we conducted field experiments, which indicated that the control efficacy of HSAF was dose dependent. Application of 40 µg/ml of HSAF increased control efficacy significantly compared with 50% carbendazim but showed similar disease incidence and DI. Therefore, we are unable to conclude that 40 µg/ml of HSAF exhibited better effects on FHB control than 50% carbendazim. The control efficacy of HSAF against FHB in the field needs more exploration, including the determination of an effective concentration, formulation, spray time, and so on. Fungicides developed for FHB control should reduce the level of disease in the field and decrease the accumulation of DON. However, the effect of HSAF on DON concentration has not been investigated.

Strategies used for managing FHB and mycotoxins include resistant cultivars, biological control, crop rotation, tillage, fungicide applications, and disease forecasting. Integrating two or more management strategies is a more effective approach to control FHB and mycotoxins (Shah et al. 2018). Although the use of biological control agents has been reported effective in the laboratory and greenhouse, their commercialization is still in slow progress. Two fungicides, pydiflumetofen and fludioxonil, which were developed by Syngenta as FHB management options in the succinate dehydrogenase inhibitor class of fungicides, have been screened and evaluated for their FHB efficacy and fungicidal activity recently (Hou et al. 2017; Qiu et al. 2018) but have yet to be widely adopted commercially. The in vitro assay and field trials of HSAF showed its strong activity against *F. graminearum*, which provided insight into the development of novel fungicides for control of FHB on wheat. For the commercialization of HSAF, it will be further evaluated as a fungicide or in combination with other integrated management tools including current fungicides against FHB. Its efficacy against other *Fusarium* species, manufacturing method, physical and chemical properties, formulation, and safety for mammals and the environment will also be studied.

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