Enhancing the Efficacy of Copper Fungicides through Synergism with Salicylaldehyde Benzoylhydrazones

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ABSTRACT

Copper salts are widely used as fungicides and serve a critical need for controlling fungal pathogens and managing resistance. However, there are concerns about the environmental impact of copper, which is used at high rates. The potential for salicylaldehyde benzoylhydrazone (SBH) compounds to increase the efficacy of copper fungicides and allow the use of lower application rates has been explored. *In vitro* fungicidal activity of 3,5-dichloro-salicylaldehyde benzoylhydrazone (SBH I) against *Stagonospora nodorum* and *Phytophthora capsici* was dramatically enhanced by Cu^{2+} in culture media. SBH compounds can form 1:1 complexes with Cu^{2+} (Cu-SBH), and Cu-SBH I was more potent than the free ligand. Cu-SBH I is proposed to act by delivering Cu^{2+} into the cell. Synergism between SBH I and Cu^{2+} salts was also demonstrated in greenhouse and field tests. Cu-SBH I, and mixtures of SBH I with Cu^{2+}, showed strong broad spectrum control of fungal diseases whereas free SBH was much less effective.

INTRODUCTION

Methods for synthesis of salicylaldehyde benzoylhydrazone (SBH) compounds and their metal complexes (1:1 molar ratio of metal ion to SBH) are well known (Ainscough *et al*. 1999). SBH compounds are tridentate ligands which chelate a variety of metal ions, including Fe^{2+} and Cu^{2+}, and have been explored as chelating agents for treatment of iron-overload disease and as anticancer drugs (Aruffo *et al*. 1982; Ainscough *et al*. 1999). Recently, activity towards human pathogenic fungi has also been demonstrated (Backes *et al*. 2014). Our studies have focused on the ability of SBH compounds to control plant disease. We report that fungicidal activity of SBH compounds is highly dependent on the presence of Cu^{2+}. Based on strong synergism, we have explored the potential use of SBH compounds to lower the rates of copper required to control disease.

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MATERIALS AND METHODS

Culture media

CS broth (Coursen & Sisler 1960) was prepared with, and without (Cu-minus CS broth), the prescribed amount (0.8 µM) of $CuSO_4.5H_2O$. Cu-minus CS broth was prepared by dissolving glucose (10 g), K_2HPO_4 (1.5 g), KH_2PO_4 (2 g) and $(NH_4)_2SO_4$ in one liter of deionized water, then adding 0.5 g Chelex 100 resin (Bio-Rad Laboratories) and stirring for 1 h. $MgSO_4.7H_2O$ (0.5 g) was added and the solution stirred for an additional hour. After allowing the resin to settle, 900 ml was transferred to a new container, then trace element and vitamin stocks were added, omitting $CuSO_4.5H_2O$. The medium was filter-sterilized. Asparagine-sucrose medium (Erwin & Katznelson 1961) was prepared without $CuSO_4.5H_2O$ in the trace element solution (Cu-minus AS broth). Asparagine (2 g), K_2HPO_4 (0.3 g), KH_2PO_4 (0.43 g), thiamine HCl (0.4 ml of a 0.5 mg/mL stock solution) and sucrose (15 g) were dissolved in one liter of deionized water. Chelex 100 resin (0.5 g) was added to the solution and stirred for 1 h. After transferring 900 ml to a new container, the pH was adjusted to 6.4, then $MgSO_4.7H_2O$ (90 mg), $FeSO_4.7H_2O$ (900 µL of freshly made 1 mg/mL solution), 900 µL trace element solution lacking $CuSO_4.5H_2O$, and $CaCl_2$ (45 mg) were added, and the medium was filter-sterilized. Plastic labware was used throughout.

In vitro assays for fungicidal activity

For assays against *S. nodorum,* test compounds in DMSO were diluted into CS broth or Cu-minus CS broth. Serial dilutions were prepared in the same broth (100 µL) in 96-well microtiter plates. Spore suspensions were prepared by adding 20 mL of Chelex 100-treated deionized water to cultures on potato dextrose agar and scraping gently. Suspensions were filtered through cheesecloth, washed twice by centrifugation then resuspended in the desired broth at 2×10^5 spores/mL. Wells were inoculated with 100 µL suspension, and incubated at 23 °C for 72 h before assessing growth using a NEPHELOstar Galaxy plate reader.

For assays against *P. capsici*, dilution series were prepared as above except that Cu-minus AS broth was used. A zoospore suspension in Chelex 100-treated deionized water was prepared at 2×10^5 spores/mL according to Young (1991). Wells were inoculated with 100 µL suspension, and incubated at 23 °C for 48 h before assessing growth.

Greenhouse evaluation against tomato late blight

Materials were SBH I (10% suspension concentrate), $CuCl_2.2H_2O$, $Cu(OH)_2$ (as Kocide® 2000), and $CuSO_4.5H_2O$. Mixtures, and materials alone, were prepared in 0.01% (v/v) Triton X-100 and applied to 14-day old tomato plants. After 24 h, plants were inoculated with *Phytophthora infestans* sporangia (5×10^4 sporangia/mL), then incubated under high humidity for 24 h before transfer to growth rooms for disease development. Disease severity was assessed 7 days after inoculation.

Field test against potato late blight

A field test was conducted in New York State, USA, to compare the efficacy of $Cu(OH)_2$ (as Kocide® 3000), mixtures of Kocide® 3000 with SBH I, Cu-SBH I, SBH I alone and Dithane®. SBH I and Cu-SBH I were formulated as 10% suspension concentrates. Three applications were made at 7-8 day intervals and disease evaluations were taken between 3 and 24 days after the last application. Data were expressed as percent control based on area under the disease progress curve.

RESULTS AND DISCUSSION

Screening of SBH analogs at Dow AgroSciences revealed potent broad spectrum activity against fungal pathogens when tested *in vitro* in complex culture media, with SBH I (Figure 1) showing particularly strong potency.
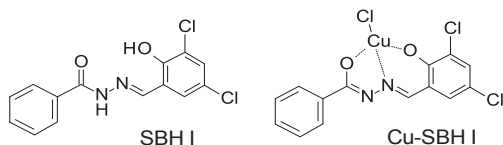

Figure 1   Structures of 3,5-dichloro-salicylaldehyde benzoylhydrazone (SBH I) and its copper (II) complex (Cu-SBH I).

Against *S. nodorum,* SBH I was 3-orders of magnitude more potent in CS broth containing Cu^{2+} as a trace element (0.8 µM) than in Cu-minus CS broth (Figure 2A). Although Cu^{2+} alone is fungicidal, concentrations >10 µM were required for growth inhibition. Enhanced potency of SBH I with Cu^{2+} was specific to this ion since other metal salts ($FeCl_3$, $MnCl_2$, $MgCl_2$ and $ZnCl_2$) at 10 µM in Cu-minus CS broth did not enhance activity.
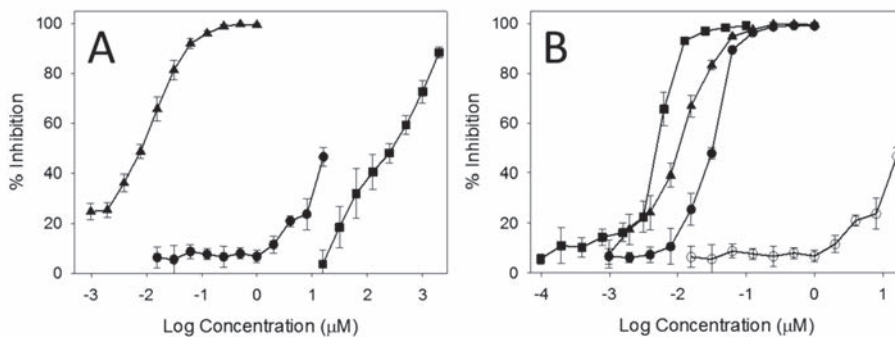

Figure 2   Fungicidal activity of SBH I (A) and Cu-SBH I (B) towards *Stagonospora nodorum*. A: SBH I in CS medium (▲), SBH I in Cu-minus CS medium (●), and $CuCl_2$ (■). B: Cu-SBH I in Cu-minus CS medium (●), Cu-SBH I in CS medium (0.8 µM $CuCl_2$,▲), Cu-SBH I in CS medium with 10 µM $CuCl_2$ (■) and SBH I in Cu-minus CS medium (○).

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Whereas SBH I had little activity in Cu-minus CS broth, the Cu-SBH I complex was highly active (Figure 2B). Activity of Cu-SBH I increased further with additional $CuCl_2$ (10 µM).

Against the Oomycete *P. capsici*, SBH I was only slightly less active than Cu-SBH I in Cu-minus AS broth (Figure 3A). However, activity of SBH I increased almost 100-fold by adding 50 µM $CuCl_2$, an amount of Cu^{2+} well below fungicidal concentrations. Synergism between SBH I and Cu^{2+} was also explored by testing the effect of low amounts of SBH I on fungicidal activity of $CuCl_2$ in dose-response experiments (Figure 3B). The EC_{50} value for growth inhibition by $CuCl_2$ alone was 759 µM. Addition of SBH I at 5, 10 and 20 nM, concentrations which had little effect in the absence of Cu^{2+}, increased sensitivity to Cu^{2+} dramatically, resulting in EC_{50} values for $CuCl_2$ of 434, 15.0 and 1.5 µM, respectively.
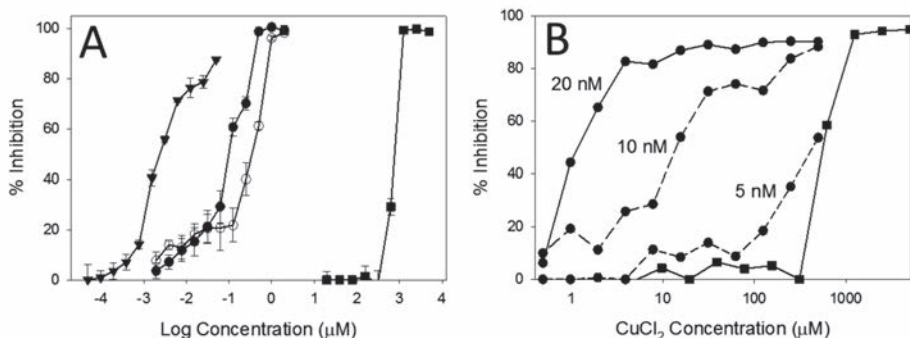

Figure 3   Fungicidal activity towards *Phytophthora capsici*. A: SBH I in Cu-minus AS medium (○), Cu-SBH I in Cu-minus AS medium (●), SBH I in AS medium with 50 µM $CuCl_2$ (▼), and $CuCl_2$ (■). B: dose response curves for $CuCl_2$ alone (■) and in the presence of SBH I (●) at 5, 10 and 20 nM.

Our working hypothesis for the mechanism responsible for synergism between SBH I and Cu^{2+} involves a shuttle system, whereby SBH-I chelates extracellular Cu^{2+} and diffuses as a complex into the cell where the intracellular environment favors dissociation. SBH I may then diffuse back out of the cell to bind additional Cu^{2+}, continuing this cycle until intracellular Cu levels reach a fungicidal concentration. This could explain the ability of small amounts of SBH I to deliver much greater potency of Cu^{2+} (Figure 3B), and why activity of the Cu-SBH I complex can be increased further by additional Cu^{2+} (Figure 3A).

These *in vitro* results prompted greenhouse experiments to explore synergism against *Phytophthora infestans* on tomato (Figure 4). $Cu(OH)_2$, $CuCl_2$ and $CuSO_4$ alone were moderately active and comparable in efficacy. In mixtures with an equimolar concentration of SBH I, Cu^{2+} salts were much more active despite the fact that SBH I alone was only weakly active. In tests against additional pathogens, isolated Cu-SBH I (200 mg/L) and mixtures of SBH I (200 mg/L) with $CuCl_2$ at 50 µM, which alone provided no control, delivered ≥ 90% control of potato and tomato late blight (*P. infestans*), cucumber anthracnose (*Colletotrichum lagenarium*), wheat glume blotch (*S. nodorum*), wheat brown rust (*Puccinia recondita*), rice blast (*Magnaporthe grisea*), barley spot blotch (*Cochliobolus sativus*), cucumber downy

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mildew (*Pseudoperonospora cubensis*), grape downy mildew (*Plasmopara viticola*) and wheat leaf blotch (*Zymoseptoria tritici*).
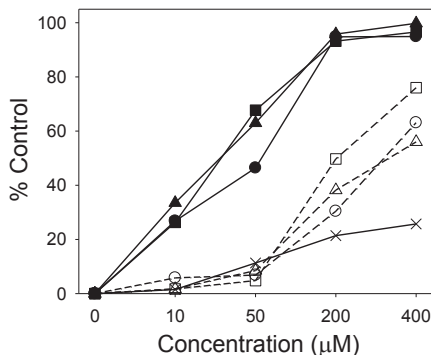

Figure 4   Control of tomato late blight by Cu^{2+} salts alone (open symbols and dashed lines) and in mixtures with SBH I (closed symbols and solid lines) under greenhouse conditions. $Cu(OH)_2$, circles; $CuCl_2$, triangles; $CuSO_4$, squares, SBH I alone, crosses.

The use of SBH compounds to lower the rates of copper needed to control disease was also explored in a field test against potato late blight (Figure 5). The test was designed to evaluate the ability of SBH I as a tank mix to improve control by $Cu(OH)_2$ (as Kocide® 3000). Treatments were chosen to allow comparisons based on the amount of copper applied, with 3 rates of copper. Control obtained with Kocide® 3000 alone at the highest rate of 1.8 kg/ha (631 g copper/ha) was 41%, whereas Kocide® 3000/SBH I mixtures (1:0.25 and 1:1 molar ratios with respect to copper) delivered 86% and 97% control, respectively, which exceeded that of Dithane® at 1.6 kg/ha. The efficacy of Cu-SBH I was similar to that of the Kocide® 3000/SBH I mixtures (1:1 molar ratio) across the 3 rates tested. Cu-SBH I and the Kocide® 3000/SBH I mixture (1:1 molar ratio) delivered a statistically significant improvement in efficacy over Kocide® 3000 alone at the medium and high rates of copper. A statistically significant improvement in efficacy was also achieved with the Kocide® 3000/SBH I mixture at the lower 0.25:1 molar ratio and the high rate of copper.

CONCLUSIONS

A strong synergistic fungicidal effect between SBH I and Cu^{2+} has been shown towards fungal pathogens *in vitro*, as well as in greenhouse and field tests. Our results suggest that SBH compounds have the potential to improve the efficacy of copper fungicides and lower the amounts of copper required to control disease. This strategy could be used to mitigate the environmental impact of copper fungicide usage.
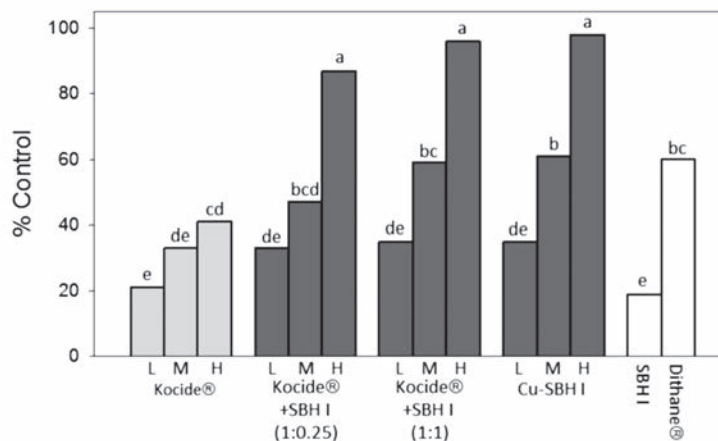
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Figure 5   Control of potato late blight in NY State by Kocide® 3000 alone, mixtures of Kocide® 3000 with SBH I at 1:0.25 and 1:1 molar ratios with respect to copper, Cu-SBH I complex, SBH I alone at the highest rate used in Kocide® 3000/SBH I mixture (1:1 molar ratio), and Dithane®. Low (L), medium (M) and high (H) rates of elemental copper were 63, 158 and 631 g/ha, respectively. A total of 3 applications were made at 7-8 day intervals and disease evaluations were taken between 3 and 24 days after the last application.

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