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DIBOA: Fate in soil and effects on root-knot nematode egg numbers

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ABSTRACT

The benzoxazinoid 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) is produced by rye (*Secale cereale*) and may contribute to plant-parasitic nematode suppression when rye plants are incorporated as a green manure. We investigated the fate of DIBOA in soil and DIBOA's effects on nematode reproduction. Soil in plastic bags was treated with DIBOA at concentrations ranging from 1.1 to 18 μg g^{-1} dry soil, and with the root-knot nematode *Meloidogyne incognita*. Control soils were treated with water or with 0.31% methanol, with or without nematodes. DIBOA concentrations extracted from the soil were measured at selected times for 5 consecutive days. The soil from each bag was then placed into a pot in the greenhouse, and a cucumber seedling was transplanted into each pot. Five weeks later, only the highest DIBOA concentration, 18 μg g^{-1} soil, reduced nematode egg numbers. At 0 h, DIBOA measured in soil ranged from 19.68 to 35.51% of the initial DIBOA concentration, and was dependent on the concentration added to the soil. DIBOA half-life was from 18 to 22 h, and very little DIBOA was present in soil after 120 h. Identified breakdown products accounted for only 4% at maximum of the initially added DIBOA. The results of our study demonstrate that high soil concentrations of DIBOA are necessary to suppress *M. incognita*; DIBOA may not be a major factor in nematode suppression by a rye cover crop.

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1. Introduction

Winter rye (*Secale cereale*) utilized as a cover crop has many beneficial attributes. Rye can be planted in the fall, grows quickly, provides abundant above ground biomass and produces a good root system to minimize erosion (Bowman et al., 2007). Rye also takes up nutrients, and in particular, captures residual nitrogen that is later released for the subsequent crop. In addition, rye cover crops suppress weeds and insect pests (Bowman et al., 2007).

Among its beneficial effects, rye has been reported to reduce gall formation or to lower populations of plant-parasitic nematodes in some cropping systems. For example, in a rye-tomato (*Solanum lycopersicum*) rotation, *Meloidogyne hapla* populations were reduced compared with populations recorded with other cover crops (McKeown et al., 1998). In other studies, fresh rye amendment decreased numbers of galls formed by *Meloidogyne incognita* on tomato (McBride et al., 2000), and rye did not increase galling on a subsequent cotton (*Gossypium hirsutum*) crop compared with galling on cotton after hairy vetch (*Vicia villosa*) cover crops (Timper et al., 2006). However, results with rye as a cover crop have been variable. When rye was used as a winter cover crop, *M. incognita* populations could increase on corn (*Zea mays*) (Wang et al., 2004). Also, a rye cover crop was not effective for suppressing populations of *Hoplolaimus columbus* on cotton nor of *M. incognita*, *Helicotylenchus dihystera*, *Heterodera glycines*, *Hoplolaimus galeatus*, *Paratrichodorus minor*, *Pratylenchus* spp. or *Tylenchorhynchus claytoni* on soybean (*Glycine max*) (Pedersen and Rodriguez-Kabana, 1991; Davis et al., 2000).

In addition to activity against nematodes, rye has been shown to exhibit antagonism to other organisms, including plants, fungi, bacteria, and insects (Friebe et al., 1998; Morrissey and Osbourn, 1999; Friebe, 2001; Zasada et al., 2005). Some of the antagonism is due to secondary metabolites, including benzoxazinoids, which are found in some plants including rye, corn and wheat (*Triticum aestivum*) (Friebe, 2001). Two of the more abundant benzoxazinoids present in intact rye plants are found as the glucosides of DIBOA (2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one) and DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one). However, the breakdown products, rather than the glucosides, are the defense compounds (Hashimoto and Shudo, 1996; Zikmundová et al., 2002).

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DIBOA and DIMBOA (which are generally found after plant injury or incorporation into soil, or as root exudates) are formed by deglucosylation of their precursors DIBOA-glucose and DIMBOA-glucose, and then break down further to form BOA (2-benzoxazolinone) and MBOA (7-methoxy-2-benzoxazolinone), respectively (Hietala and Virtanen, 1960; Wu et al., 2001; Macías et al., 2004; Belz and Hurle, 2005; Fomsgaard et al., 2006; Krogh et al., 2006). These products are the most studied. BOA can be transformed to APO (2-amino-3*H*-phenoxazin-3-one) (Macías et al., 2005a). DIBOA may also be transformed to HBOA (2-hydroxy-1,4-benzoxazin-3(2*H*)-one) (Krogh et al., 2006). A similar reaction occurs with DIMBOA, where HMBOA (2-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one) is formed. Others report that these products occur by glucosidase hydrolysis of the glycosylated forms of the compounds (Hofman and Hofmanová, 1969; Cambier et al., 1999).

In studies on activity of these compounds against plant-parasitic nematodes, DIBOA, BOA, DIMBOA and MBOA were all toxic to *M. incognita* second-stage juveniles (J2), the life stage that hatches from eggs, although only DIBOA strongly reduced egg hatch (Zasada et al., 2005). DIBOA and DIMBOA were also toxic to mixed life stages (juveniles and adults) of the plant-parasitic nematode *Xiphinema americanum*; BOA and MBOA were not. All four compounds, along with DIMBOA-glucoside, HMBOA and DIBOA-glucoside, were also present in shoots and roots of six tested cultivars of rye (Zasada et al., 2007). When compared to the results from the laboratory assays, total benzoxazinoid quantities were potentially able to affect nematode viability upon incorporation of a rye cover crop into soil, although amounts in tissues do vary with age of the plant (Reberg-Horton et al., 2005; Rice et al., 2005).

Determining the degradation rates of these compounds and the concentrations needed to adversely affect nematodes in a soil environment should aid in understanding their potential role in suppressing nematode populations, and in improving the utilization of a rye cover crop as a nematode management tool. To this end, DIBOA was selected for further study. There were two primary goals of this investigation. One was to determine whether DIBOA was active against plant-parasitic nematodes in soil, and therefore a factor in suppressing reproduction of *M. incognita* when rye is incorporated as a cover crop. This is the first investigation undertaken to correlate concentrations of DIBOA in soil with plant-parasitic nematode suppression. The second goal was to determine the fate of DIBOA in soil at environmentally relevant concentrations and under a likely exposure scenario, and to study the presence and relative concentrations of breakdown products under these conditions.

2. Materials and methods

2.1. Solutions and chemicals

Chemicals were obtained from the following sources: BOA (98% purity) was bought commercially from Sigma–Aldrich (St. Louis, MO, USA) and DIBOA (estimated >95%) was synthesized by Rice and colleagues as discussed in Rice et al. (2005). APO (100%) and AAPO (2-acetylamino-3*H*-phenoxazin-3-one) (100%) were obtained from F. Macías (University of Cádiz, Spain). Synthetic HBOA was kindly provided by D. Sicker (Institute of Organic Chemistry, University of Leipzig, Germany).

2.2. DIBOA and nematode treatment of soil

All experiments were conducted using a Norfolk A loamy sand soil (sand:silt:clay 87:6:7; pH 7.3; organic matter 0.4%). Soil was air dried, passed through a 2-mm sieve and stored at 4 °C until use. The soil was placed into sealable plastic bags (bag capacity 3.78 l; 1775 g dry soil/bag). A stock solution of 0.55 g DIBOA in 11 ml methanol was prepared, and this was diluted with water to produce a range of DIBOA concentrations. The soil was moistened to 70% of water holding capacity (0.12 ml water g^{-1} soil) by adding the DIBOA in 213 ml water (5 ml less for bags that were inoculated with nematodes suspended in water). The treatments were five DIBOA concentrations and four controls: (1) 18 μg DIBOA g^{-1} dry soil (150 μg DIBOA ml^{-1} water); (2) 9 μg DIBOA g^{-1} dry soil (75 μg DIBOA ml^{-1} water); (3) 4.5 μg DIBOA g^{-1} dry soil (37.5 μg DIBOA ml^{-1} water); (4) 2.3 μg DIBOA g^{-1} dry soil (18.8 μg DIBOA ml^{-1} water); (5) 1.1 μg DIBOA g^{-1} dry soil (9.4 μg DIBOA ml^{-1} water); (6) water control; (7) 0.31% methanol in water (v/v); (8) water, no nematodes; and (9) 0.31% methanol in water, no nematodes. For nematode inoculum, *M. incognita* Race 1, originally isolated in Maryland, was maintained in a greenhouse on pepper (*Capsicum annuum*) 'PA-136'. Eggs were extracted with 0.6% NaOCl from the roots of 3-month-old pepper plants, stored overnight at 4 °C, and placed in DIBOA-treated or untreated (control) soil the next day. All DIBOA treatments and two controls (6 and 7) received 5000 *M. incognita* eggs in 5 ml water/bag, resulting in ca. 2.8 eggs g^{-1} dry soil. The bags were incubated at 21 °C for 5 days. Soil samples of 15 g were taken from all bags at 0, 6, 12, 24, 48 and 120 h after treatment for DIBOA and DIBOA-metabolite analyses. The samples were stored at −20 °C until analyses could be completed.

2.3. Greenhouse experiments with nematodes

Five days after treatment of soil with DIBOA and inoculation with nematodes, the soil was placed into 15-cm diameter pots. Two-week old cucumber (*Cucumis sativus*) 'Sweet Slice' seedlings started in flats containing seed starter mix (Premier Pro-mix®, Premier Horticulture Inc., Quakertown, PA) were transplanted into the pots (one per pot). Pots were arranged in a randomized complete block design, and plants were fertilized after ca. 2 weeks with 3 g of Osmocote® Plus 15-9-12 NPK fertilizer (Scotts-Sierra Horticultural Products Co., Marysville, OH). The greenhouse was maintained at 24–29 °C, with natural and supplemental lighting combined for a 16 h daylength. Each treatment was replicated eight times and the experiment was repeated for a total of 16 replicates per treatment.

The experiment was terminated after 5 weeks, corresponding with approximately one *M. incognita* generation. At harvest, shoots were cut from the roots, and shoot lengths and fresh weights were measured. The shoots were dried at 60 °C for 8 days and reweighed. Fresh root weights were recorded, and the roots were processed to collect nematode eggs. Roots were cut into pieces, placed in 0.6% NaOCl and blended on a 'low' setting for 1 min, poured onto nested sieves (106 μm and 25 μm diameter openings; 140-mesh over 500-mesh, respectively), and washed with water. The eggs were collected from the sieve with 25 μm openings and stored as aqueous egg suspensions at 4 °C until counting.

2.4. Extraction and analysis of benzoxazinoids from soil

Soil samples, collected at 0, 6, 12, 24, 48, and 120 h after DIBOA addition to soil, were removed from storage and 1 g wet soil immediately weighed into a 15 ml polyethylene tube. Soils were extracted in batches of 18, and each batch included the following quality control checks: (1) a duplicate of a random sample; (2) benzoxazinoid spikes of random samples; (3) a blank dry soil not containing benzoxazinoids; and (4) a blank dry soil with a benzoxazinoid spike. The benzoxazinoid spikes were DIBOA, APO or HBOA, with 1 μg added as methanol solutions to selected samples (matrix spikes) and blank soils, and these were extracted to monitor recoveries. Acidified water (0.5% v/v glacial acetic acid) (5 ml) was added to the soil and the slurry was vortexed for 1 min. After mixing, 5 ml ethyl acetate was added and the sample was

vortexed for another 1 min. The ethyl acetate remained as the uppermost layer and was further clarified by centrifuging the sample at 1400 × *g* for 3 min. The ethyl acetate layer was removed with a Pasteur pipette and placed into a labeled 15 ml tube.

The soil was extracted a second time with 5 ml ethyl acetate and vortexed for 30 s. The sample was centrifuged as above, and the layer of ethyl acetate removed and combined with the first extraction (total volume approximately 10 ml). The ethyl acetate extracts were evaporated to dryness using a TurboVap LV Evaporation System (Zymark Corp., Hopkinton, MA) set at 40 °C and 8–10 PSI. The volumes were adjusted to either 1 ml (for the lower initial concentrations of 2.3 and 1.1 μg DIBOA g^{-1} dry soil), or 2 ml (for the remaining DIBOA initial concentrations) by adding 50:50 water:MeOH to the evaporated sample followed by vortexing for 15 s. For LC/MS-MS analysis, 1 ml of each sample was transferred to an autosampler vial.

The samples were analyzed as described by Rice et al. (2005) utilizing a Quattro LC benchtop triple quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) using the multiple reaction monitoring mode (MRM). Additional analytes were added to the list discussed in Rice et al. (2005) in order to check for additional metabolites of DIBOA, e.g., HBOA, APO, and AAPO. The conditions utilized to detect these additional compounds were similar to those used by Krogh et al. (2006), with the exception that an MRM method was used instead of a single MS. Therefore, the parent to daughter transitions were as follows: 164 > 108.4 *m/z* (negative electrospray ionization), 213 > 185.5 and 255 > 213 *m/z* (positive electrospray ionization), respectively, for HBOA, APO and AAPO with the following retention times: 8.6, 22.2 and 23.8 min. To improve separation of all analytes, methanol was replaced with acetonitrile in the running solvent and the following gradient programs were utilized. Program (1) For DIBOA, HBOA and BOA; 25:75 *A:B* (*A* = 30% acetonitrile mixed with 70% of a 1% formic acid solution and *B* = distilled deionized water). A 25:75 *A*:*B* gradient at time zero went to 65:35 *A*:*B* in 15 min, then returned to the starting conditions where it was held for 4 min before starting the next run. Program (2) APO and AAPO metabolites were measured by modifying Program 1 so that the initial gradient was the same, then at 15 min the gradient changed to 50:50 *A*:*B* up to 20 min where the solvent mix was held for the next 5 min, then returned to 25:75 *A*:*B* for 10 min.

2.5. Quality assurance/quality control (QA/QC)

Limits of detection were not directly assessed for this study. Our limits of quantitation were set by our lowest standards, e.g., 10 ng g^{-1} DIBOA, BOA and APO. Spikes were run with every set of 18 samples as were duplicate pairs and blanks.

2.6. Statistical analysis

Data were arcsine transformed or log transformed ($\log^{10}(x+1)$) prior to analyses when necessary to meet the assumptions of the models; non-transformed data are presented unless otherwise noted. A variance-weighted ANOVA was used and significant differences between treatments were determined. Means were compared with Tukey's adjustment for multiple comparisons ($P < 0.05$). The half-life of DIBOA added to soil was determined using an exponential model. All data was analyzed using the computer software JMP (SAS Institute, Cary, NC).

3. Results

3.1. M. incognita suppression and plant growth

Effects of DIBOA on reproduction of *M. incognita* varied with initial DIBOA concentration. Most of the treatments, including the water + methanol control, had fewer eggs per pot than the water control (Fig. 1). Treatment with water + methanol reduced egg numbers by 21.8% compared to the water control. Nematode egg numbers were the lowest at the highest concentration of DIBOA, 18 μg g^{-1} dry soil. This was the only DIBOA concentration that significantly suppressed reproduction compared with the water + methanol control; the egg numbers were reduced by 26.7% compared to the water + methanol control and 42.7% compared to the water control. None of the treatments affected shoot or root fresh weight or shoot length (data not shown).

3.2. Benzoxazinoids extracted from soil

The largest amount of measured DIBOA at 0 h came from soil that had received the highest initial DIBOA concentrations (9 and 18 μg DIBOA g^{-1} dry soil), and the lowest amount of DIBOA was recovered from soil that had initially been treated with the least DIBOA (1.1 μg DIBOA g^{-1} dry soil) (Table 1). In fact, the recovered concentration of DIBOA at 0 h decreased in a gradient which corresponded to the amount of DIBOA initially added to the soils. Consequently, the percentage of DIBOA accounted for in the soils at 0 h (relative to the DIBOA concentration initially added to soil) appeared to be concentration dependent (Table 1).

The degradation of DIBOA over time was similar across initial concentrations ($P = 0.4$); therefore, only the exponential loss curves for the two highest DIBOA initial concentrations are shown (Fig. 2). The concentration of DIBOA present in soil decreased very rapidly over the first 24 h. Very little DIBOA was present in soil after 120 h, regardless of the initial concentration. At the 18 μg DIBOA g^{-1} dry soil initial concentration only 0.09 (±0.03) μg DIBOA g^{-1} dry soil was accounted for after 120 h. Similarities ($P = 0.3$) in DIBOA degradation among the initial concentrations over time were also demonstrated by similar half-lives of DIBOA in soil, ranging from 17.9 to 22.2 h regardless of initial concentration (Table 2).

Soil samples were also analyzed for the presence of the DIBOA degradation products BOA, HBOA, APO and AAPO. After 0 h there

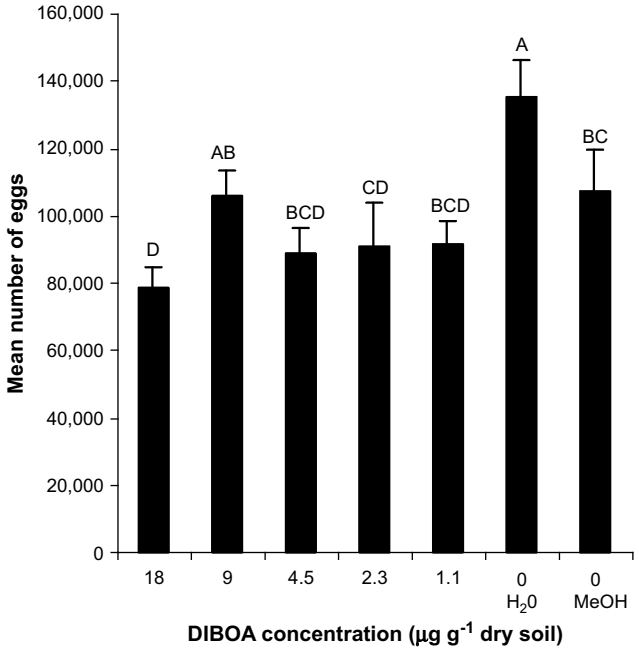

Fig. 1. Numbers of *Meloidogyne incognita* eggs recorded from cucumber plants treated with various concentrations of DIBOA and from control plants. Bars with different letters are significantly different according to Tukey's adjustment for multiple comparisons ($P < 0.05$). Error bars are ±1 standard error.

Table 1
Concentrations of DIBOA recovered from DIBOA-treated soil at 0 h, and percentages of recovered DIBOA relative to the initial DIBOA concentrations added to soil.

Initial DIBOA concentration added to soil (μg g^{-1} dry soil)	DIBOA concentration recovered from soil (μg g^{-1} dry soil)[a]	% of initial DIBOA
1.1	0.23 (±0.03)	19.68 (±2.59) a
2.3	0.61 (±0.12)	22.79 (±3.43) ab
4.5	1.09 (±0.1)	26.96 (±2.16) ab
9.0	3.16 (±0.17)	35.14 (±1.89) b
18.0	6.06 (±0.46)	35.51 (±1.67) b

[a]Data were arcsine transformed prior to analysis; non-transformed data are presented.
Values followed by different letters are not significantly different ($P = 0.01$). $N = 6$; ±1 standard error.

was significantly more ($P < 0.003$) HBOA (1.14% of DIBOA initially added) recovered from soil than BOA (0.75% of DIBOA initially added) across all DIBOA initial concentrations. The percentage of DIBOA that occurred in these transformed states as either HBOA or BOA at 0 h was not different across initial DIBOA concentrations ($P = 0.57$ and 0.61, respectively). When APO, BOA and HBOA data were normalized for percentage that was measured relative to initial DIBOA concentration and analyzed, time was the only significant factor in the model ($P < 0.001$ for both HBOA and BOA). Therefore, the data were combined across DIBOA initial concentrations for each compound (Fig. 3). The amount of BOA recovered from soil remained constant up to 24 h and then declined. The amount of HBOA recovered from soil started to decline after 12 h. At the initial concentration of 18 μg DIBOA g^{-1} dry soil, HBOA and BOA concentrations in soil were never greater than 0.3 and 0.2 μg g^{-1} dry soil, respectively, over time. Trace amounts of APO and AAPO were measured from the DIBOA-treated loamy sand soil, but only APO occurred consistently over all of the treatments (Fig. 3). Furthermore, APO was recovered at very low concentrations relative to initial DIBOA concentrations, with a maximum of only 0.04% of the initial DIBOA concentrations at 6 h. The concentrations at the various sample times did not significantly differ from each other

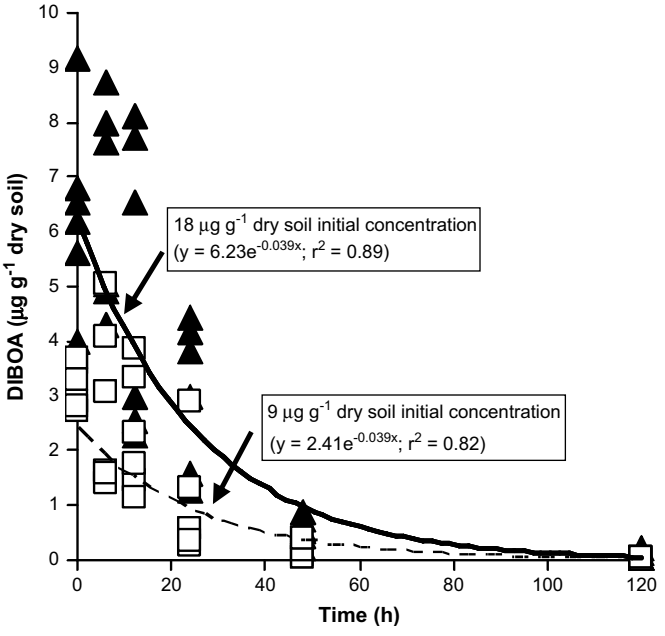

Fig. 2. DIBOA degradation over time at two initial concentrations, 18 (▲) and 9 (□) μg DIBOA g^{-1} dry soil.

Table 2
Half-life of DIBOA in a loamy sand soil when added at different initial concentrations.

Initial DIBOA concentration added to soil (μg g^{-1} dry soil)	Exponential equations	R^2 values for exponential equations	Half-life (h)[a]
1.1	$y = 0.18e^{-0.03x}$	0.83	21.8 (±1.0)
2.3	$y = 0.44e^{-0.035x}$	0.79	20.1 (±0.9)
4.5	$y = 1.05e^{-0.035x}$	0.70	22.2 (±3.5)
9.0	$y = 2.41e^{-0.039x}$	0.82	17.9 (±0.3)
18.0	$y = 6.23e^{-0.039x}$	0.89	18.2 (±1.0)

[a] Data were log transformed prior to analysis; non-transformed data are presented. Values were not significantly different ($P = 0.3$). $N = 6$; ±1 standard error.

(Fig. 3); however, this was mainly due to the high variability of the data which resulted from problems with analyzing values that were near the analytical detection limits of this compound.

3.3. QA/QC

Recoveries averaged from 72 to 90% for BOA, HBOA and DIBOA. Matrix spikes for these compounds were higher, varying from 93 to

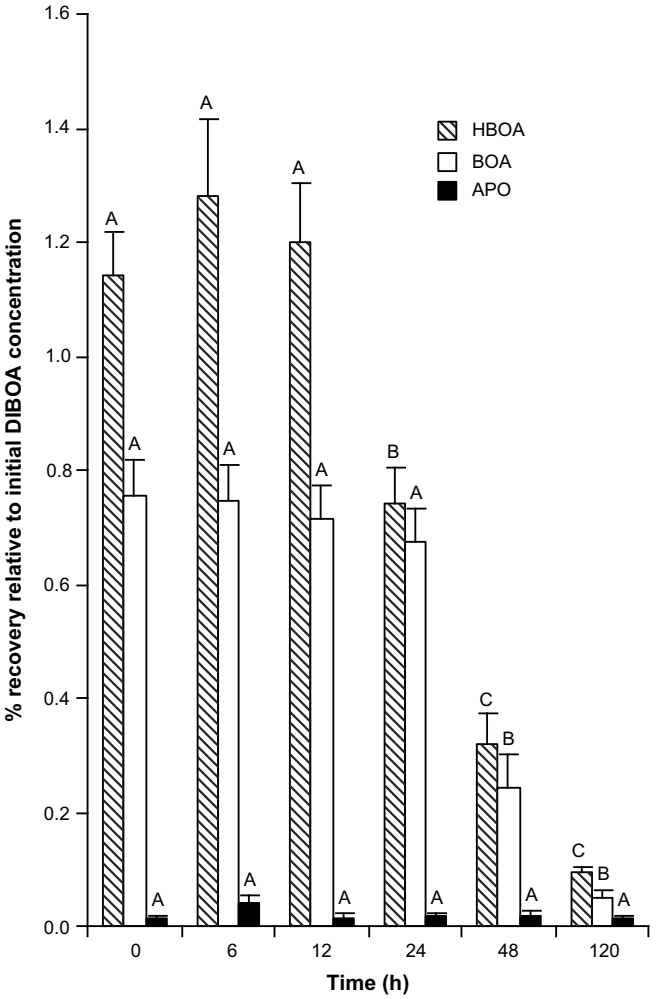

Fig. 3. Amounts of APO, BOA and HBOA recovered over time, expressed as percentage of the initial DIBOA concentrations added to a loamy sand soil. Bars with different letters within a compound are significantly different ($P < 0.001$) according to Tukey's adjustment for multiple comparisons for both HBOA and BOA; values were not significantly different ($P > 0.05$) for APO. $N = 30$. Error bars are ±1 standard error.

150%. Recovery of APO was lower, averaging 49%. All blanks lacked measureable concentrations of the monitored analytes. Duplicates averaged 23.2% relative percent difference (RPD) for the duplicate pairs in Trial 1 and 18.6% RPD for the duplicate pairs in Trial 2. This variation, while a bit high, is not unusual for soil samples.

4. Discussion

4.1. DIBOA concentrations and nematode suppression

Incubation of nematodes in DIBOA-treated soil, followed by inoculation of the nematodes onto roots of cucumber plants in the greenhouse, demonstrated that a high concentration of DIBOA was needed in soil to suppress *M. incognita* reproduction on plant roots. A rye cover crop has the potential to produce the equivalent of ca. 0.5 μg DIBOA g^{-1} soil or less, depending on such factors as the rye cultivar and the time of collection (Reberg-Horton et al., 2005; Rice et al., 2005). In our earlier laboratory assays, the lethal concentration of DIBOA necessary to result in 50% mortality (LC_{50}) of eggs was 74.3 μg DIBOA ml^{-1} water, and for J2 the LC_{50} was 40.7 μg DIBOA ml^{-1} water (Zasada et al., 2005). In our greenhouse study, the addition of 9 μg DIBOA g^{-1} dry soil (=75 μg DIBOA ml^{-1} water), which is equal to the LC_{50} for eggs and greater than the LC_{50} for J2, did not significantly suppress *M. incognita* reproduction on cucumber roots. Of the concentrations that we tested in soil, suppression of egg numbers occurred only with an initial concentration of 18 μg DIBOA g^{-1} dry soil (=150 μg DIBOA ml^{-1} water added to the soil); this is twice our calculated LC_{50} for eggs and more than three times the calculated LC_{50} for J2 (Zasada et al., 2005). We postulate that the different results in water vs. soil tests may in part be due to microbial degradation of DIBOA in the latter study and also to soil binding of DIBOA, with the ratio of soil to DIBOA concentration an important factor in the amount available in soil.

4.2. Persistence of DIBOA in soil

In our study, the amount of DIBOA recovered from a loamy sand soil immediately after application averaged < 35.5% of the DIBOA initially added, and the half-life for disappearance of DIBOA varied from 17.9 to 22.2 h, tending to decrease with increasing initial DIBOA concentration. Conversely, in a study utilizing much higher doses of DIBOA than we applied, the half-life of DIBOA increased with increasing initial DIBOA concentration (Macías et al., 2005a). The half-life of DIBOA applied at 1 mg g^{-1} soil was 28 and 24 h, depending on the soil tested. However, the half-life of DIBOA applied at 5 mg g^{-1} soil was 57 and 62 h for the same two soils (Macías et al., 2005a). The lower DIBOA concentrations added in our study were closer to estimated amounts that might be found after the incorporation of a rye cover crop into soil.

DIBOA has low stability in water and breaks down spontaneously, although microbial degradation has also been reported (Friebe et al., 1998; Macías et al., 2005a; Krogh et al., 2006). Because the DIBOA rapidly disappeared from or reacted with the soil in our investigation, and had a relatively short half-life, DIBOA may not be the most ideal plant-derived compound with which to achieve plant-parasitic nematode mortality. Incorporation of a rye cover crop into soil might not generate toxic concentrations of DIBOA that would, as a sole factor acting against nematodes, result in significant suppression of nematode numbers. However, at the highest concentration of DIBOA tested, 18 μg g^{-1} dry soil, we did achieve 43% reduction in *M. incognita* egg numbers compared to the water control. This amount of suppression, in addition to the fact that rye is a poor host for this nematode (Wang et al., 2004; Zasada et al., 2007), may combine to provide some nematode control.

4.3. Recovery and persistence of DIBOA degradation products in soil

We were also interested in the occurrence/disappearance of the DIBOA degradation products HBOA, BOA, AAPO and APO in soil and what role they might play in nematode suppression. For example, Macías et al. (2005a) suggested that as DIBOA concentration in soil increases, there might be some toxicity to the microflora, causing a reduction in bacterial activity at higher doses. They specifically mentioned that there might be effects of the degradation product APO, which is known to have toxicity towards certain microbes (Atwal et al., 1992).

Our spike recoveries of HBOA, BOA, and AAPO all averaged greater than 65%. Recovery of APO was lower, averaging 49%. Zikmundová et al. (2002) observed that some fungi degraded HBOA and BOA to additional products; however, these were not searched for in this study, mainly because standards were not available.

The time-dependent progression of the metabolites HBOA and BOA from DIBOA degradation was similar to that reported by Krogh et al. (2006), who observed the entire degradation sequence from DIBOA-glucoside to APO, starting with DIBOA-glucoside in incorporated rye tissue. The study by Krogh et al. (2006) and our research found that HBOA exceeded BOA during early stages of incubation. However, our studies differed in relative DIBOA:HBOA concentrations. Krogh et al. (2006) found an initial increase and then a decrease in DIBOA concentrations, and then observed an HBOA concentration which exceeded the highest DIBOA concentration. Our measured HBOA concentrations did not exceed DIBOA concentrations. The difference between the two investigations may have been because we recovered a higher percentage of the spiked DIBOA, averaging >70%, while Krogh et al. (2006) had a lower DIBOA recovery of ca. 5%. They did suggest that DIBOA was transformed to HBOA, and stated that this claim needs further confirmation. Our data seem to support this possibility. The effects of the formed HBOA on the *M. incognita* egg numbers cannot be determined, as it is unknown if this compound is biologically active against nematodes.

BOA was produced and declined slowly over time in our study. While Krogh et al. (2006) similarly found little BOA in their plant incorporation experiments, the production of BOA from DIBOA during incubation of soil slurries reported by Macías et al. (2005a) was much higher. BOA can be generated by a spontaneous loss of formic acid from DIBOA (Bredenberg et al., 1962) and the transformation of DIBOA to BOA in a buffered aqueous solution in a pH range of 4–8 was shown to be equimolar. The rapid appearance of BOA at 0 h in our experiment may have resulted from this rapid, spontaneous formation of BOA from DIBOA, and there also seems to have been a rapid formation of HBOA in these tests. With regard to toxicity against nematodes, BOA was generally the least toxic compound tested in our initial screening assay (Zasada et al., 2005). In fact, an LC_{50} for *M. incognita* eggs could not be generated for this compound. This, in combination with the very small concentrations of BOA that were measured from DIBOA-treated soil over time, indicates the minimal role that BOA probably plays in nematode suppression.

APO concentrations were very low in our experiment, and also seemed to be a function of the amount of BOA available for transformation. Microbial degradation of DIBOA-glucoside in soil may lead to DIBOA and BOA production, with APO as the final product (Macías et al., 2005a); the molar ratio for occurrence of APO from BOA was reported as 1:10 (APO:BOA) (Krogh et al., 2006). While APO exhibits toxicity to various organisms (Atwal et al., 1992; Macías et al., 2005b), and therefore might be active against nematodes, we detected only traces of APO in our study. Unfortunately, our detection limits were not high enough to accurately quantify this compound. The low concentrations that we measured

indicate that this compound was probably not a major factor in *M. incognita* suppression.

4.4. Conclusions

Our results demonstrate that it is extremely important to understand the fate of plant-derived compounds in soil if they are to be exploited for plant-parasitic nematode management. We initially screened DIBOA, DIMBOA, BOA and MBOA against plant-parasitic nematodes in water-based laboratory assays and demonstrated activity (Zasada et al., 2005). However, a rate of DIBOA greater than the calculated LC_{50} was required to result in a significant reduction in *M. incognita* reproduction, compared to the water control, when applied to a loamy sand soil. Clearly, while water-based assays are valuable for identifying compounds that are toxic to nematodes, the application of identified lethal concentrations to a soil environment demands caution. If a proportion of nematode suppression in a nematode management program is to be obtained by the generation of DIBOA concentrations in soil, then several factors need to be taken into consideration, including incorporation technology (i.e. residue size), irrigation, and soil type.

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