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Fate of Benzoxazinone Allelochemicals in Soil after Incorporation of Wheat and Rye Sprouts

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Growing cereals (especially rye), which are incorporated into the soil to increase soil fertility or organic matter content, is a common practice in crop rotation. The additional sanitizing effect of this incorporation has often been appreciated and is said to be due to leaching of benzoxazinones and subsequent formation of benzoxazolinones. In this study wheat (Stakado) and rye (Hacada) sprouts were incorporated into soil in amounts that simulated agricultural practice. By extraction and subsequent LC-MS analysis the disappearance and appearance of benzoxazinones, benzoxazolinones, and phenoxazinones in soil were followed. In the wheat experiments 6-methoxybenzoxazolin-2-one (MBOA) was detected as the main compound. 2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA) and 2-hydroxy-1,4-benzoxazin-3-one (HBOA) were detected as well. No phenoxazinones were detected. For the rye experiment the picture was more complex. In the first 2 days of incubation MBOA and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) were detected as the main allelochemicals along with HBOA, HMBOA, and benzoxazolin-2-one (BOA), in decreasing order. Later in the incubation period some 2-amino-3*H*-phenoxazin-3-one (APO) was detected and the amount of HBOA increased considerably and decreased again. The profiling of the benzoxazinone metabolites and their derivates in soil was dynamic and time-dependent. The highest concentrations of most of the compounds were seen at day 1 after incorporation. A maximum concentration was reached at day 4 for a few of the compounds. This study is the first of its kind that shows the dynamic pattern of biologically active benzoxazinone derivates in soil after incorporation of wheat and rye sprouts. Methods for organic synthesis of HBOA and HMBOA were developed as part of the study.

KEYWORDS: Rye; wheat; allelopathy; allelochemicals; hydroxamic acids; DIBOA; DIMBOA; HBOA; HMBOA

INTRODUCTION

Agricultural exploitation of the natural defense systems (allelochemicals) of wheat and rye or other cultivars could decrease the amounts of pesticides needed in modern farming. Extensive research on this topic has been carried out, but more is needed. Through extraction, bioassays, purification, and analysis, the benzoxazinones [mainly the hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and the lactams 2-hydroxy-1,4-benzoxazin-3-one (HBOA) and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA)] and their metabolites have been identified as substances important to the allelophatic activity seen in wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.). The research field has been extensively reviewed (*1*−*4*). The biosynthetic pathway for the formation of benzoxazinones has been investigated in maize and to some extent in wheat and rye (*2*, *5*, *6*). The synthetic pathway is believed to be identical for all of the genera of Gramineae (*2*). The benzoxazinones are present in the cereals as their inert glucoconjugates (*2*). Upon injury—or after incorporation of plant material in soil—β-glucosidase acts on the glucosidic bond in 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc) and 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-Glc), and the agluconic compounds are released (*7*). The aglucones are exudated from the roots of living plants as well (*8*, *9*). Differences in concentration levels between plants of both different age (*10*, *11*) and different variety (*8*) and between plants of the same variety grown under different conditions have been studied (*12*). The hydroxamic acids (DIBOA and DIMBOA) undergo spontaneous decomposition to the corresponding benzoxazolinone and formic acid in solution. The half-life of this decomposition has been reported to be dependent on pH, temperature, and solvent, ranging from

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little more than a minute to just above 4 days depending on the conditions (*13*−*15*). In a soil−water suspension the half-lives of DIBOA and DIMBOA have been reported to be 1 day and 30 min, respectively (*16*). Zikmundova et al. (*17*, *18*) performed degradation studies of benzoxazolin-2-one (BOA) and HBOA (0.1−1.0 mM) with endophytic fungi. She suggested some possible routes of degradation and suggested the main intermediate to be *o*-aminophenol. The main degradation products were N-acylated *o*-aminophenols [*N*-(2-hydroxyphenyl)acetamide, 2-hydroxy-*N*-(2-hydroxyphenyl)acetamide, and *N*-(2-hydroxyphenyl)malonamic acid] and phenoxazinones [2-amino-3*H*-phenoxazin-3-one (APO) and 2-acetylamino-3*H*-phenoxazin-3-one (AAPO)] (*17*, *18*). These results are supported by earlier findings (*19*, *20*). Degradation studies in which BOA was added to soil in concentrations from 0.4 μg/g of soil to 4.0 mg/g of soil were recently performed in our laboratories (*21*, *22*). They showed APO and AAPO to be formed from BOA when applied in the higher concentrations. Acylated *o*-aminophenoles were not identified. Similar experiments with 6-methoxybenzoxazolin-2-one (MBOA) showed a similar pattern (*23*−*25*). 2-Amino-7-methoxy-3*H*-phenoxazin-3-one (AMPO) and 2-acetylamino-7-methoxy-3*H*-phenoxazin-3-one (AAMPO) were formed in the high-concentration experiments. Other metabolites were detected, but their structures have not yet been confirmed (*23*). Degradation studies of DIMBOA, DIBOA, and DIBOA-Glc in a soil−water suspension at a concentration level of 1 mg/g of soil were reported recently as well (*16*, *26*). MBOA and BOA, respectively, were found to be the intermediates and AMPO and APO, respectively, the degradation products.

Use of a cover crop as green manure is very common. The cover crop is typically sown in the autumn and incorporated shortly before spring sowing. The cover crop supplies the soil with nutrients accumulated during winter. Rye is a common cover crop in Denmark because it has a dense rooting structure and is compatible with Danish soil and environmental conditions; wheat is a common crop throughout Europe. It has often been observed that the incorporation of rye reduces the amount of weeds in the field and reduces the harm caused by fungal and insect attacks on the primary crop (*27*−*29*).

The aim of the work presented here was to examine the dynamics of leaching and transformation of benzoxazinone allelochemicals from wheat and rye sprouts when incorporated into soil in concentrations and under circumstances simulating the use of green manure in modern agriculture.

METHODS AND MATERIALS

Soil Data. *Soil 1 (for the Wheat Experiment).* Sandy loam soil was sampled in September 2003 from the upper 0−10 cm of a conventionally cultivated field near the Research Centre Flakkebjerg, Denmark. Previously, potatoes (2001), spring barley (2002), and vegetables (2003) had been cultivated in the field. The characteristics of the soil were as follows: clay (<2 μm), 13.1%; silt (2−20 μm), 15.4%; coarse silt (20−63 μm), 10.4%; fine sand (63−200 μm), 27.6%; coarse sand (200−2000 μm), 31.1%; humus, 2.4%; pH 6.6 (measured in water); water concentration level, 15.4%; and water-holding capacity (WHC), 34.6% (field capacity, 17.3%). The soil was sieved through a 2.0-mm mesh-size sieve and used immediately after harvesting.

Soil 2 (for the Rye Experiment). Sandy loam soil was sampled in March 2004 from the upper 0−10 cm of a conventionally cultivated field near the Research Centre Flakkebjerg, Denmark. Previously, only spring barley (2001, 2002, and 2003) had been cultivated in the field. The characteristics of the soil were as follows: clay (<2 μm), 18.0%; silt (2−20 μm), 15.0%; coarse silt (20−63 μm), 8.8%; fine sand (63−200 μm), 31.2%; coarse sand (200−2000 μm), 24.2%; humus, 2.8%; pH 6.8 (measured in water); water concentration level, 12.9%; and WHC, 34.8% (field capacity, 17.4%). The soil was sieved through a 2.0-mm mesh-size sieve, stored at 4 °C, and preincubated for 2 days before use.

Solutions and Chemicals. *Chemicals for Analysis.* HPLC-grade methanol was purchased from Ratburn Chemicals Ltd. (Walkerburn, Scotland), glacial acetic acid from Merck (Darmstadt, Germany), 2-benzoxazolinone (BOA), purity 98%, from Acros Organics (Geel, Belgium), and 6-methoxy-2-benzoxazolinone (MBOA), purity 98+%, from Lancaster Synthesis (Lancashire, U.K.). 2-Amino-3*H*-phenoxazin-3-one (APO), purity 100%, and 2-acetylamino-3*H*-phenoxazin-3-one (AAPO), purity 100%, were synthesized in our own laboratory (*14*). 2-Amino-7-methoxy-3*H*-phenoxazin-3-one (AMPO), 2-acetylamino-7-methoxy-3*H*-phenoxazin-3-one (AAMPO), 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one (DIBOA), and 2,4-dihydroxy-7-methoxy-1,4(2*H*)-benzoxazin-3-one (DIMBOA) were obtained from F. Macías, University of Cádiz (*21*). 2-β-D-Glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc) and 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-Glc) were obtained from Prof. Dr. Hajime Iwamura (Kyoto University), and DIMBOA-Glc was obtained from Prof. Dr. Lisbeth Jonsson (Södertörn University College). 2-Hydroxy-1,4-benzoxazin-3-one (HBOA) and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA) were synthesized in our laboratory as described below.

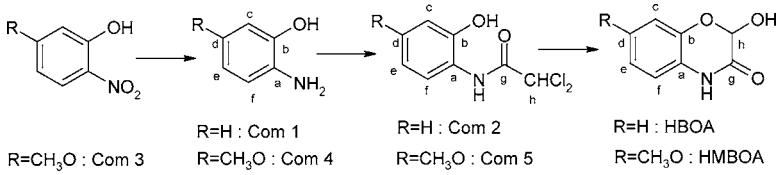
Chemicals for Synthesis. 2-Aminophenol, purity 99%, was from Acros Organics (Fair Lawn, NJ); dichloroacetyl chloride, purity 98%, from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); potassium hydroxide pellets, puris, from Riedel-de Haën (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany); formic acid, purity 98−100%, from Merck KGaA (Darmstadt, Germany); and palladium, 10 w/w %, on activated carbon from Sigma-Aldrich Chemie GmbH. 2-Methoxy-5-nitrophenol, pure by NMR, was produced in our laboratory for another project (*16*). Silica gel 60 (0.015−0.040 mm) for column chromatography was from Merck KGaA; diethyl ether, anhydrous, purity 99.5%, from J. T. Baker (Mallinckrodt Baker B.V., Deventer, The Netherlands); ethyl acetate, purity 99.8%, from Labscan Ltd. (Dublin, Ireland); heptane, purity 95%, from Labscan Ltd.; ethanol, purity 99.9%, from Danish Distillers (Aalborg, Denmark); and DMSO-d_6, purity D-99.9%, from Cambridge Isotope Laboratories Inc. (Andover, MA).

Chemical Analysis of Synthesized Compounds. GC-MS spectra were obtained on a HP GC5890-MS5972A Hewlett-Packard series II mass spectrometer. Direct inlet mass spectra were obtained on a JEOL JMS-HX110A tandem mass spectrometer. ^{1}H NMR spectra were recorded on a Varian 300 MHz instrument. ^{13}C NMR and correlated spectra (COSY, HETCOR) were recorded on a Unity 400 MHz spectrometer. Infrared spectra were recorded as a KBr sandwich using a Perkin-Elmer FT-IR spectrometer 1760X. Elementary analyses were performed with a Flash EA 1112 Series, CE Instrument.

Synthesis of HBOA. *2-Hydroxydichloroacetanilide (Compund **2**).* In a nitrogen atmosphere and with stirring, dichloroacetyl chloride (3.67 g, 2.4 mL, 25 mmol) was added dropwise to a suspension of 2-aminophenol (compound **1**) (5.45 g, 50 mmol) in diethyl ether (80 mL) at room temperature for 3 h. The mixture was filtered, the product was washed with dry diethyl ether, and the combined organic phases taken to dryness: yield 5.2 g (95%). Recrystallization from ethanol left the analytically pure compound as almost colorless crystals: mp 133−135 °C (lit. 134−135 °C) (*30*). Found: C, 43.7; H, 3.0; N, 6.3. Calculated values for $C_8H_7O_2NCl_2$: C, 43.6; H, 3.2; N, 6.4. For ^{1}H NMR data, see **Table 1**. For ^{13}C NMR, EI-MS, and IR data, see **Table 2**.

2-Hydroxy-4H-benz[1,4]oxazin-3-one (HBOA). 2-Hydroxydichloroacetanilide (3.57 g, 16 mmol) was dissolved in aqueous sodium hydrogen carbonate (0.2 M, 150 mL), boiled for 45 min, cooled, and neutralized with aqueous hydrochloric acid. The mixture was extracted with ethyl acetate (3 × 150 mL), and the combined organic phases were washed with water and taken to dryness to give the crude product (yield, 2.5 g, 15.2 mol, 95%). Recrystallization yielded the analytically pure product as brownish crystals: mp 209−211 °C. Found: C, 57.9; H, 4.3; N, 8.3. Calculated values for $C_8H_7NO_3$: C, 58.2; H, 4.2; N, 8.5. For ^{1}H NMR data, see **Table 1**. For ^{13}C NMR, EI-MS, and IR data, see **Table 2**.

Table 1. ^{1}H NMR Data for the Synthesized Compounds and the Chemical Synthetic Route of HBOA and HMBOA



	δ (^{1}H NMR, 300 MHz)				
	compound **2**	HBOA	compound **4**	compound **5**	HMBOA
NH	10.12 (s, 1H) or 9.77 (s, 1H)	10.7 (s, 1H, NH)	4.2 (br, 2H, OH/NH)	10.12 or 9.66 (s, 1H)	10.58 (s, 1H, NH)
OH	10.12 (s, 1H) or 9.77 (s, 1H)	7.89 (d, 1H, J = 6 Hz, OH)	4.2 (br, 2H, OH/NH)	10.12 or 9.66 (s, 1H)	10.58 (s, 1H, NH)
H-c	6.91 (dd, 1H, J = 2 and 8 Hz)	7.0–6.9 (m, 4H)	6.30 (d, 1H, J = 3 Hz)	6.47 (d, 1H, J = 2 Hz, H-6)	6.57 (d, 1H, J = 2 Hz)
H-d	6.80 (dt, 1H, J = 2 and 8 Hz)	7.0–6.9 (m, 4H)			
C**H**$_3$O			3.59 (s, 3H, CH_3)	3.69 (s, 3H)	3.70 (s, 3H)
H-e	7.01 (dt, 1H, J = 8 and 2 Hz)	7.0–6.9 (m, 4H)	6.16 (dd, 1H, J = 3 and 8 Hz)	6.40 (dd, 1H, J = 2 and 9 Hz)	6.58 (dd, 1H, J = 2 and 8 Hz)
H-f	7.89 (dd, 1H, J = 2 and 8 Hz)	7.0–6.9 (m, 4H)	6.49 (d, 1H, J = 8 Hz)	7.69 (d, 1H, J = 9 Hz, H-3)	6.82 (d, 1H, J = 8 Hz)
H-h	6.94 (s, 1H)	5.46 (d, 1H, J = 6 Hz)		6.86 (s, 1H)	5.42 (d, 1H, J = 6 Hz)

Table 2. Spectral Data from Chemically Synthesized Compounds

compound **2**	HBOA	compound **4**	compound **5**	HMBOA
		^{13}C NMR, δ (75 MHz, DMSO-d_6)		
161.8 (CO), 148.1, 125.6, 124.8, 121.8, 119.1, 115.3, 67.1 (CH)	162.5 (CO), 140.7 (C), 126.7 (C), 123.0 (CH), 122.4 (CH), 117.4 (CH), 115.6 (CH), 90.4 (CH)	151.4 (C), 145.0 (C), 130.0 (C), 114.9 (CH), 103.9 (CH), 102.1 (CH), 55.1 (CH_3)	161.6 (CO), 157.2, 149.6, 123.3, 118.0, 103.9, 101.5, 67.1 (CH), 55.1 (CH_3)	
		EI-MS, *m*/*z* (%)		
219/221 (M^+, 21), 136 (100), 108 (28), 80 (39)	165 (29, M^+), 136 (100, M – CHO)	139 (73, M^+), 124 (100, M – CH_3)	249/251 (M^+, 62), 166 (100), 138 (37), 110 (37)	195 (62, M^+), 166 (100, M – CHO)
		IR (KBr, cm^{-1})		
3421 vs, br (OH), 3338 s (NH), 3261 vs, br (OH), 1673 vs (CO), 1614 m, 1598 m, 1545 s, 1504 m, 1458 m, 1341 m, 1288 m, 1194 m, 752 m	3212 vs, br (OH), 3186 s (NH), 3128 s, 3066 m, 1695 vs (CO), 1610 m, 1500 s, 1452 m, 1404 m, 1277 m, 1202 m, 1077 s, 1037 s, 1018s, 830 m	3355 s and 3288 s (NH_2), 2574 vs, br (OH), 1610 m, 1586 m, 1525 s, 1449 m, 1267 s, 1202 m, 1168 s, 986 m, 958 m, 849 m	3369 s (NH), 3137 vs, br (OH), 1667 vs (CO) 1604 s, 1553 s, 1525 s, 1470 m, 1450 m, 1428 s, 1303 s, 1209 s, 1165 m, 1105 m, 954 m	

Synthesis of HMBOA. *2-Hydroxy-4-methoxyaniline (Compound **4**).* Using mechanical stirring a solution of potassium hydroxide (300 mg, 4.5 mmol) in absolute ethanol (2.25 mL) was added to a solution of formic acid (207 mg, 4.5 mmol) in degassed water (0.6 mL). Palladium on carbon (5%, 6 mg) was added followed by 2-methoxy-5-nitrophenol (compound **3**) (254 mg, 1.5 mmol). The mixture was allowed to react for 4 h at 70 °C and then taken to dryness, and the product was extracted with dry diethyl ether (5 × 15 mL). The combined ethereal phases were taken to dryness and left analytically pure 2-hydroxy-4-methoxyaniline (yield, 122 mg, 0.88 mol, 59%), mp 130−2 °C, in accordance with literature values (*31*). Solutions immediately turned red on exposure to air due to the formation of very small amounts of AMPO. Found: C, 60.6; H, 6.4; N, 10.0. Calculated values for $C_7H_9NO_2$: C, 60.4; H, 6.5; N, 10.1. For ^{1}H NMR data, see **Table 1**. For ^{13}C NMR, EI-MS, and IR data, see **Table 2**.

*2-Hydroxy-4-methoxydichloroacetanilide (Compound **5**).* In a nitrogen atmosphere and with stirring, dichloroacetyl chloride (118 mg, 0.08 mL, 0.8 mmol) was added to a solution of 2-hydroxy-4-methoxyaniline (112 mg, 0.8 mmol) in dry diethyl ether (4 mL) at room temperature. The reaction mixture immediately turned pale lilac. After 24 h, TLC indicated almost complete reaction. The hydrogen chloride was allowed to evaporate. The solid was filtered off and washed with dry diethyl ether, and the combined ethereal phases were taken to dryness, leaving 230 mg (0.92 mmol) of crude product. Recrystallization from ethanol left the analytically pure compound. Found: C, 43.5; H, 3.5; N, 5.4. Calculated values for $C_9H_9NO_3Cl_2$: C, 43.2; H, 3.6; N, 5.6. For ^{1}H NMR data, see **Table 1**. For ^{13}C NMR, EI-MS, and IR data, see **Table 2**.

2-Hydroxy-7-methoxy-4H-benz[1,4]oxazin-3-one (HMBOA). A mixture of 2-hydroxy-4-methoxy-dichloroacetanilide (174 mg, 0.7 mmol) and aqueous sodium hydrogencarbonate (16 mL, 0.1 M) was refluxed for 0.5 h, cooled to room temperature, and neutralized with hydrochloric acid to pH 6. The aqueous phase was filtered before extraction with ethyl acetate (5 × 30 mL), and the combined organic phases were washed with water and taken to dryness. The crude product (80 mg) was purified by column chromatography to give reddish HMBOA (26 mg), mp 194 °C, pure according to the ^{1}H NMR and mass spectra. For ^{1}H NMR data, see **Table 1**. For EI-MS data, see **Table 2**. Solutions of the compound rapidly turned red on exposure to air, probably because of oxidation to the aminophenoxazinone.

Incorporation Experiment. *Experiment A.* Six ordinary 1-L flower pots were filled with 900 g of soil 1. Grains of wheat (Stakado) were planted, ensuring the development of three sprouts in each pot for incorporation corresponding to field conditions (300 plants per m^2 or 33.3 cm^2 per plant). Six control samples (no grains sowed) were prepared in smaller flower pots containing 300 g of soil 1 (see **Figure 1**). The pots were placed in a greenhouse (day, 21 °C; night, 13 °C). Automatic watering in saucers kept the soil humidity at half the WHC by monitoring the weight of the pots. The pots were weeded regularly. After 11 days, the pots were taken from the greenhouse. The sprouts were freed from soil and cut into pieces of 1−2 cm and mixed back in with the soil (3 sprouts/900 g of soil 1 corresponding to 1 sprout/300 g of soil 1). The samples were returned to the greenhouse for incubation. On sampling days 0, 1, 4, 10, 15, and 21 starting from the day of incorporation of cut sprouts, a sample set (sample + control sample) was stored at −18 °C until freeze-drying. The samples were freeze-

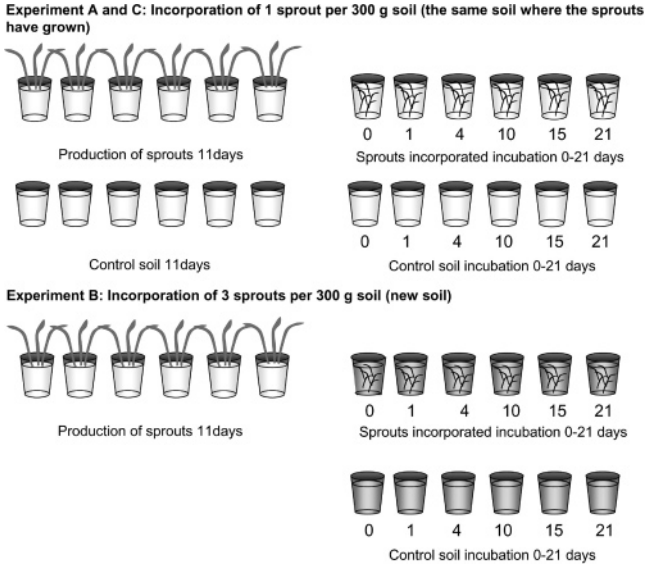


Figure 1. Setup of the incorporation experiments: (**A**, **B**) wheat; (**C**) rye. Production of sprouts and incubation were carried out under greenhouse conditions (day, 21 °C; night, 13 °C).

dried to complete dryness (24 h), sieved through a 2.0-mm mesh-size sieve, and crushed in a mortar. All larger plant parts were removed by this treatment. Samples were stored at −18 °C in PVC-free polyethylene bags until extraction and analysis.

Experiment B. The wheat sprouts were cultivated and cut as explained above. The cut sprouts were mixed with fresh soil 1 (3 sprouts/300 g of soil 1). Fresh control samples were prepared. The samples were incubated and treated as explained above.

Experiment C. This experiment was conducted using the rye variety Hacada (1 rye sprout/300 g of soil 2) as a rye analogue to incorporation experiment A.

The wheat variety Stakado was chosen on the basis of reports from a field study showing Stakado to be the one variety of three that had the highest concentration of benzoxazinones (*12*). Later, though, reports showed that when the same three varieties were grown under laboratory conditions, Stakado was the variety with the lowest concentration of benzoxazinones (*25*). The rye variety was chosen because it is commonly used as a cover crop in Denmark.

Soil Extraction and Chemical Analysis. All extractions were performed using an accelerated solvent extraction 200 system (ASE) from Dionex. The eluent used was methanol containing 0.5 v/v % of glacial acetic acid. ASE 200 setup: preheat for 5 min, heat for 5 min, static for 3 min; flush, 80%; purge, 60 s; cycles, 4; pressure, 1500 psi; and temperature, 80 °C. Thirty grams of dry crushed soil was poured into a 33-mL extraction cell. The cell was then filled to the top with chemically and microbially inert Ottawa sand. Fifty microliters of 1,2-propanediol was added to the extracts as a keeper, and the extract was taken to dryness by the use of a Turbo Vap LV evaporator from Zymark at 20 °C and 5 psi by a direct flow of nitrogen into each vial (estimated time, 8−12 h). The precipitate was suspended by adding 950 μL of methanol to a total volume of 1000 μL combined with the keeper. The methanolic suspension was diluted with 1 equiv of Milli-Q water and passed through a filter (Minisart SRP 15, 0.45 μm, from Vivascience AG) for a better LC-MS signal. The instrument was from AB Applied Biosystems. The HPLC was an Agilent 1100. The equipment was an API 2000 triple-quadrupole equipped with electrospray ionization (ESI). For HPLC a BDS Hypercil C18, 5 μm, 250 × 2.1 mm column was used. The mobile phase was composed of a gradient of A, 10% methanol in a water mixture containing 20 mM of glacial acetic acid, and B, pure methanol containing 20 mM glacial acetic acid. The injection volume was 20 μL. The program was as follows: 0−1 min, 90:10 A/B mixture; 1−8 min, a gradual decrease to 30:70; 8−15 min, 30:70 A/B mixture; 15−16 min, a gradual increase to 90:10; 16−23 min, 90:10 A/B mixture. The flow rate was 200 μL/min. BOA, MBOA, DIMBOA, DIBOA, HBOA, and HMBOA were detected in ESI^- mode, whereas APO, AAPO, AMPO, and AAMPO were detected in ESI^+ mode. Selected ion monitoring (SIM) collection was used. The retention times for the quantified compounds are shown in **Table 3** together with the molecular structure, the molecular mass of the compounds, and the detection mode. A direct injection of the solution was possible despite the heavy load of organic material, because the column eluate from the first 8 min was led to waste. The *m*/*z* values shown in **Table 4** were collected in ESI^+ and ESI^- mode, respectively, to identify possible metabolites in addition to the metabolites that were quantified.

Standard curves were made from standard solutions of 10 mg/L by continuous dilution in the area from 800 to 6.25 ng/mL. The standard curve was prepared both in pure methanol and in a soil extract. A substantial matrix effect was seen when the two standard curves were compared. Therefore, the amounts of BOA, MBOA, APO, AMPO, AAPO, AAMPO, DIBOA, DIMBOA, HBOA, and HMBOA in the samples were quantified by the use of the standard curves made in soil extract. The control samples were used to dismiss signals naturally present in the soil. The spectra from the control samples were subtracted from the spectra from the corresponding samples for more accurate quantification.

Validation of the Quantitative Analytical Method for Soil Extracts. The limit of detection (LOD) was obtained on the basis of a low-concentration standard in a blank soil extract. The LOD was determined as 3 times the standard deviation (SD) of the signal of the standard on the basis of 10 injections. For the LOD results, see **Table 5**.

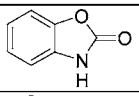
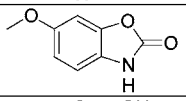
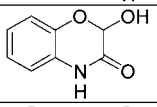
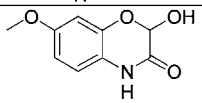
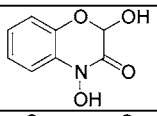
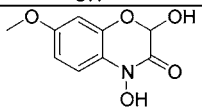
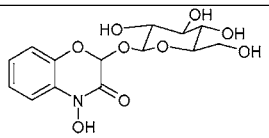
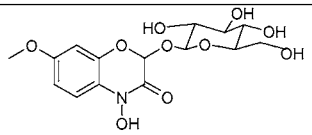
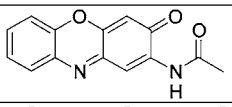
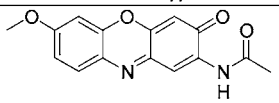
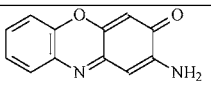
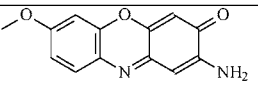
Recovery experiments were performed in soil 2. The samples were spiked with standard and adjusted to half of the WHC. A blank experiment with no standard added was prepared for control. All of the recovery experiments were carried out in six replicates.

After preparation, the samples were placed in the freezer at −18 °C for 6 days, freeze-dried overnight, and extracted. See **Table 5** for recovery results. There are various possible reasons for the low recoveries for DIBOA and DIMBOA: the compounds have short half-lives in soil, the compounds could be destroyed during sample preparation, or the compounds could be adsorbed to the soil particles. The found recovery has not been used to correct the data from the incorporation experiment.

Plant Extraction and Analysis. The sprouts for analysis were stored at −18 °C on the same day as the rest of the sprouts were cut and incorporated into the soil. Later the sprouts were freeze-dried. The sprouts were cut into pieces and crushed by the use of a mortar before extraction. The plant material (0.1004 g) was poured into an extraction cell prefilled with 5 g of ignited Ottawa sand. The cell was filled with ignited glass beads before closure. The extraction was performed identically to the soil extraction, only the eluent was changed to 80:19:1 of methanol, water, and acetic acid. The weight of the extract was regulated to 50 mL with the addition of methanol. The plant extracts were treated like the soil extracts described above prior to analysis. The plant extracts were analyzed and quantified by HPLC-MSMS using the same instrument, column, and separation method as for the soil extracts. A direct injection of the solution was possible despite the heavy load of organic material, because the column eluate from the first 8 min was led to waste. BOA, MBOA, HBOA, HMBOA, DIBOA, DIMBOA, DIBOA-Glc, and DIMBOA-Glc were all detected at ESI^-. Multiple-reaction monitoring (MRM) was used. Retention times and structures can be seen in **Table 3**. The retention times differ slightly between the soil extract analysis and the plant extract analysis due to aging of the column.

Validation of the Quantitative Plant Analytical Method. Recovery experiments were performed for BOA, MBOA, HBOA, HMBOA, DIBOA, DIMBOA, DIBOA-Glc, and DIMBOA-Glc. Plant material of the wheat variety Astron from the Zadocks stage 51 (*32*), which beforehand had been confirmed not to contain any of the above except small amounts of MBOA, was used for the recovery experiment. Recovery experiments were prepared in three repetitions. Four control recovery experiments in which no standards were added were prepared as well. An amount of 0.1 g of freeze-dried, crushed plant material was placed in an extraction cell prefilled with 5 g of ignited sand for each recovery experiment. Subsequently, the plant material was spiked with 50 μL of one of the standards. The cell was filled with ignited

Table 3. Benzoxazinones and Their Metabolites, Quantified in Soil (LC-MS Analysis, SIM Mode) and Plants (LC-MSMS Analysis, MRM Mode) with *m/z* Values and Retention Times (Rt)[a]

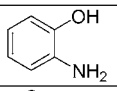
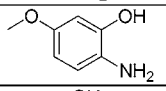
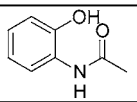
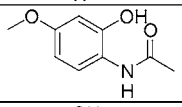
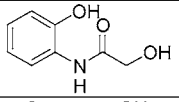
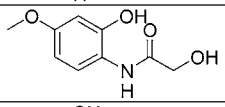
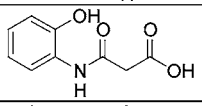
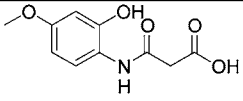
acronym	systematic name	formula	molecular mass	detection in MS (soil analysis)	detection in MSMS (plant analysis)
BOA	benoxazolin-2-one		135.12	m/z 134 Rt: 14. 4 min	m/z 136/80 Rt: 15.0 min
MBOA	6-methoxy-benzoxazolin-2-one		165.15	m/z 164 Rt: 14.8 min	m/z 166/110 Rt: 15.4 min
HBOA	2-hydroxy-1,4-benzoxazin-3-one		165.15	m/z 164 Rt: 12.8 min	m/z 166/148 Rt: 13.4 min
HMBOA	2-hydroxy-7-methoxy-1,4-benzoxazin-3-one		195.17	m/z 194 Rt: 13.4 min	m/z 196/178 Rt: 13.9 min
DIBOA	2,4-dihydroxy-1,4-benzoxazin-3-one		181.15	m/z 180 Rt: 13.0 min	m/z182/136 Rt: 13.6 min
DIMBOA	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one		211.17	m/z 210 Rt: 13.6	m/z 212/166 Rt: 14.2 min
DIBOA-Glc	2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one		343.29	NA	m/z 344/182 Rt: 12.7 min
DIMBOA-Glc	2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one		373.31	NA	m/z 374/212 Rt: 13.5 min
AAPO	2-acetylamino-3H-phenoxazin-3-one		254.23	m/z 255 Rt: 17.8	NA
AAMPO	2-acetylamino-7-methoxy-3H-phenoxazin-3-one		284.26	m/z 285 Rt: 18.3	NA
APO	2-amino-phenoxazin-3-one		212.20	m/z 213 Rt: 17.4	NA
AMPO	2-amino-7-methoxy-3H-phenoxazin-3-one		242.23	m/z 243 Rt: 17.6	NA

[a] NA, not analyzed.

glass beads, closed, and incubated for 1 h at −18 °C prior to extraction. The extracts were treated and analyzed like the other plant extracts. For completeness, recovery trials in which spiking was done prior to freeze-drying could have been considered. However, Villagrasa et al. (*33*) showed that the lowering of recovery percentages did not occur during freeze-drying. See **Table 6** for recovery rates. The detection limits were determined on the basis of the standard deviation of the signal of the standard 100 μg/L (~50 mg/kg of plant material) in foliage extract. See **Table 6** for detection limits.

Additional Collection of Masses. On the basis of the literature on the degradation of benzoxazinones (*1*), a number of other compounds could be expected to be present in the soil after incorporation of wheat and rye sprouts. A number of additional *m/z* values were therefore collected in the LC-MS analyses to perform a preliminary characteriza-

Table 4. Systematic Names and Formulas for Additional Compounds Searched for in Soil Extracts According to Their Molecular Masses[a]

acronym	systematic name	formula	molecular mass	m/z values for detection in MS
AP	2-aminophenol		109.13	110 (pos) 108 (neg)
MAP	2-amino-5-methoxyphenol		139.15	140 (pos) 138 (neg)
HPAA	N-(2-hydroxyphenyl)acetamide		151.16	152 (pos) 150 (neg)
HMPAA	N-(2-hydroxy-4-methoxyphenyl)acetamide		181.19	182 (pos) 180 (neg)
HHPAA	2-hydroxy-N-(2-hydroxyphenyl)acetamide		167.16	168 (pos) 166 (neg)
HHMPAA	2-hydroxy-N-(2-hydroxy-4-methoxy-phenyl)acetamide		197.19	198 (pos) 196 (neg)
HPMA	N-(2-hydroxyphenyl)-malonamic acid		195.17	196 (pos) 194 (neg)
HMPMA	N-(2-hydroxy-4-methoxyphenyl)-malonamic acid		225.20	226 (pos) 224 (neg)

[a] The presence of the above-mentioned compounds was analyzed qualitatively. Only mass 167 was detected with Rt 11.3 min. Confirmation of the compound is pending.

Table 5. Recovery Rates and Detection Limits (LOD) for Compounds Quantified in Soil

compound	20.0 ng/g of dried soil[a]	50.0 ng/g of dried soil[a]	100.0 ng/g of dried soil[a]	LOD[b] (ng/g)
DIBOA	BDL	5.5 [2.2]	3.9 [1.5]	4.24
DIMBOA	BDL	1.3 [1.1]	0.2 [0.2]	5.00
HBOA	23.9 [1.6]	NA	NA	0.40
HMBOA	33.0 [1.2]	NA	NA	0.32
BOA	47.3 [8.3]	74.9 [4.1]	NA	0.44
MBOA	97.5 [9.5]	95.3 [2.7]	NA	1.18
APO	50.5 [7.4]	51.9 [7.8]	NA	0.76
AMPO	49.9 [6.7]	40.0 [4.4]	NA	4.02
AAPO	93.3 [10.8]	94.7 [6.0]	NA	15.9
AAMPO	102.9 [26.1]	90.7 [5.3]	NA	2.59

[a] Recovery rates (%, mean [SD]) were obtained by adding the compounds to soil followed by our general sample preparation procedure. BDL, below detection limit; NA, not analyzed. [b] LOD (3 times SD) was determined on the basis of the standard deviation of the signal of a standard in blank soil extract.

Table 6. Recovery Rates and Detection Limits (LOD) for Compounds Quantified in the Sprouts

compound	recovery[a]	LOD[b] (μg/g)
DIBOA-Glc	118.8 [5.8]	8.7
DIMBOA-Glc	85.6 [2.3]	3.5
DIBOA	99.6 [11.3]	17.0
DIMBOA	62.4 [6.6]	9.9
HBOA	97.9 [4.9]	7.4
HMBOA	97.7 [3.9]	5.9
BOA	98.1 [4.0]	6.0
MBOA	112.1 [2.2]	3.3

[a] Recovery rates (%, mean [SD]) were obtained by adding the compounds to freeze-dried blank plant material (~50 μg/g of plant material) an hour prior to extraction. [b] LOD (3 times SD) was determined on the basis of the standard deviation of the signal of a standard in foliage extract (~50 μg/g of plant material).

tion of such additional compounds. The structures of the compounds searched for can be seen in **Table 4**.

RESULTS AND DISCUSSION

Chemical Synthesis. The investigation of the fate of phytochemicals in soil requires pure chemicals for the confirmation of the structure of the metabolites and for their accurate quantification. Thus, chemical synthesis is often needed alongside the fate studies. In this project HBOA and HMBOA were synthesized. The preparation and properties of HBOA (**Table 1**, R = H) have been amply described, but some confusion seems to exist with regard to the corresponding 7-methoxy derivative (HMBOA, **Table 1**, R = OCH_3). Thus, although the melting point of HMBOA repeatedly (*34*−*36*) has been reported to be in the interval between 196 and 200 °C, in one instance Baumeler et al. (*37*) stated the value of 179−181 °C for a sample secured from the roots of *Aphelandra tetragona*. Macías et al. (*38*) published a semisynthesis in which samarium diiodide was used for reduction of DIMBOA, isolated from plants, to HMBOA. Only one simple and reliable full synthetic method seems to have been published for this type of compound, starting with (**Table 1**) reduction of the 2-nitrophenol to the 2-aminophenol, followed by dichloroacetylation and cyclization with base. This procedure has proved to be very convenient for HBOA (*30*, *39*). Although it has been described for the preparation of HMBOA (*40*), the product was not further characterized and, in fact, the structure has never been proved

Table 7. Concentration of Benzoxazinones and Their Derivatives in the Wheat and Rye Sprouts Used for the Incorporation Experiments

	concn in dried plant material (mg/kg)		max theor concn in soil[a] (ng/g of dried soil)		
	wheat (Stakado)	rye (Hacada)	wheat (Stakado), expt A	wheat (Stakado), expt B	rye (Hacada), expt A
BOA	3.6	11.1	0.6	1.9	1.8
MBOA	391.1	19.3	70.0	209.9	3.1
HBOA	4.8	19.5	0.9	2.6	3.1
HMBOA	200.1	11.1	35.8	107.4	1.8
DIBOA	57.5	2109.5	10.3	30.9	335.2
DIMBOA	1217.7	100.3	217.8	653.5	15.9
DIBOA-Glc	6.4	104.1	1.1	3.4	16.5
DIMBOA-Glc	163.0	178.4	29.2	87.5	28.3

[a] Supposing that the total amount from the plants leached out instantaneously.

to be correct. Our results demonstrate the reliability of the method for both HBOA and HMBOA.

Content of Benzoxazinones and Derivatives in Plant Material. The content of benzoxazinones and their derivatives as measured in the plants used in this experiment is shown in **Table 7**. The wheat sprouts contained a relatively large amount of MBOA (one-third) compared with the amount of DIMBOA. HMBOA was found in a concentration half that of MBOA. The demethoxy derivatives were all found in low concentrations, although the concentration of DIBOA was a little higher than the rest.

DIBOA was found in a high concentration in the rye sprouts, whereas DIMBOA-Glc, DIBOA-Glc, and DIMBOA were found in medium concentrations (listed in decreasing order). BOA, MBOA, HBOA, and HMBOA were found in only very small amounts. It has been indicated that DIMBOA is not present in rye (*S. cereale*) (*2*). However, several authors (*4*, *41*, *42*) stated that the hydroxamic acids DIBOA and DIMBOA are found in both rye and wheat, DIMBOA being the dominating compound in wheat and DIBOA the dominating compound in rye. This is in agreement with our results. Belz and Hurle (*9*) measured the exudation of DIMBOA and DIBOA from living wheat and rye plants into hydroponics.

It has been discussed whether the aglucones could be present in the plants or whether they are formed during sampling, preparation, and analysis. Cambier et al. (*43*) stated that the benzoxazinones in maize were present only as gluconoconjugates and concluded that immediate freezing in N_2 after sampling is needed to avoid the hydrolysis of the gluconoconjugates and the liberation of the aglucones. However, a number of authors claim that both glucoconjugates and aglucones can be present in the plant simultaneously. Villagrasa et al. (*44*) and Mogensen et al. (*12*) found both aglucones and glucoconjugates in wheat, even when the sprouts were frozen immediately in liquid N_2 after harvesting. Zuñiga and Massardo (*11*) showed that the aglucones were found in the meristem and callus of wheat plants, whereas the glucoconjugates were found in the differentiated tissue. Both Zuñiga and Massardo (*11*) and Nakagawa et al. (*10*) showed that the relative amounts of DIBOA-Glc, DIMBOA-Glc, DIBOA, and DIMBOA varied by plant age.

The presence of benzoxazolinones (MBOA and BOA) in plant material has been questioned as well (*43*). However, Mogensen et al. (*12*) showed variations in the MBOA, DIMBOA, and DIMBOA-Glc concentrations in roots from organically grown wheat during the growth stages Zadocks 10, 12, 21, 31, and 51 and suggested that detection of MBOA was not due to

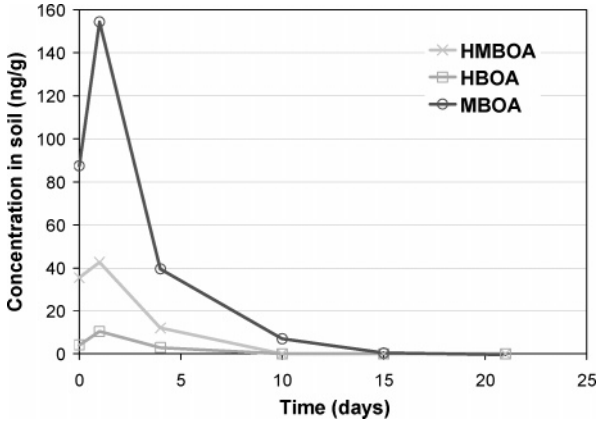


Figure 2. Concentration of allelochemicals in soil in which wheat sprouts were incorporated as a function of incubation time (1 sprout/300 g of soil 1), experiment A.

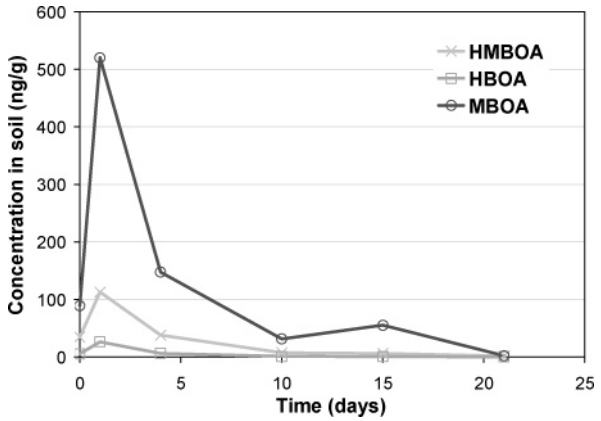


Figure 3. Concentration of allelochemicals in soil in which wheat sprouts were incorporated as a function of incubation time (3 sprouts/300 g of soil 1), experiment B.

decomposition of DIMBOA-Glc and DIMBOA during analysis. Grambow et al. (*45*) proved the existence in wheat leaves of a rarely—until then—considered compound, 2-β-D-glucopyranosyloxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA-Glc). The compound was found in *Coix lacryma-jobi* var. *ma-yuen* as well (*34*) and in corn (*46*). HDMBOA-Glc was shown to transform very quickly to MBOA (*45*, *47*). Thus, it cannot be excluded that the detection of MBOA in our plant results and in the results of Mogensen et al. (*12*), Villagrasa et al. (*44*), and Stochmal et al. (*48*) can be due to a transformation of HDMBOA-Glc, a compound that was not quantified in these studies and of which the aglucone HDMBOA has not been isolated from plants.

Content of Benzoxazinones and Derivatives in Soil in Which Wheat or Rye Was Incorporated. The control samples were used to dismiss signals naturally present in the soil under the conditions used. In the control samples there were detected no signals that correspond to a growing or decreasing concentration.

In the incorporation experiments of wheat (experiment A, **Figure 2**; and experiment B, **Figure 3**) only MBOA, HBOA, and HMBOA were quantified above the detection limit (see **Table 3** for structures and retention times). **Table 7** shows an estimate of the theoretical concentration in soil of each compound, supposing that the total amount present in the plant is immediately leached into the soil. DIMBOA was not detected in the soil due to either its fast conversion to MBOA (both spontaneously and microbial) (*16*), a strong sorption to the soil,

or degradation during sample preparation. The high amount of MBOA that was seen at day 0 (87 ng/g) and day 1 (154 ng/g) may be due to the leaching of MBOA from the plant material, a rapid transformation of HDMBOA-Glc (*45*, *47*), a rapid conversion of DIMBOA exudated during growth (*8*, *9*, *49*), or a rapid conversion of DIMBOA leached into the soil after incorporation of plant material. Both HMBOA and HBOA were present at day 0 as well and showed a small increment between days 0 and 1. Experiment B was done in a new soil, for which reason the presence of HMBOA, HBOA, and MBOA at day 0 (**Figure 3**) can only be due to leaching from the dead, incorporated plant material.

The fact that the concentrations of MBOA, HBOA, and HMBOA were approximately the same in experiments A and B at day 0, even though experiment A contained only one-third the amount of sprouts of experiment B, may indicate that exudation and transformation in the last day of growth may have occurred in experiment A. On the second day of incubation, the concentration of MBOA and HBOA increased twice for experiment A and five times for experiment B. The increase in concentration was more modest for HMBOA. After day 1, the concentration of all three compounds decreased as seen in **Figures 2** and **3**. At day 15, no MBOA was left (above the detection limit) in experiment A, and at day 21, no MBOA was left in experiment B. The application of first-order degradation kinetics on the disappearance of MBOA from the maximum concentration revealed a DT_{50} values (reduction of the concentration to half of the maximum concentration) for MBOA of 1.6 days in experiment A and 1.3 days in experiment B. Degradation studies with pure MBOA added to a similar soil at a concentration of 400 ng/g have been performed earlier in our laboratory (*24*, *25*). They resulted in a DT_{50} value of 1.4 days. Thus, the pattern for MBOA disappearance when plant or pure compound is incorporated is similar as soon as MBOA is formed in or leached into the soil. This is also an indication that MBOA is rapidly leached from the plant material because a prolonged continuous supply by leaching would probably enhance the DT_{50} value compared with the pure compound.

Degradation experiments with pure MBOA in soil (*24*, *25*) at a concentration level of 400 μg/g of soil showed that MBOA was metabolized into the phenoxazinone AMPO and that the molar relationship between added MBOA and maximum amount of AMPO was 300:1. In degradation experiments with MBOA at 400 or 4000 ng/g of soil (*24*, *25*) no AMPO was detected. The concentration of MBOA originating from the sprouts reached maximum values of 154 ng/g of soil for experiment A and 520 ng/g of soil for experiment B, and as would be expected, AMPO was not detected. MBOA, HBOA, and HMBOA were either metabolized, chemically transformed, or bound to soil particles. No metabolites were detected. Studies on metabolization of chemicals in soil need to be performed as a combination of studies with individual compounds to elucidate the transformation pathway of each compound and studies with natural plants in which naturally occurring enzymes are present as well, as is the case in the studies we are presenting here. However, the conversion between each of the detected compounds cannot be elucidated profoundly without genetically modified plant material in which the biosynthetic pathway is partly blocked. It was suggested by Macías et al. (*16*) that some DIMBOA could be detoxified by biological conversion into HMBOA in soil−water studies, but this was only seen in a small degree when DIMBOA was added to the soil−water mixture in a concentration of 5 mg/g of soil. Tipton et al. (*50*) showed that DIMBOA could be reduced to HMBOA in the presence of plant cells. The appearance of HMBOA in soil in our studies could thus partly come from DIMBOA. However, the theoretical values for HMBOA released from plant tissue (**Table 7**) and the amounts detected in **Figures 2** and **3** are roughly the same, which should indicate that the above-mentioned detoxification did not occur in our study.

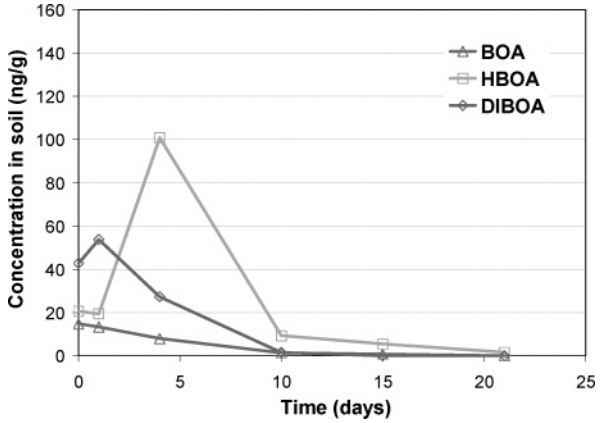


Figure 4. Concentration of BOA, HBOA, and DIBOA in soil in which rye sprouts were incorporated as a function of incubation time (1 sprout/300 g of soil 2), experiment C.

In the incorporation experiment with rye (experiment C), DIBOA was detected at days 0, 1, and 4 with the maximum level at day 1 of 53 ng/g of dried soil (see **Figure 4**). The small increase in concentration of DIBOA from day 0 to day 1 must be due to release of DIBOA from the plant material upon microbial attack in the soil matrix. It was a surprise to find that the molar concentration of BOA was much smaller than that of DIBOA. BOA can be generated by a spontaneous loss of formic acid from DIBOA (*15*), and the transformation of DIBOA to BOA in a buffered aquatic solution in a pH range of 4−8 was shown to be equimolar (*13*). However, it is probable that a microbial degradation of DIBOA leading to degradation products other than BOA can take place in soil.

The concentration of BOA was at its maximum at day 0 at the level of 15 ng/g of dried soil and did not grow as DIBOA disappeared. The level of BOA in the plant material was not high enough to make it the reason for the day 0 level of BOA in soil. Thus, BOA detected in the soil might originate from DIBOA that was leached to the soil during the growth of the plants or from other compounds in the plants not analyzed in this study.

The concentration of HBOA was ∼20 ng/g of dried soil on both days 0 and 1. On day 4 the concentration was 5 times as high. A metabolite with the mass of 167 was found that had a degradation pattern similar to that of HBOA. It is reasonable to consider it to be 2-(2-hydroxyacetamido)phenol (HHPAA) (see **Table 4**) as it is reported to be in a redoxidative microbial equilibrium with HBOA in soil (*17*). A sample of HHPAA is needed for verification. None of the masses corresponding to the other listed compounds in **Table 4** were seen in the chromatogram.

When the degradation curves of DIBOA and HBOA are combined as in **Figure 4**, it looks as if HBOA is generated from DIBOA due to the time lag between the maximum concentrations of these compounds. For the first time we may have seen a parallel to earlier reports on the conversion of DIMBOA to HMBOA (*16*, *50*). However, a confirmation of this with labeled DIBOA in the presence of plant material is required. Another explanation for the rise in HBOA concentration in soil could be that the glucoside of HBOA is present in

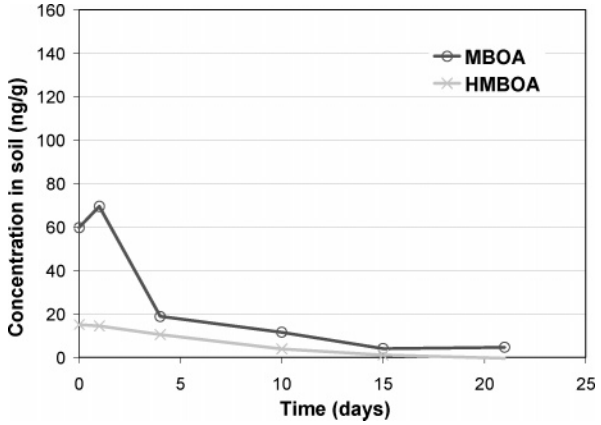


Figure 5. Concentration of MBOA and HMBOA in soil in which rye sprouts were incorporated as a function of incubation time (1 sprout/300 g of soil 2), experiment C.

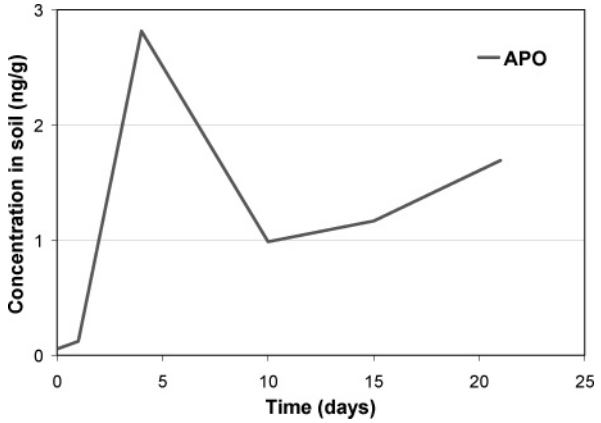


Figure 6. Concentration of APO in soil in which rye sprouts were incorporated as a function of incubation time (1 sprout/300 g of soil 2), experiment C.

the plant, although it is not a part of the biosynthesis of DIBOA-Glc (*51*), and that the aglucone is released slowly compared with DIBOA.

Both HMBOA and MBOA were detected in the rye incorporation experiment, experiment C (See **Figure 5**). Reports on rye benzoxazinones have generally focused on two compounds: DIBOA as the main benzoxazinone compound and DIMBOA as a minor compound (*4*, *41*, *42*). The concentration of HMBOA was at the level of BOA (15 ng/g of dried soil). The concentration of MBOA peaked at day 1 at the level of 70 ng/g of dried soil and thereby far exceeded the concentration of BOA. The concentration of MBOA in soil in which rye was incorporated exceeds the theoretical concentration in rye (**Table 7**), even if a complete conversion of DIMBOA and HMBOA took place. Thus, similarly to the wheat incorporation experiment, MBOA in soil may originate from a compound in the plant that was not quantified.

A small amount of APO was detected just above the limit of detection (see **Figure 6**) with a maximum at day 4 but without disappearing for the incubation period. To verify this, a MSMS analysis of the day 4 sample was done, and APO was identified by the mass pair 213/185. Degradation studies for BOA made at our laboratory (*21*, *22*) showed that BOA at a concentration level of 400 μg/g of soil was metabolized into the phenoxazinone APO and that the molar relationship between added BOA and maximum amount of APO was 10:1. In degradation experiments with BOA at 400 ng/g of soil, no APO was detected. Similar to the MBOA−AMPO discussion in the wheat experiment above, we did not expect to find APO in the rye experiment, due to the low concentrations of BOA. Detecting APO anyway could be explained by the different molar relation between added BOA and formed APO (*21*) and, thus, a possible formation of high local concentrations around the incorporated cut sprouts as DIBOA and BOA are leached into the soil.

This project proved that several of the biologically active benzoxazinone derivatives can be found in soil after incorporation including the 2-aminophenoxin-3-one. We have shown that degradation experiments with individual compounds for a start can give a picture of the degradation of allelochemicals from plants. The largest differences in the variation of metabolites detected are probably due to the differences in concentration between the two types of studies: degradation studies of isolated compounds and incorporation of plant material into soil simulating natural conditions properly. For a more detailed picture, incorporation of plant material into soil will give answers on factors such as concentration differences in a single sample and the role of the plant material itself. More of these types of experiments are needed. Experiments with more sampling points would give a more accurate picture of the leaching and degradation. Experiments with a wide number of wheat and rye varieties would elucidate both varietal differences and differences in concentration of the allelochemicals leached to the soil.

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LITERATURE CITED

(1) Fomsgaard, I. S.; Mortensen, A. G.; Carlsen, S. C. K. Microbial transformation products of benzoxazolinone and benzoxazinone allelochemicals—a review. *Chemosphere* **2004**, *54*, 1025−1038.

(2) Sicker, D.; Frey, M.; Schulz, M.; Gierl, A. Role of natural benzoxazinones in the survival strategy of plants. *Int. Rev. Cytol.* **2000**, *198*, 319−346.

(3) Hashimoto, Y.; Shudo, K. Chemistry of biologically active benzoxazinoids. *Phytochemistry* **1996**, *43*, 551−559.

(4) Niemeyer, H. M. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defence chemicals in the Gramineae. *Phytochemistry* **1988**, *27*, 3349−3358.

(5) Gierl, A.; Frey, M. Evolution of benzoxazinone biosynthesis and indole production in maize. *Planta* **2001**, *213*, 493−498.

(6) Frey, M.; Huber, K.; Park, W. J.; Sicker, D.; Lindberg, P.; Meeley, R. B.; Simmons, C. R.; Yalpani, N.; Gierl, A. A 2-oxoglutarate-dependent dioxygenase is integrated in DIMBOA-biosynthesis. *Phytochemistry* **2003**, *62*, 371−376.

(7) Nikus, J.; Jonsson, L. M. V. Tissue localization of β-glucosidase in rye, maize and wheat seedlings. *Physiol. Plant.* **1999**, *107*, 373−378.

(8) Wu, H.; Haig, T.; Pratley, J.; Lemerle, D.; An, M. Allelochemicals in wheat (*Triticum aestivum* L.): production and exudation of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one. *J. Chem. Ecol.* **2001**, *27*, 1691−1700.

(9) Belz, R. G.; Hurle, K. Differential exudation of two benzoxazinoids—one of the determining factors for seedling allelopathy of Triticeae species. *J. Agric. Food Chem.* **2005**, *53*, 250−261.

(10) Nakagawa, E.; Amano, T.; Hirai, N.; Iwamura, H. Non-induced cyclic hydroxamic acids in wheat during juvenile stage of growth. *Phytochemistry* **1995**, *38*, 1349−1354.

(11) Zuniga, G. E.; Massardo, F. Hydroxamic acid content in undifferentiated and differentiated tissues of wheat. *Phytochemistry* **1991**, *30*, 3281−3283.

(12) Mogensen, B. B.; Krongaard, T.; Mathiassen, S.; Kudsk, P. Quantification of benzoxazinone derivatives in wheat (*Triticum aestivum*) varieties grown under contrasting conditions in Denmark. *J. Agric. Food Chem.* **2006**, *54*, 1023−1030.

(13) Bredenberg, J. B.; Honkanen, E.; Virtanen, A. I. The kinetics and mechanism of the decomposition of 2,4-dihydroxy-1,4-benzoxazin-3-one. *Acta Chem. Scand.* **1962**, *16*, 135−141.

(14) Woodward, M. D.; Corcuera, L. J.; Helgeson, J. P.; Upper, C. D. Decomposition of 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one in aqueous solution. *Plant Physiol.* **1978**, *61*, 796−802.

(15) Bravo, H. R.; Niemeyer, H. M. Decomposition in aprotic solvents of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, a hydroxamic acid from cereals. *Tetrahedron* **1985**, *41*, 4983−4986.

(16) Macías, F. A.; Oliveros-Bastidas, A.; Marin, D.; Castellano, D.; Simonet, A. M.; Molinillo, J. M. G. Degradation studies on benzoxazinoids. Soil degradation dynamics of 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and its degradation products, phytotoxic allelochemicals from Gramineae. *J. Agric. Food Chem.* **2004**, *52*, 6402−6413.

(17) Zikmundova, M.; Drandarov, K.; Bigler, L.; Hesse, M.; Werner, C. Biotransformation of 2-benzoxazolinone and 2-hydroxy-1,4-benzoxazin-3-one by endophytic fungi isolated from *Aphelandra tetragona*. *Appl. Environ. Microbiol.* **2002**, *68*, 4863−4870.

(18) Zikmundova, M.; Drandarov, K.; Hesse, M.; Werner, C. Hydroxylated 2-amino-3*H*-phenoxazin-3-one derivatives as products of 2-hydroxy-1,4-benzoxazin-3-one (HBOA) biotransformation by *Chaetosphaeria* sp., an endophytic fungus from *Aphelandra tetragona*. *Z. Naturforsch. C* **2002**, *57C*, 660−665.

(19) Yue, Q.; Bacon, C. W.; Richardson, M. D. Biotransformation of 2-benzoxazolinone and 6-methoxy-benzoxazolinone by *Fusarium moniliforme*. *Phytochemistry* **1998**, *48*, 451−454.

(20) Friebe, A.; Vilich, V.; Hennig, L.; Kluge, M.; Sicker, D. Detoxification of benzoxazolinone allelochemicals from wheat by *Gaeumannomyces graminis* var. *tritici*, *G. graminis* var. *graminis*, *G. graminis* var. *avenae*, and *Fusarium culmorum*. *Appl. Environ. Microbiol.* **1998**, *64*, 2386-2391.

(21) Gents, M. B.; Nielsen, S. T.; Mortensen, A. G.; Christophersen, C.; Fomsgaard, I. S. Transformation products of 2-benzoxazolinone (BOA) in soil. *Chemosphere* **2005**, *61*, 74−84.

(22) Understrup, A. G.; Ravnskov, S.; Hansen, H. C. B.; Fomsgaard, I. S. Biotransformation of 2-benzoxazolinone to 2-amino-(3*H*)-phenoxazin-3-one and 2-acetylamino-(3*H*)-phenoxazin-3-one. *J. Chem. Ecol.* **2005**, *31*, 1205−1222.

(23) Etzerodt, T.; Nielsen, S. T.; Mortensen, A. G.; Christophersen, C.; Fomsgaard, I. S. Elucidating the transformation pattern of the cereal allelochemical 6-methoxy-2-benzoxazolinone (MBOA) and the trideuteriomethoxy analogue [D_3]-MBOA in soil. *J. Agric. Food Chem.* **2006**, *54*, 1075−1085.

(24) Fomsgaard, I. S.; Mortensen, A. G.; Gents, M. B.; Understrup, A. G. Time-dependent transformation of varying concentrations of the hydroxamic acid metabolites MBOA and BOA in soil. *Proceedings, Second European Allelopathy Symposium. Allelopathy—from Understanding to Application, FATEALLCHEM Workshop ("Fate and Toxicity of Allelochemicals (Natural Plant Toxins) in Relation to Environment and Consumer")*, June 2004;I nstitute of Soil Science and Plant Cultivation Press Services: Pulawy, Poland; 2004; pp 61−63.

(25) Fomsgaard, I. S.; Mortensen, A. G.; Nielsen, S. T. Transformation of 6-methoxy-2-benzoxazolinone (MBOA) in soil under influence of concentration and temperature. In preparation.

(26) Macías, F. A.; Oliveros-Bastidas, A.; Marin, D.; Castellano, D.; Simonet, A. M.; Molinillo, J. M. G. Degradation studies on benzoxazinoids. Soil degradation dynamics of (2*R*)-2-*O*-β-D-glucopyranosyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-Glc) and its degradation products, phytotoxic allelochemicals from Gramineae. *J. Agric. Food Chem.* **2005**, *53*, 554−561.

(27) Hartwig, N. L.; Ammon, H. U. Cover crops and living mulches. *Weed Sci.* **2002**, *50*, 688−699.

(28) Smeda, R.; Weller, S. Potential of rye (*Secale cereale*) for weed management in transplant tomatoes (*Lycopersicon esculentum*). *Weed Sci.* **1996**, *44*, 596−602.

(29) Worsham, A. Allelopathic cover crops to reduce herbicide input. *Proceedings of the 44th Annual Meeting of the Southern Weed Science Society*; SWSS: Champaign, IL, 1991; pp 58−69.

(30) Chatterjee, A.; Sharma, N.; Bnaerji, J.; Basa, S. Studies on Acanthaceae-benzoxazine glucoside and benzoxazolone from *Blepharis edulis* Pers. *Indian J. Chem.* **1990**, *29B*, 132−134.

(31) Nachman, R. J. Convenient preparation of 2-benzoxazolinones with 1,1-carbonyldiimidazole. *J. Heterocycl. Chem.* **1982**, *19*, 1545−1547.

(32) Zadoks, J. C.; Chang, T. T.; Konzak, C. F. A decimal code for the growth stages of cereals. *Weed Res.* **1974**, *14*, 415−421.

(33) Villagrasa, M.; Guillamón, M.; Eljarrat, E.; Barceló, D. Determination of benzoxazinone derivatives in plants by combining pressurized liquid extraction−solid-phase extraction followed by liquid chromatography−electrospray mass spectrometry. *J. Agric. Food Chem.* **2006**, *54*, 1001−1008.

(34) Nagao, T.; Otsuka, H.; Kohda, H.; Sato, T.; Yamasaki, K. Benzoxazinones from *Coix lachryma-jobi* var. *ma-yuen*. *Phytochemistry* **1985**, *24*, 2959−2962.

(35) Atkinson, J.; Morand, P.; Arnason, J. T.; Niemeyer, H. M.; Bravo, H. R. Analogues of the cyclic hydroxamic acid 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (DIMBOA): decomposition to benzoxazolinones and reaction with beta-mercaptoethanol. *J. Org. Chem.* **1991**, *56*, 1788−1800.

(36) Tipton, C. L.; Klun, J. A.; Husted, R. R.; Pierson, M. D. Cyclic hydroxamic acids and related compounds from maize. Isolation and characterization. *Biochemistry* **1967**, *6*, 2866−2870.

(37) Baumeler, A.; Hesse, M.; Werner, C. Benzoxazinoids—cyclic hydroxamic acids, lactams and their corresponding glucosides in the genus *Aphelandra* (Acanthaceae). *Phytochemistry* **2000**, *53*, 213−222.

(38) Macías, F. A.; Marín, D.; Oliveros-Bastidas, A.; Chinchilla, D.; Simonet, A. M.; Molinillo, J. M. G. Isolation and synthesis of allelochemicals from Gramineae: benzoxazinones and related compounds. *J. Agric. Food Chem.* **2006**, *54*, 991−1000.

(39) Yalcin, I.; Tekiner, B. P.; Oren, I. Y.; Arpaci, O. T.; Aki-Sener, E.; Altanlar, N. Synthesis and antimicrobial activity of some novel 2,6,7-trisubstituted-2*H*-3,4-dihydro-1,4-benzoxazin-3-one derivatives. *Indian J. Chem.* **2003**, *42B*, 905−909.

(40) Gahagan, H. E.; Mumma, R. O. The isolation of 2-(2-hydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranoside from *Zea mays*. *Phytochemistry* **1967**, *6*, 1441−1448.

(41) Petho, M. Occurrence and physiological role of benzoxazinones and their derivatives. IV. isolation of hydroxamic acids from wheat and rye root secretions. *Acta Agron. Hung.* **1992**, *41*, 167−175.

(42) Wilkes, M. A.; Marshall, D. R.; Copeland, L. Hydroxamic acids in cereal roots inhibit the growth of take-all. *Soil Biol. Biochem.* **1999**, *31*, 1831−1836.

(43) Cambier, V.; Hance, T.; Hoffmann, D. E. Non-injured maize contains several 1,4-benzoxazin-3-one related compounds but only as glucoconjugates. *Phytochem. Anal.* **1999**, *10*, 119−126.

(44) Villagrasa, M.; Guillamón, M.; Labandeira, L.; Taberner, A.; Eljarrat, E.; Barceló, D. Benzoxazinone allelochemicals in wheat: distribution among foliage, roots, and seeds. *J. Agric. Food Chem.* **2006**, *54*, 1009−1015.

(45) Grambow, H. J.; Lückge, J.; Klausener, A.; Müller, E. Occurrence of 2-(2-hydroxy-4,7-dimethoxy-2*H*-1,4-benzoxazin-3-one)-β-D-glucopyranoside in *Triticum aestivum* leaves and its conversion into 6-methoxy-benzoxazolinone. *Z. Naturforsch.* **1986**, *41C*, 684−690.

(46) Hedin, P.; Davis, F.; Williams, W. 2-Hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (N-O-Me-DIMBOA), a possible toxic factor in corn to the southwestern corn borer. *J. Chem. Ecol.* **1993**, *19*, 531−542.

(47) Oikawa, A.; Ishihara, A.; Tanaka, C.; Mori, N.; Tsuda, M.; Iwamura, H. Accumulation of HDMBOA-Glc is induced by biotic stresses prior to the release of MBOA in maize leaves. *Phytochemistry* **2004**, *65*, 2995−3001.

(48) Stochmal, A.; Kus, J.; Martyniuk, S.; Oleszek, W. Concentration of benzoxazinones in roots of field-grown wheat (*Triticum aestivum* L.) varieties in two cultivation systems. *J. Agric. Food Chem.* **2006**, *54*, 1016−1022.

(49) Macías, F. A.; Oliveros-Bastidas, A.; Castellano, D.; Marín, D.; Simonet, A. M.; Molinillo, J. M. G. Evidences of the allelopathic phenomenon, *Proceedings, Second European Allelopathy Symposium. Allelopathy—from Understanding to Application, FATEALLCHEM Workshop ("Fate and Toxicity of Allelochemicals (Natural Plant Toxins) in Relation to Environment and Consumer")*, June 2004; Institute of Soil Science and Plant Cultivation Press Services: Pulawy, Poland; 2004; pp 5−6.

(50) Tipton, C. L.; Ming-Chung, W.; Tsao, F. H.; Chang-chu, L. T.; Husted, R. R. Biosynthesis of 1,4-benzoxazin-3-ones in *Zea mays*. *Phytochemistry* **1973**, *12*, 347−352.

(51) Leighton, V.; Niemeyer, H. M.; Jonsson, L. M. V. Substrate specificity of a glucosyltransferase and an *N*-hydroxylase involved in the biosynthesis of cyclic hydroxamic acids in Gramineae. *Phytochemistry* **1994**, *36*, 887−892.

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