Effects of the Novel Fungicide Benzothioestrobin on *Sclerotinia sclerotiorum* in the Laboratory and on Sclerotinia Stem Rot in Rape Fields

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Abstract

Xu, C., Liang, X., Hou, Y., and Zhou, M. 2015. Effects of the novel fungicide benzothioestrobin on *Sclerotinia sclerotiorum* in the laboratory and on Sclerotinia stem rot in rape fields. Plant Dis. 99:969-975.

We determined the effects and efficacy of benzothioestrobin, a new strobilurin-derived fungicide, against the plant-pathogenic fungus *Sclerotinia sclerotiorum* (the causal agent of Sclerotinia stem rot). Mycelial growth and sclerotial germination in vitro were strongly inhibited by benzothioestrobin in the presence of salicylhydroxamic acid. On detached rapeseed leaves, benzothioestrobin at 40 µg/ml reduced lesion development by 87%. No cross-resistance was detected between

benzothioestrobin and carbendazim, iprodione, fludioxonil, or boscalid. A formulated mixture of benzothioestrobin and fluazinam at 1:1 had synergistic activity against *S. sclerotiorum* in vitro. In field trials, benzothioestrobin alone or formulated with fluazinam at 1:1 (150 g a.i. ha⁻¹) was significantly ($P < 0.05$) superior to iprodione in controlling Sclerotinia stem rot of rapeseed. These results suggest that benzothioestrobin has substantial potential for the control of Sclerotinia stem rot.

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic plant pathogen with a worldwide distribution and a broad host range. The fungus can infect >405 plant species, including economically important crops such as rapeseed, sunflower, lettuce, and soybean. These and other crops routinely suffer substantial losses due to *S. sclerotiorum* (4,7). The fungus causes Sclerotinia stem rot of rapeseed, which is a very damaging disease that occurs in all rapeseed-growing areas in China and especially in the area of the Yangtze River. The average incidence of Sclerotinia stem rot of rapeseed in this region was estimated at 10 to 20% but can be as high as 80% (17).

At present, application of fungicides is the principal tool for controlling Sclerotinia diseases on most crops (5,27). However, the extensive use of benzimidazole fungicides (carbendazim) and dicarboximide fungicides (iprodione or dimethachlon) has selected for resistant individuals and resulted in control failures (20,25,36). Therefore, it is necessary to find more effective fungicides with contrasting modes of action to replace the compounds that are currently used to control Sclerotinia stem rot. It is also important to screen mixtures of fungicides with different modes of action in order to minimize selection for fungicide resistance and to increase disease control. In the present study, we investigated the potential of a new fungicide, benzothioestrobin, for the control of *S. sclerotiorum*.

The strobilurin fungicides are a class of fungicidal compounds modeled after natural compounds isolated from several Basidiomycetes species that inhabit decaying plant material in woodland soils (3). Their mode of action is to inhibit electron transport between cytochrome *b* and cytochrome *c1* in the mitochondrial respiratory chain, leading to disruption of the production of ATP (3,6). Benzothioestrobin [(E)-2-[2-(5-methoxy-benzothiazol-2-methylthio)phenyl]-3-methoxyacrylate] (Fig. 1; development code number: Y5247) is a novel strobilurin fungicide that was developed by the Key Laboratory of Pesticide & Chemical Biology of Ministry of Education, Central China Normal University. Our previous study showed that benzothioestrobin had a strong antifungal activity against *S. sclerotiorum* in vitro (34).

In the current study, we determined how benzothioestrobin performed when combined with fluazinam. Fluazinam is a protective fungicide and belongs to the phenylpyridinamine group of chemicals. It has a broad spectrum of activity and is effective against diseases caused by species of *Sclerotinia*, *Botrytis*, *Alternaria*, and *Phytophthora* (2).

To our knowledge, the use of benzothioestrobin alone or in combination with fluazinam for control of Sclerotinia stem rot has not been previously reported. Therefore, the objectives of this study were to (i) obtain more information concerning how benzothioestrobin inhibits *S. sclerotiorum*, (ii) determine whether there is cross-resistance between benzothioestrobin and other fungicides, (iii) determine the optimum ratio of benzothioestrobin and fluazinam for inhibiting mycelial growth of *S. sclerotiorum*, and (iv) determine the efficacy of benzothioestrobin alone and in combination with fluazinam for controlling Sclerotinia stem rot of rapeseed in the field.

Materials and Methods

Fungicides, media, and isolates. Stock solutions of the following fungicides were prepared by dissolving them in methanol to 10⁴ or 10⁵ µg/ml: technical-grade benzothioestrobin (a.i. 97%; Central China Normal University), azoxystrobin (a.i. 97.8%; Syngenta China Co., Ltd., China), fluazinam (a.i. 97.5%; Suli Chemical Co., Ltd., China), iprodione (a.i. 96.2%; Wenzhou Pesticide Factory, China), fludioxonil (a.i. 97.9%; Syngenta Biotechnology Co. Ltd.), boscalid (a.i. 98.4%; BASF Co., China) and salicylhydroxamic acid (SHAM) (a.i. 99%; Sigma China Co., Ltd., China). Technical-grade carbendazim (MBC) (a.i. 98%; Shenyang Study Institute of Chemical Industry, China) was dissolved in 0.1 N HCl to make a stock solution of 10⁴ µg/ml. Iprodione (50% WP), fluazinam (40% SC), and benzothioestrobin (5% EC) were purchased in Nanjing. The fungicides were stored at 4°C in the dark.

Alkyl ester agar (AEA) medium contained 5 g of yeast extract, 6 g of NaNO₃, 1.5 g of KH₂PO₄, 0.5 g of KCl, 0.25 g of MgSO₄, 20 ml of glycerin, and 20 g of agar per liter of distilled water. Alkyl ester broth (AEB) medium was identical to AEA medium except that it lacked agar. Potato dextrose agar (PDA) was prepared with 200 g of potato, 20 g of dextrose, and 20 g of agar per liter of distilled water.

All 24 isolates of *S. sclerotiorum* used in this study were collected from oilseed rape fields in Jiangsu Province of China or were derived from isolates collected from field. Their relevant characteristics are listed in Table 1. The isolates that were collected from field were tested for sensitivity to MBC on PDA by the discriminatory dosage

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Accepted for publication 10 January 2015.

method (18,19); isolates that showed normal growth with MBC at 5 µg/ml were considered resistant to MBC, while isolates that were completely inhibited were considered sensitive to MBC (Table 1). The following fungicide-resistant isolates, which were collected from the field or induced in our laboratory, were used to determine cross-resistance: JK19R1, JK19R2, and NT16R1 are resistant to boscalid (32); SZ10R and HA61R are resistant to fludioxonil (9,14); ZJ288-1 and CZ138-1 are resistant to iprodione (31); and HA4D and XH17D are resistant to MBC (collected from field). The others were used to determine the inhibitory activity of benzothiofostrobin or synergistic interaction of benzothiofostrobin and fluzazinam in mixture. These isolates were maintained on PDA slants in airtight centrifuge tubes at 4°C.

Mycelial growth of *S. sclerotiorum* as affected by benzothiofostrobin. The effect of benzothiofostrobin on in vitro mycelial growth of *S. sclerotiorum* was determined on agar and in shake culture. The experiment on agar used isolates WX7S, XH1S, SA2D, and HA22D. Plugs (5 mm in diameter) from the leading edge of an actively growing colony were transferred to a series of AEA plates containing benzothiofostrobin at 0, 0.0078, 0.015625, 0.03125, 0.0625, 0.125, 0.25, or 0.5 µg/ml. The plates were also amended or not amended with SHAM at 50 µg/ml; SHAM was included in order to inhibit the alternative respiratory pathway (23,31). This experiment also included azoxystrobin as a standard, with and without SHAM, at the same concentrations used for benzothiofostrobin. Azoxystrobin was used as a standard because a previous study showed that it is extremely effective against *S. sclerotiorum* (10). Each treatment was represented by three replicate plates. Colony diameters were measured after 2 days of incubation at 25°C in

darkness. Growth inhibition, expressed as a percentage of the control (plates without fungicide), was calculated. The median effective benzothiofostrobin and azoxystrobin concentrations (EC₅₀ values) were calculated based on linear regression of colony diameter on log-transformed fungicide concentration (28). The experiment was performed twice.

The experiment in shake culture (broth) used three randomly selected *S. sclerotiorum* isolates: NT41S, LYG112S, and SA2D. Ten mycelial plugs (5 mm in diameter) were transferred to a 250-ml conical flask containing 100 ml of AEB with benzothiofostrobin at 0.015 µg/ml and SHAM at 50 µg/ml or with azoxystrobin at 0.015 µg/ml (as a standard) and SHAM at 50 µg/ml. Cultures without benzothiofostrobin or azoxystrobin but with SHAM were used as the control. After the flasks were shaken at 175 rpm and 25°C for 3 days, the mycelia were harvested, washed three times with distilled water, dried at 80°C for 8 h, and weighed. The percentage of growth inhibition was determined based on mycelial dry weight relative to that of the control. Each treatment was represented by three replicate flasks. The experiment was performed twice.

Sclerotial germination as affected by benzothiofostrobin. Sclerotia were produced by growing isolates SA2D and LYG112S on PDA at 20°C. After 2 weeks, the sclerotia were gently harvested, surface disinfested in 0.5% NaOCl for 3 min, rinsed three times with sterile distilled water, and dried with sterilized filter paper at room temperature. Then, sclerotia (3 to 5 mm in diameter) were imbedded in the agar in 9-cm-diameter petri dishes (five sclerotia per dish) containing 20 ml of AEA with benzothiofostrobin or azoxystrobin (as a standard) at 0, 0.5, 1, 2, or 5 µg/ml and with SHAM at 50 µg/ml. Four of the sclerotia in each dish were spaced at equal intervals along the edge of the agar, and one was placed in the center. Twenty sclerotia were used in each treatment. Each treatment was represented by three replicate groups. The dishes were incubated at 25°C. Sclerotia were examined under a light microscope for myceliogenic germination and hyphal growth after 3, 4, and 5 days. Sclerotia that produced hyphae ≥2 mm long were considered to have germinated. Inhibition of sclerotial germination was calculated as previously described (13). The experiment was performed twice.

Efficacy of benzothiofostrobin on rapeseed leaves. Benzothiofostrobin inhibition of *S. sclerotiorum* on rapeseed leaves was determined using previously described methods (15,31). Fully expanded leaves of the same age and area were excised from greenhouse-grown oilseed rape plants ('Ningyou 16' from Jiangsu Fengqing Seed Science Company). The leaves were surface disinfested in 0.5% sodium hypochlorite for 1 min, rinsed three times with sterile distilled water, blotted with sterile filter paper to remove excess water, and allowed to dry. The benzothiofostrobin stock solution was diluted with distilled water containing 0.1% Tween 80 to obtain benzothiofostrobin at 0, 5, 10, 20, and 40 µg/ml; these solutions were applied to the leaves as a spray. Azoxystrobin at 20 µg/ml was included as a standard. Mycelial plugs (5 mm in diameter, from PDA plate) of *S. sclerotiorum* isolate YZ6S were placed on detached leaves when the leaves were dry. The leaves were maintained on moistened filter papers in a 20-cm-diameter petri dish, and the dishes were kept at 25°C with a 16-h photoperiod and 80% relative humidity. After 3 days, lesion size was determined by taking the mean of two diameters that were measured at a right angle to each other. Disease control was calculated as follows (14): disease control = (lesion diameter in the water control – lesion diameter in the treatment)/lesion diameter in the water control × 100. Each treatment was represented by 10 replicate leaves, and the experiment was performed three times.

Cross-resistance. To determine cross-resistance between benzothiofostrobin and fungicides that have been extensively used to control *Sclerotinia* stem rot in China, wild-type fungicide-sensitive isolates of *S. sclerotiorum* and isolates resistant to iprodione (a dicarboximide fungicide), fludioxonil (a phenylpyrrole fungicide), boscalid (a carboxamide fungicide), or carbendazim (a benzimidazole fungicide) were assessed for sensitivity to benzothiofostrobin. The Fungicide Resistance Action Committee rates these as medium to high resistance risk and places them in resistance groups 2 for iprodione, 12

Fig. 1. Chemical structure of benzothiofostrobin.

Table 1. Isolates of *Sclerotinia sclerotiorum* used in this study

Isolate	Characteristic	Source of material
WX7S	Wild type	Oilseed rape field in Jiangsu Province
XH1S	Wild type	Oilseed rape field in Jiangsu Province
SA2D	MBC resistant	Oilseed rape field in Jiangsu Province
HA22D	MBC resistant	Oilseed rape field in Jiangsu Province
NT41S	Wild type	Oilseed rape field in Jiangsu Province
LYG112S	Wild type	Oilseed rape field in Jiangsu Province
YZ6S	Wild type	Oilseed rape field in Jiangsu Province
JK19	Wild type	Oilseed rape field in Jiangsu Province
NT16	Wild type	Oilseed rape field in Jiangsu Province
JK19R1	Boscalid resistant	Induced in laboratory
JK19R2	Boscalid resistant	Induced in laboratory
NT16R1	Boscalid resistant	Induced in laboratory
SZ10S	Wild type	Oilseed rape field in Jiangsu Province
HA61S	Wild type	Oilseed rape field in Jiangsu Province
SZ10R	Fludioxonil resistant	Induced in laboratory
HA61R	Fludioxonil resistant	Induced in laboratory
ZJ288-1	Iprodione resistant	Induced in laboratory
CZ138-1	Iprodione resistant	Induced in laboratory
HA4D	MBC resistant	Oilseed rape field in Jiangsu Province
XH17D	MBC resistant	Oilseed rape field in Jiangsu Province
WX1S	Wild type	Oilseed rape field in Jiangsu Province
XH8D	MBC resistant	Oilseed rape field in Jiangsu Province
YZ10S	Wild type	Oilseed rape field in Jiangsu Province
NY28S	Wild type	Oilseed rape field in Jiangsu Province

for fludioxonil, 7 for boscalid, 9 for cyprodinil, and 1 for carbendazim (11). Mycelial plugs (5 mm in diameter) were transferred from the margins of PDA colonies to PDA or AEA plates containing a series of concentrations of iprodione, fludioxonil, boscalid, carbendazim, or benzothiothiobin. Each concentration of each fungicide was represented by three replicate plates, and the experiment was performed three times. The plates were incubated for 2 to 3 days at 25°C in the dark, and the EC₅₀ values were determined as described above.

Synergistic in vitro inhibition of *S. sclerotiorum* by mixtures containing different proportions of benzothiothiobin and fluazinam. Mycelial plugs (5 mm in diameter) were removed from the edge of a 2-day-old colony of isolate WX1S growing on PDA. The plugs were placed face up in the center of a petri dish (9 cm in diameter) containing AEA with benzothiothiobin alone, fluazinam alone, or mixtures containing various concentrations of both fungicides. The mixtures were prepared at ratios (benzothiothiobin/fluazinam [vol/vol]) of 6:1, 4:1, 2:1, 1:1, 1:2, 1:4, and 1:6. The solutions were then diluted to obtain a series of concentrations. The final concentrations for fluazinam alone were 0, 0.00625, 0.0125, 0.025, 0.05, and 0.1 µg/ml. The final concentrations for benzothiothiobin alone or in mixture with fluazinam were 0, 0.0039, 0.0078, 0.015625, 0.03125, 0.0625, and 0.125 µg/ml. SHAM at 50 µg/ml was also added to the AEA plates amended with benzothiothiobin or the mixtures including control. Each concentration of each treatment was represented by four replicate plates. After the plates were incubated for about 2 days at 25°C in the dark, the EC₅₀ values were determined as described earlier. The experiment was performed twice.

The synergy ratio (SR) of fungicide mixtures was calculated according to Wadley (29) and Gisi et al. (12). The EC_{50(th)} values, which are the theoretical EC₅₀ values of the mixtures, were calculated as follows: $EC_{50(th)} = (a + b)/(a/EC_{50(A)} + b/EC_{50(B)})$, where *A* and *B* represent the individual components of the mixture, and *a* and *b* represent the ratios of these components in the mixtures. SR was calculated as $SR = EC_{50(th)}/EC_{50(me)}$, where EC_{50(me)} values are the measured EC₅₀ values of the mixtures.

An SR <0.5 indicates antagonistic interactions between the fungicides in mixture, an SR between 0.5 and 1.5 indicates additive interactions, and an SR >1.5 indicates synergistic interactions.

Variation among isolates in their response to the optimal mixture of benzothiothiobin and fluazinam. The response of six *S. sclerotiorum* isolates from different areas of Jiangsu Province to the optimal mixture of benzothiothiobin and fluazinam (i.e., the mixture with the largest SR value as determined in the previous section) was determined. AEA medium was amended with benzothiothiobin alone, fluazinam alone, or the optimal mixture (which was 1:1; see Results) at a series of concentrations, as described in the previous section. SHAM at 50 µg/ml was added to the AEA plates amended with benzothiothiobin or the mixtures including control. EC₅₀ values were determined and SR values were calculated as described above. Each concentration was represented by four replicate plates, and the experiment was repeated.

Effect of the optimal mixture of benzothiothiobin and fluazinam on mycelial growth over time. Fresh mycelial plugs (5 mm diameter) from the edge of a 2-day-old colony (isolate WX1S) on PDA were transferred to AEA plates containing benzothiothiobin, fluazinam, or the optimal mixture at 0.01 µg/ml (1:1; see Results). SHAM

at 50 µg/ml was also added to all plates with benzothiothiobin and the mixture. Plates without fungicide or SHAM or with SHAM alone served as controls. After 12, 24, 48, or 54 h at 25°C, colony diameters (minus the diameter of the inoculation plug) were measured, and growth inhibition as a percentage of the controls was calculated. Each treatment was represented by four replicate plates, and the experiment was performed two times.

Control of Sclerotinia stem rot by benzothiothiobin alone and a 1:1 mixture of benzothiothiobin and fluazinam in the field. The control efficacy of different treatments with benzothiothiobin and fluazinam on Sclerotinia stem rot was evaluated under field conditions. The field trials were conducted in two oilseed rape fields (about 1 km away from each other) in Jiangyan, Jiangsu Province in 2014. The soil type in fields is classified as clay loam. Ningyou 16 oilseed rape is a hybrid of 'Yangyou 1' and 'Zhen 8901'. Planting date was in mid-September 2013 and planting density was 25,000 plants/acre.

Each field was divided into 33 plots; each plot was 4 by 5 m². Eleven treatments with three replicates were arranged in a completely randomized design. The treatments were (i) water control, 750 liters ha⁻¹ (ii); iprodione (50% WP), 150 g a.i. ha⁻¹; (iii) benzothiothiobin (5% EC), 75 g a.i. ha⁻¹; (iv) benzothiothiobin, 112.5 g a.i. ha⁻¹; (v) benzothiothiobin, 150 g a.i. ha⁻¹; (vi) fluazinam (40% SC), 75 g a.i. ha⁻¹; (vii) fluazinam, 112.5 g a.i. ha⁻¹; (viii) fluazinam, 150 g a.i. ha⁻¹; (ix) a 1:1 mixture of benzothiothiobin and fluazinam, 75 g a.i. ha⁻¹; (x) a 1:1 mixture of benzothiothiobin and fluazinam, 112.5 g a.i. ha⁻¹; and (xi) a 1:1 mixture of benzothiothiobin and fluazinam, 150 g a.i. ha⁻¹. Fungicides, which were applied as foliar sprays with a Jacto Heavy-Duty HD400 sprayer (Agrolex, Singapore), were applied twice, with 7 days between sprays, when 90% of the major stems were flowering. On 27 March 2014, the first fungicide application was conducted. No other fungicides were applied. About 1 week before harvest, disease severity was assessed in both experiments by examining 120 randomly selected plants in each plot for Sclerotinia stem rot. Severity was assessed using the following scale: 0 = no lesions, 1 = lesions on <5% of the stem, 3 = lesions on 6 to 15% of the stem, 5 = lesions on 16 to 30% of the stem, 7 = lesions on 31 to 50% of the stem, and 9 = lesions on >50% of the stem (30,35).

Statistical analysis. Statistical Package for the Social Science (SPSS 14.0; SPSS Inc., Chicago) and SAS 9.2 (SAS Institute, Cary, NC) were used for statistical analysis. The analysis of variance procedure of SPSS and Fisher's least significant difference (*P* = 0.05) were used to determine significant differences between treatments. Data of field test were analyzed nonparametrically using Proc Mixed of SAS, and relative treatment effects and their confidence intervals were calculated using the LD_CI macro (24). Cross-resistance between fungicides was analyzed using Spearman's rank correlation coefficient for the log-transformed EC₅₀ values.

Results

Sensitivity of mycelial growth of *S. sclerotiorum* to benzothiothiobin.

Based on EC₅₀ values, the inhibition of mycelial growth of four *S. sclerotiorum* isolates by benzothiothiobin and azoxystrobin (the standard) was greater with SHAM than without SHAM (Table 2). Among the four isolates, the EC₅₀ values of benzothiothiobin with SHAM ranged from 0.014 to 0.029 µg/ml, with a mean 0.021 µg/ml. The EC₅₀ values of azoxystrobin with SHAM ranged from 0.050 to 0.118 µg/ml, with a mean of 0.077 µg/ml. Although there was

Table 2. Effect of benzothiothiobin and azoxystrobin ± salicylhydroxamic acid (SHAM) on mycelial growth of *Sclerotinia sclerotiorum* on agar

Fungicide	EC ₅₀ (µg/ml) ^a			
	Isolate WX7S	Isolate XH1S	Isolate SA2D	Isolate HA22D
Benzothiothiobin (–SHAM)	0.360 ± 0.036 b	0.649 ± 0.010 a	0.249 ± 0.024 b	0.401 ± 0.049 b
Benzothiothiobin (+SHAM)	0.014 ± 0.001 c	0.026 ± 0.004 b	0.014 ± 0.001 c	0.029 ± 0.019 c
Azoxystrobin (–SHAM)	0.879 ± 0.189 a	0.664 ± 0.133 a	1.330 ± 0.452 a	0.949 ± 0.158 a
Azoxystrobin (+SHAM)	0.050 ± 0.002 bc	0.075 ± 0.008 b	0.065 ± 0.007 c	0.118 ± 0.040 c

^a EC₅₀ = effective concentration causing 50% mycelial growth inhibition. Values are means ± standard deviations. Means in a column followed by the same letter are not different according to Fisher's least significant difference test (*P* = 0.05).

no significant statistics difference between benzoethiostrobin and azoxystrobin on the EC₅₀ values against *S. sclerotiorum*, benzoethiostrobin was more effective on the inhibition of mycelial growth than azoxystrobin at the same concentration (Fig. 2A).

Benzoethiostrobin also greatly decreased the dry weight of mycelium of *S. sclerotiorum* isolates growing in AEB liquid medium (Fig. 2B and C). Inhibition in the liquid medium was greater with benzoethiostrobin than with azoxystrobin (Fig. 2C).

Effect of benzoethiostrobin on sclerotial germination. Benzoethiostrobin inhibition of germination of *S. sclerotiorum* sclerotia on agar increased with benzoethiostrobin concentration. The highest concentration tested (5 µg/ml) strongly inhibited sclerotial germination and mycelial growth from sclerotia (Table 3). Inhibition of sclerotial germination by benzoethiostrobin was comparable with that by azoxystrobin.

Efficacy of benzoethiostrobin on detached rapeseed leaves. The control of *S. sclerotiorum* on detached rapeseed leaves increased with the concentration of benzoethiostrobin (Table 4). At 20 µg/ml, benzoethiostrobin and azoxystrobin provided similar levels of control.

Cross-resistance. Depending on EC₅₀ values, all *S. sclerotiorum* strains which were sensitive or resistant to the commonly used fungicides iprodione, fludioxonil, boscalid, or carbendazim, were sensitive to benzoethiostrobin (Table 5). Spearman rank correlations for cross-resistance between benzoethiostrobin and other fungicides were analyzed. The values of Spearman's ρ (r_s) for iprodione, fludioxonil, boscalid, or carbendazim were 0.664, 0.435, -0.749, and 0.399, respectively ($P > 0.05$). Therefore, the sensitivities of *S. sclerotiorum* isolates to benzoethiostrobin were not positively correlated with their sensitivities to iprodione,

Fig. 2. Effect of benzoethiostrobin and azoxystrobin on mycelial growth of *Sclerotinia sclerotiorum*. Note that all results presented in this figure were derived with addition of salicylhydroxamic acid. **A**, Mycelial growth on agar. Fungicide concentrations (µg/ml) are listed along the top. Plates of a representative isolate (WX7S) were photographed after 2 days of culture. **B**, Mycelial growth in shake culture. Isolates are listed on the left and fungicide concentrations are listed at the top. Flasks were photographed after 3 days of culture. **C**, Inhibition of mycelial growth in shake culture. Fungicide rates were the same as in B. The inhibition rate is based on dry weight of the mycelium relative to that of the control. Values are means $\pm$ standard errors of two experiments. For a given isolate, bars with the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.05$).

Table 3. Inhibition of sclerotial germination of *Sclerotinia sclerotiorum* by benzoethiostrobin (Ben) and azoxystrobin (Azo)^z

Fungicide	Inhibition (%) of SA2D sclerotial germination			Inhibition (%) of LYG112S sclerotial germination		
	3 days	4 days	5 days	3 day	4 days	5 days
Ben (0.5 µg/ml)	78 $\pm$ 12 b	53 $\pm$ 12 c	14 $\pm$ 9 e	47 $\pm$ 12 cd	46 $\pm$ 11 c	18 $\pm$ 8 d
Ben (1 µg/ml)	100 a	85 $\pm$ 5 b	17 $\pm$ 4 de	68 $\pm$ 18 bc	49 $\pm$ 15 c	44 $\pm$ 10 c
Ben (2 µg/ml)	100 a	92 $\pm$ 8 ab	38 $\pm$ 13 c	85 $\pm$ 14 ab	75 $\pm$ 24 b	56 $\pm$ 10 bc
Ben (5 µg/ml)	100 a	100 a	80 $\pm$ 9 ab	100 a	95 $\pm$ 5 a	82 $\pm$ 4 a
Azo (0.5 µg/ml)	81 $\pm$ 12 b	64 $\pm$ 5 c	17 $\pm$ 4 de	39 $\pm$ 13 d	37 $\pm$ 12 c	16 $\pm$ 8 d
Azo (1 µg/ml)	93 $\pm$ 7 ab	81 $\pm$ 11 b	31 $\pm$ 13 cd	54 $\pm$ 19 cd	52 $\pm$ 14 c	49 $\pm$ 8 bc
Azo (2 µg/ml)	100 a	90 $\pm$ 10 ab	76 $\pm$ 6 b	85 $\pm$ 14 ab	78 $\pm$ 23 ab	60 $\pm$ 13 b
Azo (5 µg/ml)	100 a	100 a	93 $\pm$ 1 a	100 a	93 $\pm$ 7 ab	89 $\pm$ 4 a

^z Inhibition was assessed for two isolates (SA2D and LYG112S) after 3, 4, and 5 days on agar. All plates contained salicylhydroxamic acid at 50 µg/ml. Inhibition was calculated relative to control plates that did not contain Ben or Azo. Values are the means ($\pm$ standard deviations) of two independent experiments, each with three replications per treatment. Means in a column followed by the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.05$).

fludioxonil, boscalid, or carbendazim. In other words, cross-resistance between benzothiofuran and these other fungicides was not detected.

Synergistic interaction between benzothiofuran and fluazinam against isolate WX1S. Depending on the ratio of the fungicides in the mixture, inhibition of mycelial growth of isolate WX1S after 48 h on agar differed among mixtures of benzothiofuran and fluazinam (Table 6). The EC₅₀ value was numerically smallest, with the 1:1 ratio, although this value was not statistically different from that obtained with ratios of 6:1 (benzothiofuran/fluazinam), 4:1, or 2:1. The SR values indicated that inhibition by benzothiofuran/fluazinam at 4:1, 2:1, and 1:1 was synergistic. Mixtures at 6:1, 1:2 1:4, and 1:6 had additive effects (Table 6).

At a 1:1 ratio of benzothiofuran to fluazinam, which was the optimal ratio against isolate WX1S (Table 6), the EC₅₀ values of six *S. sclerotiorum* isolates ranged from 0.0034 to 0.0094 µg/ml, with a mean of 0.0062 µg/ml (Table 7). The SR values indicated that the interaction was synergistic for all six isolates of *S. sclerotiorum* (Table 7).

Inhibition of mycelial growth of isolate WX1S over time by a 1:1 mixture of benzothiofuran and fluazinam. The inhibition of mycelial growth was greater with benzothiofuran than with fluazinam at 0.01 µg/ml at 12 and 54 h. The inhibition was greater with the mixture than with benzothiofuran alone at 54 h. However, the mixture was less effective than benzothiofuran alone at 12 h. This may be because benzothiofuran worked earlier than fluazinam against *S. sclerotiorum* (Fig. 3).

Control of Sclerotinia stem rot by benzothiofuran alone and a 1:1 mixture of benzothiofuran and fluazinam in the field. Results from both field experiments were similar and have been independently presented (Table 8). The mean disease incidence in control plots were 43.7%, while application of benzothiofuran and the mixture at 150 g a.i. ha⁻¹ reduced the mean incidence by 54 and 69.9%, respectively (data not shown). The level of control

obtained with the 1:1 mixture at 150 g a.i. ha⁻¹ did not significantly differ from that obtained with benzothiofuran alone at 150 g a.i. ha⁻¹ ($P > 0.05$). Iprodione (the standard) also controlled the disease but not well as benzothiofuran alone and the mixture ($P < 0.05$).

Discussion

The fungus *S. sclerotiorum* that is the causative agent of Sclerotinia stem rot has developed resistance to benzimidazole and dicarboximide fungicides worldwide, and high levels of resistance to these fungicides had developed in the field (19,25). Because of these pesticide resistance problems, novel alternative fungicides should be used for managing Sclerotinia stem rot. Therefore, we determined the effects of the novel fungicide benzothiofuran on *S. sclerotiorum* in the laboratory and on Sclerotinia stem rot in rape fields.

When combined with SHAM in the current study, the novel fungicide benzothiofuran had higher activity against mycelial growth of *S. sclerotiorum* than the standard, azoxystrobin. Benzothiofuran also exhibited excellent antifungal activity on detached rapeseed leaves.

Because sclerotia of *S. sclerotiorum* serve as the primary survival structures and inoculum sources during seasonal crop infection cycles (1,26,33), sclerotia are crucial in the life cycle of *S. sclerotiorum*.

Table 6. Synergistic effects of mixtures of benzothiofuran (Ben) and fluazinam (Flu) on mycelial growth of *Sclerotinia sclerotiorum* (isolate WX1S) as indicated by EC₅₀ values^w

Fungicide	EC _{50(me)} (µg/ml) ^x	EC _{50(th)} (µg/ml) ^y	SR ^z
Ben alone	0.0111 ± 0.0008 cde	—	—
Flu alone	0.0129 ± 0.0030 cd	—	—
Ben:Flu (6:1)	0.0089 ± 0.0021 def	0.0113 ± 0.0004 a	1.2872 ± 0.2481 c
Ben:Flu (4:1)	0.0048 ± 0.0006 f	0.0113 ± 0.0002 a	2.3818 ± 0.3305 a
Ben:Flu (2:1)	0.0064 ± 0.0002 ef	0.0115 ± 0.0002 a	1.7883 ± 0.0898 b
Ben:Flu (1:1)	0.0042 ± 0.0001 f	0.0118 ± 0.0008 a	2.7735 ± 0.1404 a
Ben:Flu (1:2)	0.0149 ± 0.0034 bc	0.0121 ± 0.0014 a	0.8227 ± 0.0906 d
Ben:Flu (1:4)	0.0188 ± 0.0018 ab	0.0124 ± 0.0020 a	0.6553 ± 0.0453 d
Ben:Flu (1:6)	0.0226 ± 0.0052 a	0.0125 ± 0.0023 a	0.5561 ± 0.0264 d

^w Values are means (± standard deviations). Means in a column followed by different letters are not significantly ($P = 0.05$) different according to Fisher's least significant difference test; — indicates no data.

^x EC₅₀ = effective concentration causing 50% mycelial growth inhibition. EC_{50(me)} indicates the measured EC₅₀ values of the fungicides.

^y EC_{50(th)} indicates the theoretical EC₅₀ values of the mixtures.

^z Synergy ratio (SR) = ratio between expected and observed EC₅₀ values. SR = EC_{50 (th)}/EC_{50 (me)}. An SR < 0.5 indicates an antagonistic interaction between the fungicides, an SR between 0.5 and 1.5 indicates an additive interaction, and an SR > 1.5 indicates a synergistic interaction.

Table 4. Control of *Sclerotinia sclerotiorum* by benzothiofuran and azoxystrobin on detached rapeseed leaves^z

Treatment	Lesion diameter (cm)	Lesion area (cm ²)	Control (%)
Ben (5 µg/ml)	2.27 ± 0.14 ab	4.05 ± 0.50 b	31 ± 3 c
Ben (10 µg/ml)	1.87 ± 0.17 bc	2.75 ± 0.48 bc	53 ± 1 b
Ben (20 µg/ml)	1.51 ± 0.22 c	1.81 ± 0.53 cd	68 ± 14 ab
Ben (40 µg/ml)	0.98 ± 0.05 d	0.76 ± 0.07 d	87 ± 3 a
Azo (20 µg/ml)	1.69 ± 0.23 c	2.26 ± 0.61 cd	60 ± 17 b
0.1% Tween 80	2.73 ± 0.24 a	5.91 ± 1.01 a	...

^z Values are means (± standard deviations) of three experiments. Means followed by the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.05$).

Table 5. Sensitivity of four other fungicide-sensitive and -resistant *Sclerotinia sclerotiorum* strains to benzothiofuran^z

Isolate	EC ₅₀ (µg/ml)				
	Benzothiofuran	Iprodione	Fludioxonil	Boscalid	Carbendazim
JK19	0.0179 ± 0.0075 abc	0.2684 ± 0.0035 c	0.0146 ± 0.0011 c	0.2677 ± 0.0223 b	0.1534 ± 0.0047 b
NT16	0.0195 ± 0.0035 abc	0.3054 ± 0.0163 b	0.0189 ± 0.0014 b	0.2683 ± 0.0284 b	0.1565 ± 0.0504 b
SZ10S	0.0112 ± 0.0005 bc	0.1835 ± 0.0106 d	0.014 ± 0.0002 c	0.3247 ± 0.0520 b	0.1088 ± 0.0191 b
HA61S	0.0172 ± 0.0045 abc	0.2749 ± 0.0058 c	0.0092 ± 0.0016 d	0.2936 ± 0.0873 b	0.1044 ± 0.0132 b
JK19R1	0.0045 ± 0.0019 c	—	—	>30 a	—
NT16R1	0.0074 ± 0.0035 bc	—	—	>30 a	—
JK19R2	0.0103 ± 0.0042 bc	—	—	>30 a	—
ZJ288-1	0.0200 ± 0.0023 abc	>100 a	—	—	—
CZ138-1	0.0196 ± 0.0094 abc	>100 a	—	—	—
SZ10R	0.0122 ± 0.0007 bc	—	>100 a	—	—
HA61R	0.0323 ± 0.0248 a	—	>100 a	—	—
HA4D	0.0202 ± 0.0059 abc	—	—	—	>100 a
XH17D	0.0239 ± 0.0078 ab	—	—	—	>100 a

^z Values are means (± standard deviations) of three experiments. Means followed by the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.05$); — indicates no data.

Sclerotia germinate and form long-stalked apothecia. These apothecia release large numbers of ascospores, which are the main source of inoculum for infecting susceptible plants (22). However, the eruptive germination of sclerotia followed by mycelial growth also causes stem rot in rapeseed fields. Therefore, suppression of sclerotial germination and subsequent mycelial growth should reduce the ability of the pathogen to cause disease in the field. We found that benzothiofuran could inhibit sclerotial germination and that inhibition by benzothiofuran was comparable with inhibition by azoxystrobin. Higher concentrations of benzothiofuran were required to inhibit sclerotial germination than to inhibit mycelial growth. This is in agreement with many reports that indicated that sclerotia are

usually less sensitive than mycelium to fungicides (4). At lower concentrations, benzothiofuran inhibition of sclerotial germination but not of mycelial growth declined with time.

Generally, analysis of cross-resistance between a fungicide and other fungicides should use resistant mutants of the fungicide and the parent strains. However, our efforts to induce benzothiofuran-resistant mutants of *S. sclerotiorum* have been unsuccessful. Therefore, we used the resistant mutants and sensitive strains of other commonly used fungicides for our analysis of cross-resistance to benzothiofuran. No cross-resistance was detected, probably because benzothiofuran and the other commonly used fungicides have different modes of action

Table 7. Synergistic effects of a 1:1 mixture of benzothiofuran and fluazinam on mycelial growth of six isolates of *Sclerotinia sclerotiorum*^y

Isolate	EC ₅₀ (μg/ml) of benzothiofuran	EC ₅₀ (μg/ml) of fluazinam	EC ₅₀ (μg/ml) of the mixture	SR ^z
XH8D	0.0115 ± 0.0005	0.0153 ± 0.0006	0.0069 ± 0.0021	2.0048 ± 0.6933
YZ10S	0.0198 ± 0.0052	0.0159 ± 0.0010	0.0079 ± 0.0002	2.2080 ± 0.3925
NT28S	0.009 ± 0.0016	0.0095 ± 0.0008	0.0053 ± 0.0006	1.7252 ± 0.1189
LYG112S	0.0399 ± 0.0024	0.0143 ± 0.0008	0.0094 ± 0.0021	2.2951 ± 0.4643
SA2D	0.0079 ± 0.0023	0.0086 ± 0.0023	0.0034 ± 0.0002	2.4780 ± 0.8432
WX7S	0.0121 ± 0.0006	0.0101 ± 0.0006	0.0045 ± 0.0004	2.4434 ± 0.2559

^y EC₅₀ = effective concentration causing 50% mycelial growth inhibition. Values are means (± standard deviations).

^z Synergy ratio = ratio between expected and observed EC₅₀ values. An SR > 1.5 indicates a synergistic interaction.

Fig. 3. Inhibition of growth (%) of isolate WX1S in alkyl ester agar medium containing benzothiofuran alone at 0.01 μg/ml, fluazinam alone at 0.01 μg/ml, or a 1:1 mixture of benzothiofuran and fluazinam at 0.01 μg/ml. Salicylhydroxamic acid (SHAM) at 50 μg/ml was added to all treatments except the treatments of fluazinam. Inhibition was calculated relative to a control that contained SHAM or not but did not contain benzothiofuran or fluazinam. Values are means ± standard errors of two experiments. At each time, bars with different letters are significantly different according to Fisher's least significant difference test ($P = 0.05$).

Table 8. Median disease rating (MDR), mean rank (R_{ij}), and relative treatment effects ($\hat{p}_{ij}$) along with 95% confidence intervals (CI) for benzothiofuran (Ben) alone and a 1:1 mixture of Ben and fluazinam (Flu) on the control of *Sclerotinia* stem rot of rape in the field^y

Treatment ^z	Dosage (g a.i. ha ⁻¹)	Field 1				Field 2			
		MDR	R_{ij}	$\hat{p}_{ij}$	95% CI for $\hat{p}_{ij}$	MDR	R_{ij}	$\hat{p}_{ij}$	95% CI for $\hat{p}_{ij}$
5% Ben (EC)	75	1.00	21.67	0.64	(0.49, 0.77)	1.00	22.17	0.67	(0.47, 0.80)
5% Ben (EC)	112.5	0.50	16.33	0.48	(0.25, 0.72)	0.00	19.33	0.57	(0.39, 0.73)
5% Ben (EC)	150	0.00	13.33	0.39	(0.17, 0.69)	0.00	13.33	0.39	(0.15, 0.71)
40% Flu (SC)	75	1.00	18.67	0.55	(0.27, 0.79)	1.00	19.17	0.57	(0.26, 0.82)
40% Flu (SC)	112.5	0.50	16.33	0.48	(0.25, 0.72)	0.50	16.33	0.48	(0.24, 0.73)
40% Flu (SC)	150	0.00	13.33	0.39	(0.17, 0.69)	0.00	13.33	0.39	(0.15, 0.71)
Ben:Flu (1:1)	75	0.00	13.33	0.39	(0.17, 0.69)	0.00	13.33	0.39	(0.15, 0.71)
Ben:Flu (1:1)	112.5	0.00	8.00	0.23	(0.17, 0.31)	0.00	7.50	0.21	(0.16, 0.29)
Ben:Flu (1:1)	150	0.00	8.00	0.23	(0.17, 0.31)	0.00	7.50	0.21	(0.16, 0.29)
50% Ipr (WP)	150	1.00	26.33	0.78	(0.63, 0.87)	1.00	25.00	0.74	(0.65, 0.81)
Water (check)	...	3.00	31.67	0.94	(0.87, 0.95)	3.00	30.00	0.89	(0.61, 0.95)

^y MDR = severity of *Sclerotinia* stem rot assessed visually on an ordinal 0-to-9 scale, where 0 = no lesions and 9 = lesions on >50% of the stem.

^z Ipr = iprodione.

(16). Because of the limitation of resistant isolates provided by our lab, further work with a larger number of isolates is needed.

Fungicides may provide better disease control when used in combinations than alone (8,21). Azoxystrobin and thiram, for example, have synergistic effects against *S. sclerotiorum* (10). In this study, we found that a 1:1 mixture of benzothiofuran and fluzazinam resulted in a strong synergistic inhibition of mycelial growth of *S. sclerotiorum* isolates.

Management of insensitivity development depends on an integrated pest management (IPM) approach, including cultural practices and resistant cultivars as well as chemical and biological control. As an important approach of IPM, the reduction of selection pressure is accomplished by restricting the use of fungicides with the same or a similar mode of action, by alternating fungicides that differ in mode of action, and by using mixtures of fungicides which differ in mode of action. The results in this study showed that the application of benzothiofuran alone at 150 g a.i. ha⁻¹ or a 1:1 mixture of benzothiofuran and fluzazinam at 150 g a.i. ha⁻¹ was more effective than iprodione in controlling *Sclerotinia* stem rot in the field.

In conclusion, the current study demonstrates that benzothiofuran or the mixture of benzothiofuran and fluzazinam at the ratio of 1:1 greatly inhibits mycelial growth of *S. sclerotiorum* in vitro and provides good control of *Sclerotinia* stem rot in the field. We also found that benzothiofuran inhibits the germination of *Sclerotinia* in vitro and has no cross-resistance with other common fungicides. Therefore, benzothiofuran combined with fluzazinam or alternated with other fungicides will be a valuable tool for controlling *Sclerotinia* stem rot and for limiting the development of fungicide resistance.

Acknowledgments

This study was supported by the Commonweal Specialized Research Fund of China Agriculture (201303023) and the open fund of Key Laboratory of Pesticide and Chemical Biology of Ministry of Education (201301A03).

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