Activity of Hydroxamic Acids from *Secale cereale* Against the Plant-Parasitic Nematodes *Meloidogyne incognita* and *Xiphinema americanum*

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ABSTRACT

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Cyclic hydroxamic acids are secondary metabolites found in the family Poaceae and have been implicated in the allelopathy of rye (*Secale cereale*). The toxicity of these compounds against plant-parasitic nematodes is unknown. DIBOA (2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one), DIMBOA (2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one), and their degradation products BOA (benzoxazolin-2(3*H*)-one) and MBOA (6-methoxy-benzoxazolin-2(3*H*)-one) were screened in vitro against *Meloidogyne incognita* second-stage juveniles (J2) and eggs and mixed-stages of *Xiphinema americanum*. *Xiphinema americanum* was more sensitive to DIBOA and DIMBOA than *M. incognita* J2, with a maximum apparent mortality of 96 and 92% compared to 73 and 72% at 90 µg/ml. Eggs of *M. incognita* were less sensitive to the hydroxamic acids than J2; only DIBOA resulted in a 50% reduction in egg hatch, with a lethal concentration (LC_{50}) of 74 µg/ml compared to 21 µg/ml for J2. When *M. incognita* J2 were exposed to DIBOA for 48 h and the compound was removed and replaced with water, the LC_{50} value increased from 21.0 to 40.7 µg/ml. MBOA was not toxic to *X. americanum* or *M. incognita* eggs, but was toxic to *M. incognita* J2, with LC_{50} values of 44 and 20 µg/ml before and after the compound was removed and replaced with water. BOA was the least toxic hydroxamic acid tested; it did not reduce *M. incognita* egg hatch after 1 week of exposure or increase *X. americanum* mortality after 24 h of exposure. While in vitro studies provide a valuable starting point in determining the toxicity of the chemical component of rye, the relevance of the data to soil remains to be determined.

Additional keywords: allelopathy, cover crop, dagger nematode, root-knot nematode.

The use of cover crops is recognized as a management practice that reduces crop damage caused by plant-parasitic nematodes (5,6). Cover crops can be used most effectively and reliably as a nematode management tool when the mechanisms of nematode suppression are understood. One potential mechanism involves the release of nematicidal or nematistatic compounds during cover crop decomposition. In this case, factors that are usually unknown include the chemical identities of the active components, the lethal concentrations of the active components for specific target nematodes, and the impact of the material on and influence of soil physical, biological, and chemical properties.

The winter annual cover crop rye (*Secale cereale*) has been used to reduce soil erosion, recycle nutrients, enhance soil tilth, and reduce inputs of chemical fertilizers and pesticides. Rye also produces allelochemicals that suppress weeds, insects, and nematodes (1,2,4,18–20). The incorporation of a rye cover crop before planting cotton reduced damage caused by *Meloidogyne incognita* (12), but the effectiveness of a rye treatment declined with time (9). While rye reduced *M. incognita* activity, there was no correlation between the production of low molecular weight aliphatic organic acids and nematode suppression, suggesting that other mechanisms were responsible (10).

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Another potential mechanism responsible for suppression of nematodes by a rye cover crop is the production of cyclic hydroxamic acids. Cyclic hydroxamic acids are secondary metabolites found in the family Poaceae including corn, wheat, and rye. The compound 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) and its breakdown product benzoxazolin-2(3*H*)-one (BOA) have each been implicated in the allelopathy of rye to weeds (1). Another allelopathic compound identified in rye is 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and its degradation product 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA) (20). In intact rye, the cyclic hydroxamic acids DIBOA and DIMBOA occur as glucosides. Upon tissue disruption, β-glucosidase is released and the glucosides are rapidly hydrolyzed to DIBOA and DIMBOA, which subsequently decompose in water to form BOA and MBOA, respectively. While these compounds have been shown to be allelopathic, little is know about their effect on nematodes or the fate of these compounds in soil. The objective of this study was to determine the in vitro toxicity of DIBOA, DIMBOA, BOA, and MBOA against different stages of the plant-parasitic nematodes *M. incognita* and *Xiphinema americanum*.

MATERIALS AND METHODS

Nematodes. *M. incognita* race 1 originally isolated from a field near Salisbury, MD, and cultured on greenhouse-grown pepper (*Capsicum annuum*) cv. PA-136 was used in all assays. Individual egg masses were picked from the roots, placed in water for

30 min, and rinsed with sterile deionized water (DI) three times. Egg masses were transferred to a 25-ml scintillation vial, 0.5% sodium hypochlorite was added, and the eggs were agitated for 3 min and allowed to settle for 30 s. Sterilized eggs were retained on a 500-mesh sieve, collected in sterile DI water, and used immediately in assays. To obtain second-stage juveniles (J2), sterilized eggs were placed on a Baermann funnel to hatch; 72 h later J2 were collected and used. *X. americanum* was obtained from a greenhouse population maintained on sudangrass (*Sorghum sudanense*) in Arendtsville gravelly loam soil; the nematodes were extracted on a Baermann funnel and used immediately.

Chemicals. The following chemicals were prepared as 100 µg/ml stock solutions in sterile DI water and stored at 4°C until use: BOA and MBOA, bought commercially from Sigma-Aldrich (St. Louis, MO); DIMBOA, isolated from corn according to procedures described by Klun et al. (8); and DIBOA, synthesized following the methods of Sicker et al. (23). Dilutions of 0, 1, 5, 10, 25, 50, 75, and 100 µg/ml were prepared for each chemical immediately before use in assays. These concentrations represented a range of hydroxamic acid concentrations previously measured in plant material (20). To confirm the starting concentration and to quantify changes in concentration during the assays, samples of the highest concentration of all chemical dilutions were collected at the beginning and end of an assay and analyzed by liquid chromatography coupled to triple quadruple mass spectrometry using electrospray ionization methods (LC/ESI/MS-MS) following previously described methods (20). The LC instrument was a Waters 2690 XE Separation module and the LC column was a 5-µm Waters X-Terra MS C-18 column (2.1 × 150 mm) (Waters Corp., Milford, MA). The pH of all solutions was measured.

Assays. *Meloidogyne incognita* eggs and J2 were exposed to chemicals in polystyrene 24-well cell culture plates (Costar, Corning, NY); each well contained 1 ml of solution. After chemical dilutions were added in 900 µl of sterile DI water, each well received approximately 200 eggs or 50 J2 of *M. incognita* in 100 µl of sterile DI water. In separate assays, juveniles and adults of *X. americanum* were exposed to chemicals in 2-ml polystyrene sample cups (VWR, Bridgeport, NJ), each containing 500 µl of solution; chemical dilutions were added to each cup in 450 µl of sterile DI water and made up to 500 µl with DI water. Twenty mixed-stage *X. americanum* were hand picked into each cup. The final concentration of chemical to which nematodes were exposed was 0, 0.9, 4.5, 9, 22.5, 45, 67.5, and 90 µg/ml. A water-only control was included for each nematode and life stage. All chemical dilutions were replicated five times and assays were conducted twice. The plates and cups were covered and incubated at 24 and 25°C for *M. incognita* and *X. americanum*, respectively. Exposure periods depended on the nematode life stage: 1 week for *M. incognita* eggs (16), 48 h for *M. incognita* J2 (13), and 24 h for *X. americanum* juveniles and adults.

Egg hatch of *M. incognita* was determined by counting the number of hatched J2 in wells that had received eggs. Motility of *M. incognita* J2 added to wells was assessed by counting moving versus straight nematodes. To determine the effects of the compounds on J2 mortality, the chemicals were removed after 48 h and replaced with sterile DI water, and moving versus straight nematodes were counted again 24 h later; J2 that moved were considered alive and those that did not move were considered dead. *Xiphinema americanum* juveniles and adults were considered dead if they were not moving and did not move when gently probed with a #08 root canal file.

Statistical analyses. Results from repeated assays were similar; therefore they were combined for analysis. All nematode mortality data were expressed as a percentage decrease of the number of nematodes surviving in the water controls. For chemical–nematode dose-response curves, PROC NLIN (SAS Institute, Cary, NC) was used to fit Gompertz and log-linear regression models to the percentage of inactive or unhatched nematodes relative to concentrations. Nonlinear regressions for each data set were used to estimate the concentration that caused 50% nematode mortality (LC_{50}, 50% lethal concentration). Significant differences among LC_{50} values between treatments and nematode life stages were determined using PROC MIXED. Means were compared with Sidak P value adjustments ($P < 0.05$).

RESULTS

The pH of the water control was 6.5. As the concentration of BOA and MBOA increased, the pH remained relatively constant, ranging from 6.5 to 6.8. The pH decreased with increasing concentrations of DIBOA and DIMBOA. The pH for these compounds decreased from 6.7 for 0.9 µg/ml to 5.7 for 90 µg/ml. The concentrations of BOA and MBOA to which nematodes were exposed remained constant over the duration of the assay (Table 1). At the beginning of the assay, however, there was 27% MBOA in the DIMBOA standard. Furthermore, DIMBOA converted to MBOA during the assay, with about half of the DIMBOA standard being MBOA after 168 h. This 1-week period was the length of time *M. incognita* eggs were exposed to the compounds. Low concentrations of BOA were present in the DIBOA standard at the beginning and end of the assay (Table 1). Recovery of DIBOA at the end of the experiment was 89%, indicating that unknown degradation products may have been present.

Neither MBOA nor BOA was toxic to *X. americanum* at 90 µg/ml (the highest concentration tested) after 24 h, although nematode movement was noticeably sluggish after exposure to MBOA (data not shown). Based upon their LC_{50} values, DIBOA and DIMBOA differed in their toxicity against *X. americanum* ($P < 0.001$) (Table 2). Near the LC_{50} values for both compounds, toxicity increased greatly and followed a Gompertz model (Fig. 1). For both compounds, there was no increase in nematode mortality up to a concentration of 9 µg/ml. At the highest concentration, however, nematode mortality was more than 80% compared with that of the water control.

Because exposure of *M. incognita* eggs to BOA, MBOA, and DIMBOA did not result in >50% reduction in egg hatch relative

TABLE 1. Concentrations of hydroxamic acids measured at the beginning and end of assays[y]

Compound added	Time (h)	Compound detected (µg/ml)				Total hydroxamic acids (µg/ml)	% Recovery of parent compound[z]
		BOA	DIBOA	MBOA	DIMBOA		
BOA	0	0.49	…	…	…	0.49	98
	168	0.60	…	…	…	0.60	120
DIBOA	0	0.03	0.61	…	…	0.65	122
	168	0.05	0.42	…	…	0.47	84
MBOA	0	…	…	0.62	…	0.62	123
	168	…	…	0.63	…	0.63	125
DIMBOA	0	…	…	0.07	0.19	0.26	38
	168	…	…	0.14	0.16	0.30	32

[y] LC/ESI/MS-MS method from Rice et al. (20).
[z] Based upon an expected recovery of 0.5 µg/ml of each individual compound.

to the water control, LC_{50} values could not be calculated for these compounds and means could not be compared. The LC_{50} value against *M. incognita* eggs for DIBOA was 74.3 µg/ml (Table 2).

After a 48-h exposure, all of the tested compounds increased *M. incognita* J2 mortality (Table 2; Fig. 2). At the highest concentration, DIBOA and DIMBOA resulted in 73 and 71% apparent mortality, respectively (Fig. 2). Based upon the LC_{50}, DIBOA was the most toxic hydroxamic acid to *M. incognita* J2 ($P < 0.001$) (Table 2). An LC_{50} value could not be calculated for DIBOA's degradation product BOA because apparent mortality was <50%. There was no difference in the LC_{50} values after 48-h exposure for DIMBOA and its degradation product MBOA. Both DIBOA and DIMBOA demonstrated similar Gompertz dose-response curves with very steep increases in toxicity around the LC_{50} (Fig. 2). In contrast, the data for MBOA and BOA were better described by log-linear response curves, which indicated gradual increases in toxicity across the concentrations tested.

Some of the effects of DIBOA against *M. incognita* J2 appeared to be nematistatic rather than nematicidal, because some J2 recovered when the compound was replaced with water. At 90 µg DIBOA/ml, mortality was 73% before rinsing but only 59% after rinsing (Figs. 2 and 3), and rinsing reduced mortality and increased the LC_{50} (Table 2). Because rinsing is unlikely to revive dead J2, we infer that some of the nematodes scored as dead before rinsing were alive, and we have used the term "apparent mortality" in Figures 2 and 3. Whereas DIBOA had some nematistatic activity, the other tested compounds did not. At 90 µg DIMBOA/ml, rinsing did not reduce mortality and did not change the LC_{50}. For MBOA, rinsing increased J2 mortality and decreased the LC_{50} (Table 2). Rinsing also reduced the LC_{50} for BOA (Table 2). After rinsing, the DIBOA and DIMBOA dose-response curves continued to follow a Gompertz model, with a rapid increase in mortality around the LC_{50} (Fig. 3). In contrast, MBOA caused gradual increases in mortality, whether the J2 were rinsed (Fig. 3) or not (Fig. 2).

TABLE 2. Hydroxamic acid concentrations causing 50% mortality (LC_{50}) of *Meloidogyne incognita* and *Xiphinema americanum*

Nematode and life stage	Compound	LC_{50} (µg/ml)[t]
M. incognita egg[u]	DIBOA	74.3 ± 0.5 a
	BOA	*[v]
	DIMBOA	*
	MBOA	*
M. incognita J2[w]	DIBOA	20.9 ± 0.1 d
	BOA	*
	DIMBOA	46.1 ± 1.7 bc
	MBOA	49.1 ± 1.4 b
M. incognita J2 rinsed[x]	DIBOA	40.7 ± 1.7 c
	BOA	10.1 ± 0.1 f
	DIMBOA	43.1 ± 1.0 c
	MBOA	16.7 ± 0.3 e
X. americanum[y]	DIBOA	18.4 ± 0.6 de
	BOA	NE[z]
	DIMBOA	48.2 ± 1.0 b
	MBOA	NE

[t] Values are the means (± one standard error) of 10 replications (data from two assays combined). Values followed by different letters are different ($P < 0.001$) according to Sidak P value adjustments.
[u] After 168 h exposure.
[v] An asterisk indicates that a 50% reduction in hatch or mortality was not obtained.
[w] After 48 h exposure.
[x] Rinsed with sterile deionized water and counted 24 h later.
[y] After 24 h exposure.
[z] NE = no effect on nematode mortality.

DISCUSSION

Rye cover crops have been used for *Meloidogyne* spp. management with mixed results. The incorporation of rye suppressed *M. incognita* J2 temporarily (7) and limited the damage to cotton caused by *M. incognita* (12). Conversely, rye was not particularly effective in decreasing *M. arenaria* (11) or *M. incognita* (14) populations. To intelligently use rye cover crops for plant-parasitic nematode management, it is necessary to understand how these crops suppress nematodes. One possible mechanism concerns nematicidal compounds released during decomposition. These nematicides may include low molecular weight organic acids (10), phenolic compounds (22,27), or hydroxamic acids (3, 4,19,20). The current study reports the acute toxicity of hydroxamic acids in vitro against *X. americanum* and *M. incognita*; their direct application to a soil environment demands caution and will be discussed.

Xiphinema americanum adults and juveniles were more sensitive to DIBOA and DIMBOA than *M. incognita* J2. The LC_{50} values were similar for both nematodes, but maximum mortality was greater for *X. americanum*. These plant-parasitic nematodes are in

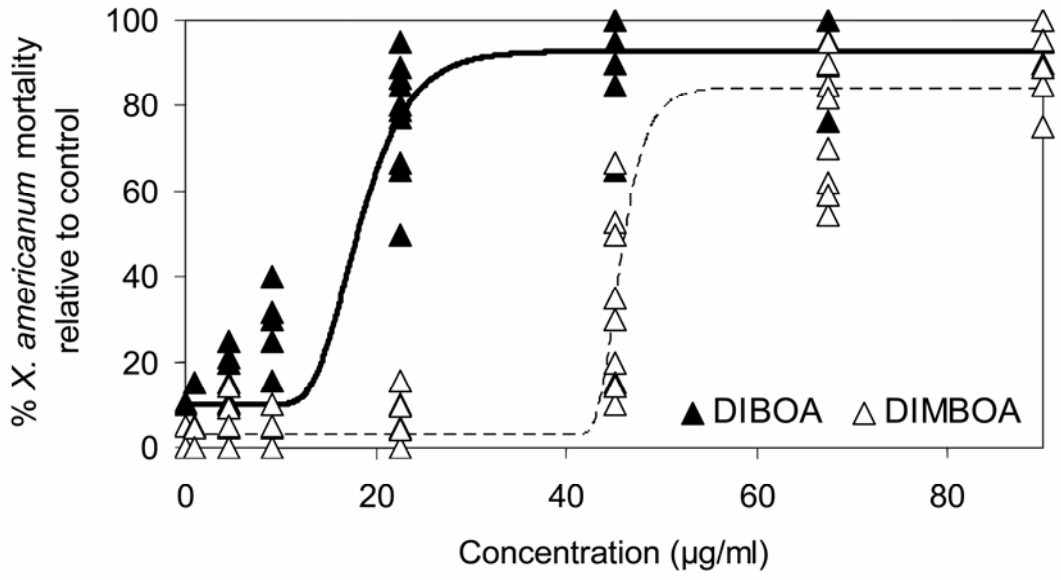

Fig. 1. Mortality of *Xiphinema americanum* after 24 h incubation in 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) or 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) in vitro. Data are from two assays, and each data point is from one replicate. Lines represent Gompertz response curves.

different taxonomic classes (class Adenophorea for *X. americanum* and class Secernentea for *M. incognita*). Nematodes in these two classes often respond differently to tillage, fertilizers, and nematicides (21,24,25), with adenophorean nematodes in general being more sensitive.

In this study, the hydroxamic acids reduced *M. incognita* J2 motility and to a lesser extent egg hatch. Differences in susceptibility of life stages to control practices have been reported (30). The egg is often one of the most resistant stages in the nematode life cycle, possibly due to its three-layer shell (26). If the egg stage is the target, our data indicate that higher concentrations of DIBOA would need to be present.

In general, DIBOA was more toxic than DIMBOA, particularly at lower concentrations. DIBOA is the predominant hydroxamic acid in rye, while DIMBOA occurs at very low concentrations in rye but at high concentrations in corn and wheat (4,20). No other studies on the toxicity of DIBOA against plant-parasitic nematodes have been conducted. It was interesting that the LC_{50} for DIBOA against *M. incognita* J2 increased when the compound was removed and replaced with water, indicating that this compound was nematistatic rather than nematicidal. Higher exposure concentrations will be needed for this compound to be toxic. DIBOA was also more toxic than its degradation product BOA. Although a low LC_{50} for rinsed *M. incognita* J2 was calculated for BOA, the maximum mortality was greater for DIBOA. DIBOA is more phytotoxic to weeds than BOA (1,2).

The toxicity of DIMBOA against *X. americanum* and *M. incognita* was more difficult to assess than that of the other chemicals. This was due to the conversion of DIMBOA to MBOA over the course of the assays. DIMBOA and MBOA resulted in similar *M. incognita* J2 mortality after 48 h, and while DIMBOA's efficacy did not change after the compound was removed, MBOA continued to cause mortality even after removal. Although DIMBOA was toxic to the nematodes tested in our study,

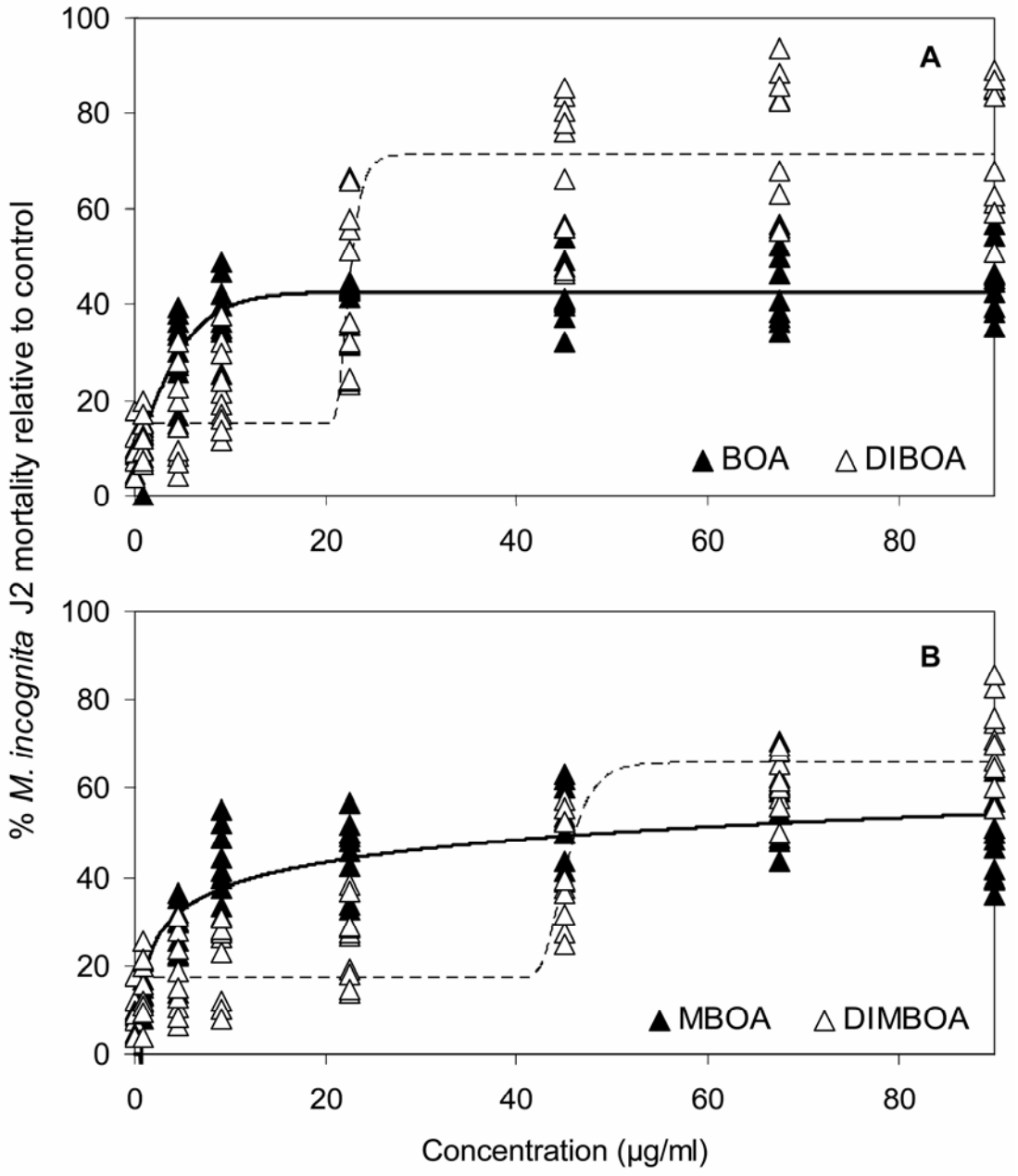

Fig. 2. Apparent mortality of *Meloidogyne incognita* second-stage juveniles (J2) after 48 h incubation in **A,** 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) or benzoxazolin-2(3*H*)-one (BOA) and **B,** 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) or 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA) in vitro. Data are from two assays, and each data point is from one replicate. Lines represent Gompertz response curves (for DIBOA and DIMBOA) or log-linear response curves (for BOA and MBOA).

DIMBOA at low concentrations behaved as an attractant for *Pratylenchus zeae* (4).

The response curves for the hydroxamic acids against the different nematode species and life stages demonstrated differential activity across the range of concentrations tested. DIBOA and DIMBOA followed a Gompertz model with very rapid increases in toxicity around the predicted LC_{50} values for *X. americanum* and *M. incognita* J2. In contrast, MBOA resulted in a gradual increase in *M. incognita* J2 mortality across concentrations. The practical implication of this finding is that a higher concentration of DIBOA and DIMBOA will have to be present to result in appreciable nematode mortality, while greater mortality will occur at lower concentrations of MBOA.

It is possible that other degradation chemicals contributed to nematode mortality in this study. At the end of the assays, DIBOA comprised less than 100% of the sample analyzed by LC/ESI/MS-MS, indicating that other undetected compounds were present. Additional compounds, including phenolic acids, have been identified in rye (22,27), but their toxicity against nematodes is unknown. The only other compounds produced by rye that have been investigated for their effects on nematodes were low molecular weight organic acids. A 2% (dry wt/wt) application of rye suppressed *M. incognita*, but the production of organic acids was not solely responsible for this suppression (10).

These assays have shown that selected hydroxamic acids found in rye were highly toxic to plant-parasitic nematodes in vitro. An essential question is whether nematodes can be exposed to lethal concentrations of these compounds in soil (5). Several studies have estimated hydroxamic acid content of plant material (1,3,15, 19,20), but few have determined whether these concentrations were maintained upon the incorporation of plant material into soil (17). Hydroxamic acids are also added to the soil as root exudates. For example, root exudates from rye contained 25 mmol DIBOA/kg fresh weight and were allelopathic against *Avena fatua*

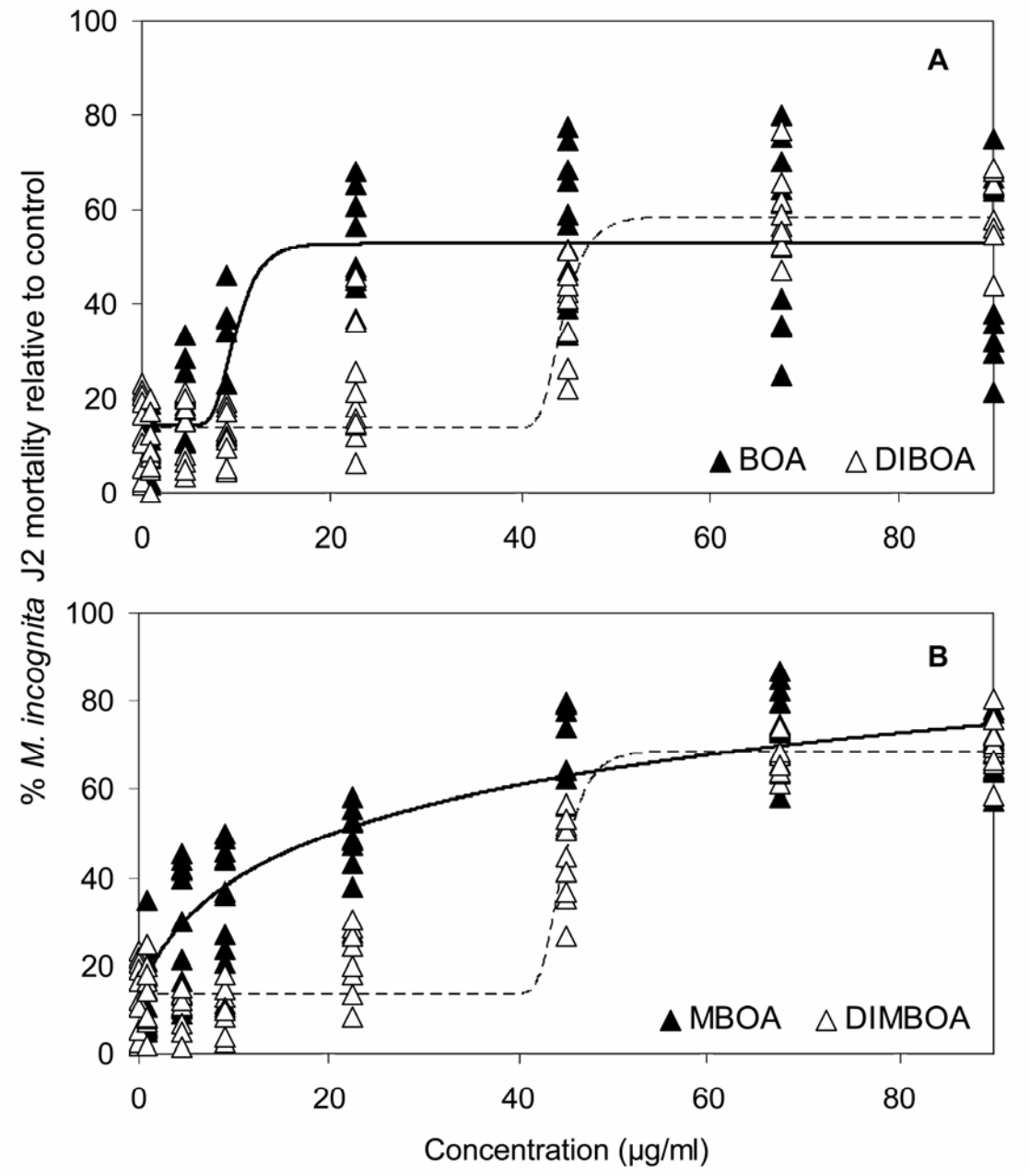

Fig. 3. Apparent mortality of *Meloidogyne incognita* second-stage juveniles (J2) that were incubated for 48 h in **A,** 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) or benzoxazolin-2(3*H*)-one (BOA) and **B,** 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) or 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA) and then rinsed and incubated in water for 24 h in vitro. Data are from two assays, and each data point is from one replicate. Lines represent Gompertz response curves (for DIBOA and DIMBOA) or log-linear response curves (for BOA and MBOA).

(17). Moreover, the concentration of DIBOA was greater in the exudates than in the root extracts, indicating that it may be possible to achieve toxic concentrations of hydroxamic acids in soil. Further research is needed to determine the activity of hydroxamic acids against nematodes in soil solution.

Hydroxamic acids in soil can probably be quantified with the LC/ESI/MS-MS method used in this study. Using LC/ESI/MS-MS to determine hydroxamic acid concentrations in plant material, Rice et al. (20) reported minimum detection limits ranging from 0.1 to 1.1 µg/g dry weight values which were more than 10 times lower than those reported using more conventional analytical methods. This LC/ESI/MS-MS method was designed to rapidly and accurately analyze crude extracts rather than to maximize detection of small concentrations of hydroxamic acids. It should be possible to modify this method to increase sample size (10- to 100-g soil samples) and to substantially increase the sensitivity with solid phase clean-up of the soil organic extracts. Thus, it may be possible to detect concentrations of hydroxamic acids as small as 0.005 to 0.01 µg/g dry weight soil, concentrations which are relevant to the toxicity of these compounds to nematodes.

Demonstration of nematicidal compounds in rye suggests the potential of developing a practical nematode management strategy using rye as a green manure crop. But determining the toxicity of specific compounds involved in nematode suppression is only part of the information needed to optimize this management tactic. In addition to determining the concentration of these compounds in soil, an appropriate cultivar containing large concentrations of hydroxamic acids must be used. 'Wrens Abruzzi' is the most common cultivar evaluated in previous nematode studies (7,10,11, 14); other cultivars higher in hydroxamic acids such as 'Wheeler' and 'Bonel' require evaluation (3,19). A high content of hydroxamic acids becomes even more important because rye cover crops are usually incorporated at a time when hydroxamic acid content is less than maximum (19,20). Timing of incorporation should coincide with the activity of *M. incognita* J2 and *X. americanum* in the soil. Timing is critical because hydroxamic acids do not persist for long periods. For example, DIBOA and BOA concentrations in rye residue declined 50% after 10 days, and the compounds were undetectable beyond 170 days (29). DIMBOA's degradation rate varied greatly with temperature and pH (28). Further research is needed to understand the fate of hydroxamic acids in soil and their use for control of plant-parasitic nematodes.

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