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In vitro inhibition of *Sclerotinia sclerotiorum* by mixtures of azoxystrobin, SHAM, and thiram

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ABSTRACT

The necrotrophic fungal phytopathogen *Sclerotinia sclerotiorum* (Lib.) de Bary has a broad host range and frequently causes destructive diseases. The extensive use of common fungicides to control these diseases has selected for resistance in populations of *S. sclerotiorum*. In this study, 105 isolates of *S. sclerotiorum* from different geographical regions in Jiangsu Province of China were characterized for baseline sensitivity to azoxystrobin, and the average EC_{50} value was 0.2932 μg/mL for mycelial growth. Of the mixtures of the fungicides thiram and azoxystrobin that were tested using an *in vitro* mycelial growth assay, the 1:4 ratio provided the greatest inhibition of *S. sclerotiorum*. When tested against nine isolates, the 1:4 mixture resulted in a mean synergy ratio of 2.31, indicating synergistic inhibition. Mycelial respiration was inhibited for about 2 h by azoxystrobin alone but for 48 h by the mixture of thiram and azoxystrobin. Salicylhydroxamic acid (SHAM, a known inhibitor of alternative respiration) also increased the inhibition of mycelial growth and respiration caused by azoxystrobin. These results suggest the need for further study of effects of combinations of azoxystrobin with thiram or SHAM in planta to evaluate their potential for management of diseases caused by *S. sclerotiorum*.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic, phytopathogenic, filamentous ascomycete with a broad host range and a worldwide distribution. Over 400 species of plants are susceptible to this pathogen, including many agronomic and horticultural crops [1–3]. The sclerotium of *S. sclerotiorum* is a pigmented, multicellular asexual resting structure composed of condensed vegetative hyphal cells that become interwoven and firm; sclerotia can remain viable for up to 8 years in soil [4–8]. The sclerotia are crucial in the life cycle of *S. sclerotiorum* because they serve as the primary survival structure and inoculum source during seasonal crop-infection cycles [8]. The survival of sclerotia is influenced by moisture, aeration, and temperature [9].

Owing to inadequate levels of host resistance, application of fungicides is the principal tool for controlling Sclerotinia diseases on most crops [10,11]. Unfortunately, the extensive use of a single fungicide selects for resistant individuals within a population and leads to control failures. In recent years, populations of *S. sclerotiorum* with resistance to benzimidazole fungicides (carbendazim, MBC) and dicarboximide fungicides (dimethachlon) have been frequently reported [12–15].

Selection for fungicide resistance can be minimized and disease control can be increased by using mixtures of fungicides with different modes of action. These fungicide mixtures are used to broaden the spectrum of activity of a compound or to achieve higher levels of activity by means of synergistic interaction [16]. Mixtures of two commercial fungicides, thiram and azoxystrobin, were investigated in the current study.

Thiram is one of the dithiocarbamate fungicides used for the control of plant diseases [17]. It is known to inhibit a number of sulfhydryl (SH) enzymes [18]. Unlike thiram, the strobilurin fungicide azoxystrobin inhibits mitochondrial electron transfer by binding to the Qo center of cytochrome bc_1 complex and interfering with ATP synthesis [19–22]. Because the fungicides have different modes of action cross-resistance is unlikely, the strobilurin fungicides do not have cross-resistance with demethylation inhibitors (DMIs), dicarboximide fungicides, or benzimidazoles (unpublished data). Some fungi, however, use an alternative respiration pathway to avoid inhibition by azoxystrobin [23].

In this study, azoxystrobin and thiram were tested *in vitro* for their individual and combined effects on mycelial growth and respiration of *S. sclerotiorum*. Mixtures containing salicylhydroxamic acid (SHAM), which is an inhibitor of the alternative respiration (oxidase) pathway [24], were also tested. Although spore germination

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has been considered an important criterion for testing fungicidal effects, *in vitro* mycelial growth and respiration are also considered useful criteria [25].

The objectives of this study were: (i) to determine the optimum ratio of thiram and azoxystrobin for inhibiting mycelial growth of *S. sclerotiorum*; (ii) to determine the rate of mycelial respiration of *S. sclerotiorum* when treated with fungicides alone or with fungicides plus SHAM; and (iii) to investigate the relationship between the initiation of the alternative oxidase pathway and the sensitivity to azoxystrobin or to fungicide mixtures.

2. Materials and methods

2.1. Media, strains, and fungicides

Three media were used. PDA was made from 200 g of potato, 20 g of agar, and 20 g of dextrose L^{-1} of distilled water. AEA medium consisted of 5 g of yeast extract, 6 g of $NaNO_3$, 1.5 g of K_2HPO_4, 0.25 g of $MgSO_4$, 0.5 g of KCl, 20 mL of glycerol, and 20 g of agar L^{-1} of distilled water. AEB medium contained 5 g of yeast extract, 6 g of $NaNO_3$, 1.5 g of K_2HPO_4, 0.25 g of $MgSO_4$, 0.5 g of KCl, and 20 mL of glycerol L^{-1} of distilled water.

Between 2007 and 2008, 105 isolates of *S. sclerotiorum* were collected from infected oilseed rape plants in Jiangsu Province, China. It is unlikely that these isolates had been previously exposed to azoxystrobin. Pure cultures were obtained by transfer of a single sclerotium and were maintained on PDA slants at 4 °C [26–28].

Stock solutions were prepared by dissolving technical grade azoxystrobin (Azo) (a.i. 97.8%; Syngenta China Co., Ltd., Shanghai, China) or SHAM (a.i. 99%; Sigma China Co., Ltd., Shanghai, China) in methanol. Thiram (Thi) (a.i. 96%; Baolin Chemicals, Jiangsu, China) was dissolved in acetone. The two fungicides were used alone and as mixtures. Solutions were diluted as required and stored at 4 °C in the dark. The methanol and acetone concentration never exceeded 1% of the testing solution (fungicide-amended medium). This concentration of methanol or acetone did not affect the different life stages of *S. sclerotiorum* (data not shown). Controls always contained the same methanol or acetone concentration as the test samples. Fungicides were added to AEA or AEB after autoclaving, when the agar had cooled to approximately 50 °C.

2.2. In vitro baseline sensitivity to azoxystrobin

The baseline sensitivity of *S. sclerotiorum* to azoxystrobin (i.e., the sensitivity of field isolates that have never been exposed to the fungicide), was determined with the 105 *S. sclerotiorum* isolates collected in Jiangsu Province during 2007–2008. Mycelial plugs (5 mm diameter) from the edge of 2-day-old colony were transferred to a series of AEA plates containing 0.0, 0.0625, 0.125, 0.25, 0.5, 1.0, and 4 μg of azoxystrobin per mL. Each isolate was represented by four replicate plates. After 2 days at 25 °C, colony diameters (minus the diameter of the inoculation plug) were measured, and the growth inhibition as percent of control was calculated. The median effective azoxystrobin concentration (EC_{50}) for each isolate was calculated by based on linear regression of colony diameter on log-transformed fungicide concentration [29]. The experiment was performed twice.

2.3. Synergistic interaction of mixtures containing different proportions of thiram and azoxystrobin

Synergistic interactions always decrease rapidly with increasing control levels of the single components [30–32] and may be almost nil at high control levels. According to the Wadley approach for quantifying synergistic interactions [30,33], dose–response curves of the single components (A and B) and the mixture (A + B) are constructed. With a logit–log transformation, the dose–response curves are linearized by regression and then are used to calculate EC (effective concentration) values for different control levels, e.g., EC_{50} or EC_{90}. When a and b are the proportion of the components in the mixture, the expected effective concentration (EC_{exp}) at any control level can be calculated [33] as:

$$EC_{exp} = (a + b)/[a/EC(A) + b/EC(B)].$$

The synergy ratio (SR) is the ratio of the expected and the observed EC_{50} values. When SR > 1.5, the interaction is defined as synergistic. When 1.5 > SR > 0.5, the interaction is defined as additive. When SR < 0.5, the interaction is defined as antagonistic [34]. Individual agar discs (5 mm diameter) were removed from the edge of a 2-day-old colony (isolate CZ162) on PDA and placed face up in the center of a Petri dish (9 cm diameter) containing AEA amended with the test mixture or each fungicide at various concentrations. The final concentrations tested for thiram were 0, 1.25, 2.5, 5, 10, 20, and 40 μg/mL. The final concentrations tested for azoxystrobin (alone or in mixtures) were 0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, and 4 μg/mL. The ratios (Thi:Azo) for the mixtures were 8:1, 4:1, 2:1, 1:1, 1:2, 1:4, and 1:8. The plates were incubated for 2 days at 25 °C in the dark, and the EC_{50} values were determined as described above. Each fungicide or mixture at each concentration was represented by four replicate plates, and the experiment was repeated.

2.4. Variation among isolates to the mixture of thiram and azoxystrobin

The SR for the optimal mixture (as determined for isolate CZ162 in the previous section) was determined for nine *S. sclerotiorum* isolates collected from different geographical regions of Jiangsu Province. Autoclaved AEA was amended with thiram alone, azoxystrobin alone, or a mixture at various concentrations. The final concentrations were: 0, 1.25, 2.5, 5, 10, 20, and 40 μg/mL for thiram, and 0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, and 4 μg/mL for azoxystrobin (alone or in mixture). EC_{50} values were determined as described in Section 2.2. The mixture at each concentration was represented by four replicate plates, and the experiment was repeated.

2.5. Effect of the optimal mixture (giving greatest inhibition) of thiram and azoxystrobin on mycelial growth in AEB

Fresh mycelial plugs (5 mm diameter) from the edge of a 2-day-old colony (isolate CZ162) on PDA were placed in 250-mL flasks (eight plugs per flask) containing 100 mL of AEB; the flasks were placed on a rotary shaker (175 rpm, 25 °C) for 36 h. The optimal mixture (Thi:Azo, 1:4) or the optimum concentration of each component alone was then added to the cultures. The final concentrations tested for the optimal mixture (Thi:Azo, 1:4), azoxystrobin or thiram were respectively 50 μg/mL. Cultures without fungicide served as the control. After an additional 24, 48, 72, or 96 h at 25 °C, the mycelia were washed with distilled water three times to remove medium, dried in an air drier at 80 °C for 12 h, and weighed. There were four replications for each treatment, and the test was repeated.

2.6. Inhibition of mycelial respiration

Mycelial plugs (5 mm diameter) from the margins of a 2-day-old colony (isolate CZ162) on PDA were placed in 250-mL conical flasks (12 plugs per flask) containing 100 mL AEB. They were placed on a rotary shaker (175 rpm at 25 °C). After 36 h, flasks were