JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Effects of Some Benzoxazinoids on in Vitro Growth of *Cephalosporium gramineum* and Other Fungi Pathogenic to Cereals and on *Cephalosporium* Stripe of Winter Wheat

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The benzoxazolinones benzoxazolin-2(3*H*)-one (BOA) and 6-methoxybenzoxazolin-2(3*H*)-one (MBOA) and selected degradation products of these compounds were examined for their in vitro antifungal activity against *Cephalosporium gramineum*, *Gaeumannomyces graminis* var. *graminis*, and *Fusarium culmorum*. BOA was also applied to the soil-incorporated inoculum of *C. gramineum* to test its capability of reducing *Cephalosporium* stripe disease in winter wheat. MBOA reduced the mycelial growth of *G. graminis* var. *tritici*, *C. gramineum*, and *F. culmorum* by 50% ($EC_{50}$) at the concentrations of 77, 134, and 271 $\mu$g/mL of corn meal agar, respectively, and the corresponding BOA $EC_{50}$ values for the fungi were 11, 189, and 456 $\mu$g/mL. BOA degradation products 2-amino-3*H*-phenoxazin-3-one (APO), 2-acetylamino-3*H*-phenoxazin-3-one (AAPO), and *o*-aminophenol (*o*-AP) were much more inhibitory to the growth of *C. gramineum* and *G. graminis* var. *tritici* than the parent compounds. APO, AAPO, and *o*-AP $EC_{50}$ values were found to be as low as 0.58, 4.57, and 1.4 $\mu$g/mL, respectively, for *C. gramineum* and 0.78, 2.18, and 0.80 $\mu$g/mL for *G. graminis* var. *tritici*. These compounds applied at the corresponding concentrations did not significantly affect the mycelial growth of *F. culmorum*. The treatment of *C. gramineum* inoculum with a 1% water solution of BOA resulted in a significant reduction infection of winter wheat with *C. gramineum* as compared to the control with the untreated inoculum, but this treatment was not as effective as the application of a commercial fungicide.

**KEYWORDS: Benzoxazinoids; benzoxazolinones; derivatives; antifungal activity**

## INTRODUCTION

Cyclic hydroxamic acids, the core compounds of plant benzoxazinoids, occur constitutively as glucosides in some plant families and are considered to be an important factor in plant−parasite interactions, particularly in the case of cereals (Poaceae) such as corn, rye, and wheat (*1*−*3*). In response to pest attacks or physical tissue damage these glucosides are hydrolyzed to form the more toxic aglycones, which in turn decompose in aqueous solutions to the benzoxazolinones: benzoxazolin-2(3*H*)-one (BOA) and 6-methoxybenzoxazolin-2(3*H*)-one (MBOA) (*1*−*6*). Inhibitory activity of BOA and MBOA has been demonstrated for several fungal pathogens of plants, including *Stagonospora nodorum* (*3*), *Fusarium moniliforme* (*4*), *Fusarium subglutinans* (*5*), *Fusarium culmorum* (*6*), and *Gaeumannomyces graminis* (*6*, *7*). It was also shown that some of these fungi, for example, *F. moniliforme* (*4*) or *G. graminis* var. *graminis* (*6*), are able to degrade BOA and MBOA with accumulation of less toxic metabolites *N*-(2-hydroxyphenyl)-malonamic acid (HPMA) and *N*-(2-hydroxy-4-methoxyphenyl)-malonamic acid (HMPMA), respectively. Other transformation products of BOA have been also identified in fungal cultures, including 2-amino-3*H*-phenoxazin-3-one (APO) and 2-acetylamino-3*H*-phenoxazin-3-one (AAPO), known as the naturally occurring antibiotics (*6*, *8*). Moreover, Friebe et al. (*9*) reported a rapid degradation of BOA in liquid media by root-colonizing bacteria of *Avena sativa*, with APO and AAPO as the main transformation products and *o*-aminophenol as an intermediate. The same compounds (APO and AAPO) have been also identified as degradation products of BOA in soil incubated under laboratory conditions and, similarly, MBOA was transformed to AMPO and 2-acetylamino-7-methoxy-3H-phenoxazin-3-one (AAMPO) in the soil environment (*10*−*17*).

*Cephalosporium gramineum* Nisikado and Ikata (sporodochial stage, *Hymenula cerealis* Elis and Everh), the causal agent of *Cephalosporium* stripe disease of winter cereals, is an economically damaging vascular pathogen occurring worldwide (*18*−

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10.1021/jf050901x CCC: $33.50 
Published on Web 01/24/2006

*21*). It infects roots of winter cereals (however, causing no symptoms on these organs) and spreads further through water-conducting vessels into stems and leaves. Symptoms include striping on leaves and finally premature blighting of the infected plants (*18*, *21*). The pathogen survives in soil as a saprophyte on the previously infected host residues, on which it produces numerous, small conidia serving as the primary inoculum for plant infection during wet and cool seasons of the year (*21*). This pathogen can be controlled by growing winter cereals in rotation with other nonhost crops, for example, leguminous plants, but fungicides, including those applied in-furrow, are ineffective (*20*). Results of our preliminary pot and microplot experiments indicate that commercial fungicides applied to the inoculum of *C. gramineum* significantly decreased sporulation of the pathogen and thus reduced numbers of triticale plants with *Cephalosporium* stripe symptoms (*19*).

The objectives of this research were to assess the in vitro sensitivity of *C. gramineum* and two other cereal-infecting fungi [*G. graminis* (Sacc.) Arx and Olivier var. *tritici* Walker and *F. culmorum* (W. G. Sm.) Sacc.] to benzoxazolinones and their derivatives and to find out whether treatment of *C. gramineum* inoculum with BOA results in a reduction of winter wheat infection by the pathogen in a greenhouse pot experiment.

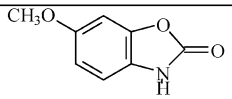
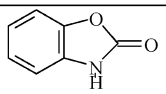
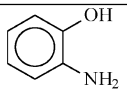
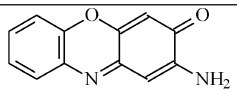
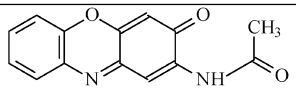
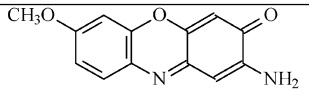
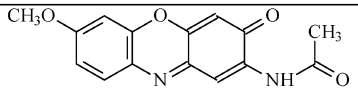
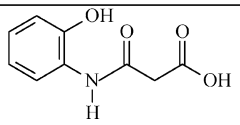
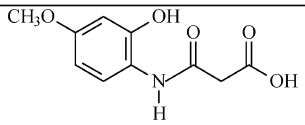
## MATERIALS AND METHODS

**Allelochemicals.** BOA, MBOA, and *o*-aminophenol (*o*-AP) were purchased from Aldrich-Sigma. Other transformation products of BOA and MBOA tested in these studies were supplied by F. A. Macias and co-workers (*16*, *22*). In this group the following compounds were included: APO, AAPO, AAMPO, HPMA, and HMPMA. The purity and structures of these allelochemicals were determined by $^1$H NMR and HPLC analyses (*16*).The structures and the molecular weights of the tested compounds are given in **Table 1**.

**Fungal Cultures, Media, and Testing of Antifungal Activity.** The fungal species used in this study [*C. gramineum* Nisikado & Ikata, *G. graminis* (Sacc.) Arx & Oliv. var. *tritici* Walker, and *F. culmorum* (W.G.Sm.) Sacc.] were isolated from diseased winter wheat plants and stored on potato dextrose agar (PDA; Difco) slants at −20 °C in a cryoprotectant. Fresh cultures of these fungi were obtained by transferring mycelial plugs to Petri plates containing 20 mL of corn meal agar (CMA; Becton & Dickinson) and incubation for 7−14 days at 20 °C (*C. gramineum*) or 25 °C (other fungi). CMA was also used to test the antifungal activity of all the allelochemicals. Portions of the CMA medium were enriched after autoclaving with different volumes of stock solutions (in 96% ethanol) of the tested compounds to give various concentrations of the compounds in CMA. All treatments contained the same amounts of ethanol, usually 1 mL per 100 mL of the medium (2 mL in the case of AAMPO, HPMA, and HMPMA), including the control treatment containing pure ethanol. The tested concentrations of the allelochemicals in CMA depended on their antifungal activity in preliminary range-finding tests. In the case of BOA and MBOA the tested concentrations ranged from 0.125 to 4.0 mM, whereas metabolites or derivatives of these benzoxazolinones were examined at markedly lower concentrations, ranging from 0.00125 to 0.1 mM.

Aliquots of 15 mL of warm CMA containing different concentrations of the tested compounds were poured into Petri plates (90 mm in diameter) and allowed to solidify. The plates were inoculated in the center with 5 mm disks cut from actively growing colonies of the fungi on CMA without any amendments. There were three or four replicated plates for each tested concentration of the compounds. The inoculated plates were incubated in the dark at the optimal temperature for the growth of a particular fungus: 20 °C for *C. gramineum* and 25 °C for *G. graminis* var. *tritici* and *F. culmorum*. After different periods of incubation, 4, 7, and 10 days in the case of *F. culmorum*, *G. graminis* var. *tritici*, and *C. gramineum*, respectively, colony diameters were measured. Pooled data of two independent experiments are presented. For the calculation of the percentage of inhibition the diameter of the inoculum disk (5 mm) was subtracted from the measured colony diameters. Results are expressed as percentages of inhibition in relation to the control, and for each compound the 50% effect concentration ($EC_{50}$, the concentration at which 50% inhibition occurred) was calculated from dose−response curves using linear regression analysis (Microsoft Excel).

**Table 1.** Molecular Weights and Structures of the Tested Compounds

| Compound* | mol wt | Structure |
|---|---|---|
| MBOA | 165 |  |
| BOA | 135 |  |
| o-AP | 109 |  |
| APO | 212 |  |
| AAPO | 254 |  |
| AMPO | 242 |  |
| AAMPO | 284 |  |
| HPMA | 195 |  |
| HMPMA | 225 |  |

*BOA, benzoxazolin-2(3*H*)-one; MBOA, 6-methoxybenzoxazolin-2(3*H*)-one; *o*-AP, *o*-aminophenol; APO, 2-amino-3*H*-phenoxazin-3-one; AAPO, 2-acetylamino-3*H*-phenoxazin-3-one; AAMPO, 2-acetylamino-7-methoxy-3*H*-phenoxazin-3-one; HPMA, *N*-(2-hydroxyphenyl)malonamic acid; HMPMA, *N*-(2-hydroxy-4-methoxyphenyl)malonamic acid.

**Pot Experiment.** Mitcherlich pots were filled with 7 kg of a brown soil at 40% water-holding capacity (WHC) and fertilized with 0.5 g of $NH_4NO_3$, 1.1 g of $K_2SO_4$, and 1.9 g of $KH_2PO_4$ per pot. The soil had the following basic characteristics: 1.1% organic C, 0.134% total N, 19% clay, and $pH_{KCl}$ 6.5. Each pot received 5 g (air-dry) of grass seed inoculum of *C. gramineum* prepared as described previously (*19*). The inoculum was mixed into the surface (4−5 cm) soil layer in the pots. Before its application, the inoculum was treated with water or water solutions of the test chemicals at the rate of 1:1 (w/v) and left for 2 h at room temperature to absorb the liquid. The following treatments were performed: I, water-soaked inoculum; II, as in I but autoclaved; III, inoculum soaked in 0.5 and 1.0% water solution of BOA; IV, inoculum soaked in 0.5 and 1.0% water suspension of a commercial fungicide containing 45% thiuram plus 20% carbendazim in the commercial

**Table 2.** Percentages of Inhibition of the Radial Growth of the Tested Fungi (*C. gramineum, G. graminis* Var. *tritici*, and *F. culmorum*) on Corn Meal Agar Enriched with Different Concentrations of BOA and MBOA in Relation to the Control Medium without Amendments and the Estimated $EC_{50}$ Values

| concn (mM) | *C. gramineum* | G. *graminis* var. *tritici* | *F. culmorum* |
|---|---|---|---|
| | | BOA | |
| 4.0 | 100[a] | | 59.6[a] |
| 2.0 | 73.1[a] | | 29.0[a] |
| 1.0 | 34.5[a] | 64.7[a] | 15.7[a] |
| 0.5 | 4.9 | 22.5[a] | 1.9 |
| 0.25 | 0 | 11.2[a] | 0 |
| 0.125 | 0 | 2.8 | 0 |
| | | $EC_{50}$ ($\mu$g/mL) | |
| | 189 | 111 | 456 |
| | | MBOA | |
| 4.0 | | | |
| 2.0 | | | 62.7[a] |
| 1.0 | 63.7[a] | 100[a] | 27.5[a] |
| 0.5 | 21.9[a] | 54.9[a] | 16.8[a] |
| 0.25 | 8.4[a] | 19.7[a] | 1.9 |
| 0.125 | 4.7 | 19.7[a] | 0 |
| | | $EC_{50}$ ($\mu$g/mL) | |
| | 134 | 77 | 271 |

[a] Significant growth reduction in relation to the controls with the following colony diameters (in mm ± SD): 27.3 ± 0.52, 23.0 ± 0.5, and 30.5 ± 0.55 for *C. gramineum*, *G. graminis* var. *tritici*, and *F. culmorum,* respectively.

formulation. Each treatment consisted of five replicated pots. At the beginning of October 2003, the pots were sown with 18 seeds of winter wheat (cv. Zyta), and each pot was watered to 60% WHC. The pots were kept in an unheated glasshouse. After germination, the seedlings were thinned to 15 per pot. At flowering (GS 50−52), all plants were inspected for *Cephalosporium* stripe symptoms and infected plants counted. After harvest at full ripeness (July 2004), grain yields per pot and per ear were determined. One-way analysis of variance (ANOVA) and Tukey's test were used to determine the significance of differences.

## RESULTS AND DISCUSSION

The antifungal activity of the tested benzoxazolinone compounds was dependent both on their structure and on the fungus tested. Inhibitory activity of BOA toward the tested fungi was weaker than that of MBOA (**Table 2**). Accordingly, MBOA $EC_{50}$ values were lower than those of BOA for all of the fungi. MBOA reduced the mycelial growth of *G. graminis*, *C. gramineum*, and *F. culmorum* by 50% at the concentrations of 77, 134, and 271 $\mu$g/mL, respectively, and the corresponding values of BOA $EC_{50}$ for the fungi were 111, 189, and 456 $\mu$g/mL. Of the compared fungi, *G. graminis* var. *tritici* was the most sensitive to both compounds and *F. culmorum* the most resistant one. With respect to *F. culmorum* and *G. graminis* var. *tritici*, similar results were obtained by Friebe et al. (*6*), who reported higher antifungal activity of MBOA than of BOA toward these fungi, with *F. culmorum* being substantially less sensitive to both compounds than three varieties of *G. graminis* tested by these authors. In those studies neither BOA nor MBOA $EC_{50}$ values were given for *F. culmorum*, but it was shown that this fungus was not able to metabolize MBOA, contrary to BOA. In our studies MBOA $EC_{50}$ values for *F. culmorum* were almost 2-fold lower than BOA $EC_{50}$ values (**Table 2**). It is possible that the much higher sensitivity of *F. culmorum* to MBOA was related to the inability of this fungus to metabolize and thus to detoxify MBOA. The response of *C. gramineum* to benzoxazolinones has not been tested so far. Because amounts of BOA and MBOA detected in winter wheat root tissue can range up to 250 $\mu$g/g of dry weight (*23*, *24*), it seems that these compounds can play an important role in reducing the infection of wheat roots by the tested fungi.

**Table 3.** Percentages of Inhibition of the Radial Growth of the Tested Fungi (*C. gramineum, G. graminis* Var. *tritici*, and *F. culmorum*) on Corn Meal Agar Enriched with Different Concentrations of BOA Transformation Products in Relation to the Control Medium without Amendments and the Estimated $EC_{50}$ Values

| concn (mM) | *C. gramineum* | | | *G. graminis* var. *tritici* | | | *F. culmorum* | | |
|---|---|---|---|---|---|---|---|---|---|
| | APO | AAPO | *o*-AP | APO | AAPO | *o*-AP | APO | AAPO | *o*-AF |
| 0.02 | 100[a] | 54[a] | 65[a] | 100[a] | 94[a] | 100[a] | 11 | 0 | 0 |
| 0.01 | 87[a] | 34[a] | 43[a] | 100[a] | 89[a] | 87[a] | 0 | 0 | 0 |
| 0.005 | 73[a] | 24[a] | 17[a] | 98[a] | 20[a] | 50[a] | 0 | 0 | 0 |
| 0.0025 | 57[a] | 20[a] | 3 | 35[a] | 16[a] | 11 | 0 | 0 | 0 |
| 0.00125 | 32[a] | | | 18[a] | | | | | |
| | $EC_{50}$ ($\mu$g/mL) | | | $EC_{50}$ ($\mu$g/mL) | | | | | |
| | 0.58 | 4.57 | 1.4 | 0.78 | 2.18 | 0.80 | | | |

[a] Significant growth reduction in relation to the controls with the following colony diameters (in mm ± SD): 39.7 ± 0.8, 54.8 ± 2.5, and 51.0 ± 7.8 for *C. gramineum*, *G. graminis* var. *tritici*, and *F. culmorum,* respectively.

The antifungal activity of the transformation products of BOA was examined at markedly lower concentrations, mainly due to higher toxicity of some of these compounds to *C. gramineum* and *G. graminis* var. *tritici*. Of the tested compounds APO was found to be the most inhibitory to the mycelial growth of *C. gramineum* and *G. graminis* var. *tritici*, with $EC_{50}$ values as low as 0.58 and 0.78 $\mu$g/mL, respectively (**Table 3**). *o*-AP also showed high antifungal activity with $EC_{50}$ amounting to 0.80 $\mu$g/mL in the case of *G. graminis* and 1.4 $\mu$g/mL in the case of *C. gramineum*. AAPO reduced the growth of *G. graminis* by 50% at the concentration of 2.18 $\mu$g/mL and that of *C. gramineum* at 4.57 $\mu$g/mL. It is interesting to note that none of the above-mentioned transformation products of BOA affected the growth of *F. culmorum* at the tested concentrations (**Table 3**). This is the first study showing marked, species-dependent differences in the in vitro response of soilborne fungal pathogens of cereals to BOA transformation products such as *o*-aminophenol APO and AAPO. Markedly higher resistance of *F. culmorum* to benzoxazolinones (**Table 2**) and its transformation products in soil (**Table 3**) is probably related to their ability to infect a broader spectrum of host plants, including roots and green parts of plants, than more specialized pathogens, for example, the cereal root infecting fungus *G. graminis* (*6*, *7*).

Other derivatives such as AAMPO, HPMA, and HMPMA tested at concentrations up to 0.1 mM had no substantial effect on the mycelial growth of any of the fungi used in the study (data not shown).

Because APO, a strong inhibitor of *C. gramineum* in vitro growth (**Table 3**), was found to be the main transformation product of BOA by soil microorganisms (*9*−*17*), we treated the inoculum of *C. gramineum* with a BOA water solution to see whether this treatment reduces infection of winter wheat by the pathogen. Results of this pot experiment are presented in **Table 3**, which shows that only treatment of the inoculum with 1% BOA solution resulted in a significant increase in the number of healthy wheat plants and grain yields as compared to the control with the untreated inoculum. These positive effects were, however, markedly lower than those achieved with the application of a commercial fungicide, which completely protected winter wheat against *C. gramineum* (**Table 4**). It is possible that concentrations of BOA >1% would give results comparable to those of commercial fungicides, but more

**Table 4.** Numbers of Uninfected Winter Wheat Plants and Grain Yields As Influenced by *C. gramineum* Inoculum Treatment with BOA and a Fungicide[a]

| inoculum treatment | no. of uninfected plants per pot | grain yield per pot (g) | grain yield per ear (g) |
|---|---|---|---|
| autoclaved | 15.0 a | 61.1 a | 2.06 a |
| untreated | 4.6 c | 31.6 c | 0.87 c |
| 0.5% BOA | 5.2 c | 33.7 c | 0.91 c |
| 1.0%BOA | 8.0 b | 39.3 b | 1.19 b |
| 0.5% fungicide | 15.0 a | 56.1 a | 1.75 a |
| 1.0% fungicide | 15.0 a | 58.0 a | 1.88 a |

[a] Numbers followed by the same letter are not significantly different at $\alpha = 0.05$ (ANOVA).

research is needed on the different aspects of a practical use of benzoxazinoid allelochemicals and their derivatives.

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**Received for review April 19, 2005. Revised manuscript received July 27, 2005. Accepted December 6, 2005. This work was performed as a part of the project "FATEALLCHEM", "Fate and Toxicity of Allelochemicals in Relation to Environment and Consumer" (http://www.fateallchem.dk), a project carried out with financial support from The Commission of the European Communities under the work program Quality of Life, Contract QLK5-CT-2001-01967.**

JF050901X