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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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

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Wheat (*Triticum aestivum* L.) has been found to possess allelopathic potential and studies have been conduced to apply wheat allelopathy for biological weed control. 2,4-Dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) is a common product found in wheat, corn, and rye exudates and it can be released to the environment by that way. In this report, the stability of DIBOA is studied in two soils from crop lands of wheat cv. Astron and cv. Ritmo. These varieties were selected by their concentrations of DIBOA and 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) from aerial parts and by the bioactivities of their aqueous extracts in the growth of wheat coleoptile sections. The degradation rate of DIBOA in these soils was measured in laboratory tests during 90 h by high-pressure liquid chromatography methods. These analyses demonstrate that DIBOA was transformed primarily into 2-benzoxazolinone (BOA). This transformation was similar in both soil types with an average half-life of 43 h. The degradation studies for BOA show its biotransformation to 2-aminophenoxazin-3-one (APO) with a half-life of 2.5 days. Therefore, BOA is an intermediate product in the biotransformation from DIBOA to APO in these wheat crop soils and is consistent with previous findings. APO was not degraded after three months in soil, suggesting that its degradation rate in soil is very slow.

KEYWORDS: Benzoxazinoids; DIBOA-Glc; DIBOA, BOA; biodegradations; soil; *Triticum aestivum*; bioactivity

INTRODUCTION

Some cereal plants produce a series of benzoxazinoid compounds (cyclic hydroxamic acids). The number of this group of natural products is small (*1*), but they possess diverse biological activities. These compounds are involved in the defense of plants against fungi (*2*) and insects (*3*) as well as in allelopathic interactions (*4*,*5*). The most important benzoxazinoids reported (**Figure 1**) are 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA), which are present in wheat, maize, and rye and have been found in members of families *Acanthaceae*, *Rannunculaceae*, and *Scrophulariaceae* (*6*). These compounds are present as glycosides (**Figure 1**) in plants, being released as aglycones by the activity of the enzyme *β*-glucosidase (*7*, *8*).

Moreover, these aglycones are unstable in solution and soil, being transformed to 2-benzoxazolinone (BOA), 7-methoxy-2-benzoxazolinone (MBOA), and other degradation products (*1*) (**Figure 1**). These transformations depend on the chemical and biological conditions. Some of these transformation products are more biologically active than the original ones (*9*).

Transport of allelochemicals to the soil can occur mainly by leaching of the foliar parts (*10*, *11*), exudation from root (*12*), decomposition of plant residues by microbial action (*13*), or by direct transformation by microbes associated to the roots (*14*).

Previous publications have dealt with the isolation, characterization, and biological activity of the degradation products (*15*−*17*). However, the dynamic aspects of the degradative processes of these compounds such as half-life, concentration dependence of the half-life, or the influence of other microbial substrate on degradation have not been researched. Here, we report the stability and degradation studies of DIBOA-Glc and its derivatives in different conditions and soils used to cultivate two wheat varieties (*Triticum aestivum* cv. Astron and cv. Ritmo). Our objectives were to study the influence of soil microbes in the degradation dynamics of these compounds. Understanding this process will allow us to elucidate the influence of these microbes on chemical defense strategies in

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10.1021/jf048702l CCC: $30.25 © 2005 American Chemical Society
Published on Web 01/04/2005

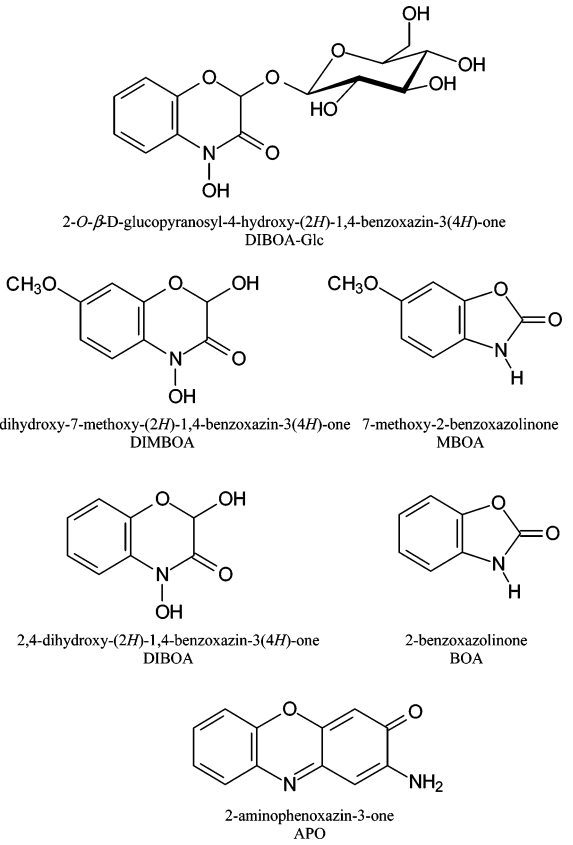

Figure 1. Structures of benzoxazinoids and their degradation products mentioned in the text.

plants that produce hydroxamic acids and to propose which chemical structures are responsible for the allelopathic behavior observed. To ensure the maximum accuracy in the study, soils from different wheat varieties were used and compared, and the influence of soil chemical reactivity and spontaneous degradation processes was taken into account by using the suitable standards for the different analysis.

MATERIALS AND METHODS

Contents of DIBOA and DIMBOA in Six *Triticum aestivum* Varieties. The contents of DIBOA and DIMBOA were determined in six wheat varieties (*Triticum aestivum* cv. Hill, Portal, Ritmo, Astron, Stakado, and Solist). The varieties were cultivated in a growth chamber at controlled temperature and humidity (25 °C and 68%) with a photoperiod of 16 h light/8 h dark provided by white light fluorescent lamps. After 7 days of growth, seedlings were extracted with acidified MeOH (1% AcOH) in an ultrasonic bath for 5 min. The resulting mixture was filtered, and the residue was extracted again with acidified MeOH. The two solutions were combined and the solvents were eliminated by distillation in vacuo at 30 °C. The residue was dissolved in water and loaded in a Sep-Pak C18. Then, it was washed with acidified water and with an acidified solution of aqueous methanol (MeOH:H_2O:AcOH, 60:40:1). The resulting solutions were combined, the solvents were eliminated by distillation in vacuo, and the residue was dissolved in 1 mL of MeOH:H_2O:HOAc (60:40:1) for their HPLC analysis. The six wheat varieties were cultivated in experimental crop lands (see below). They were harvested in their stage 21 (BBCH scale) (*18*) and were immediately stored at −20 °C until their study. One kilogram of the complete plant was extracted with 3 L of distilled water by rain simulation (24 h) by means of a Pluviotron Rain Simulation Equipment (*19*). The resulting aqueous extract was filtered (<22 ím, Millipore Express PLUS) before the bioactivity evaluations. Dilutions at 1/5, 1/10, 1/20, and 1/40 v/v concentrations were prepared from the original extracts. This extraction procedure intended to simulate the natural process of release to soil and to obtain the compounds that could affect the soil conditions and degradation capacity.

Bioassays of General Activity. The wheat coleoptile bioassay was used as protocol for general bioactivity evaluation of wheat extracts. Wheat seeds (*Tricticum aestivum* L. cv. Duro) were sown in Petri dishes (15-cm diameter), moistened with water, and grown in the dark at 22 ± 1 °C for 3 days in a Memmert ICE 700 growth chamber. The roots and caryopsis were removed from the shoots. The latter was placed in a Van der Weij guillotine and the apical meristem (2 mm) was cut and discarded. The next 4 mm of the coleoptile sections were removed and used for bioassay. All manipulations were performed under green safelight (*20*). The aqueous extract obtained from wheat lixiviating to be assayed (2 mL at 1 mg/15 mL initial concentration) for biological activity was added to four 10-mL test tubes for each dilution. The assay was done in duplicate. Phosphate-citrate buffer (2 mL) containing 2% sucrose (*21*) at pH 5.6 was added to each test tube. Following the placement of five coleoptile sections in each test tube, they were placed in the growth chamber mentioned above and rotated at 0.25 rpm in a roller tube apparatus (24 h) at 22 ± 1 °C in the dark. The coleoptile sections were measured automatically by generation of digital images of them that were processed in a Photomed Equipment Software (*19*). Data were statistically analyzed using Welch's test (*22*). Results are presented as cluster analysis.

Sources of Hydroxamic Acids and their Derivatives. 2-Benzoxazolinone (BOA) was purchased from Fluka Chemika. The benzoxazinone standards (2*R*)-2-*O*-β-D-glucopyranosyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-Glc) and 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) were obtained from natural sources.

DIBOA-Glc was isolated from 7-day-old rye (*Secale sereale* L.) seedlings. They were grown in darkness at 22 °C and soaked and homogenized in acetone (3 mL/g seedling). The resulting mixture was filtered and centrifuged. The remaining solid was extracted twice with acetone:water (1:5 v/v). The solutions are combined and the acetone removed by distillation in vacuo at 35 °C. The remaining aqueous solution is extracted with hexane (×5) to eliminate lipophilic material and ethyl acetate. Organic layers were discarded, leaving aqueous phase containing DIBOA-Glc as well as other hydrophilic substances. This phase was extracted with *n*-butanol (×4). The organic phase was dried by distillation at reduced pressure, and the resulting solid was chromatographed to select fractions enriched in hydroxamic acid glycosides. This extract was purified in a medium-pressure liquid chromatography (MPLC) system by using an RP-C18 stationary phase. The mobile phase was MeOH:H_2O (3.5:6.5) with 1% of AcOH. Eighty-eight milligrams of DIBOA-Glc was obtained by processing 1 kg of rye seedlings (0.0088%).

To obtain DIBOA, the ethyl acetate extract discarded in the isolation of DIBOA-Glc was purified by column chromatography (normal phase) by using chloroform:hexane increasing polarity solutions (0−30% $CHCl_3$). Fractions containing DIBOA were combined and dried by distillation at reduced pressure. Recrystallization from ethyl acetate/hexane afforded pure DIBOA.

2-Aminophenoxazin-3-one (APO) was synthesized according to previously described procedures and its structure confirmed by ^{1}H, ^{13}C NMR, and MS experiments, which were in agreement with those previously reported (*14*, *17*).

The purity of the isolated standards and synthetic was determined by NMR and HPLC analyses and was >98%. ^{1}H and ^{13}C NMR spectra were recorded using MeOH-d_4 as solvent in a Varian INOVA spectrometer at 399.99 and 100.577 MHz, respectively. The resonance of residual methanol for ^{1}H and ^{13}C was set to δ_H 3.30 and δ_C 49.00, respectively, and used as internal reference. HPLC data were acquired by using a Varian ProStar system, equipped with two solvent delivery modules (Model 210), an autosampler (Model 410), a PDA detector (diode array UV-vis system, Model 330), a Quadrupole MS/MS detector (Model 1200L), and a Phenomenex SYNERGI 4 micron Fusion RP-80 column (250−460 mn).

***Triticum aestivum* Cultivars (cv. Astron and cv. Ritmo).** The soil samples were collected in November 2002 from fields where wheat crops of cv. Astron and Ritmo were grown at the Institute of Agriculture and Development Center, Rancho de la Merced (Jerez de la Frontera, Spain). Conventional fertilization procedures were used without using herbicides and fungicides. The crop terrains were mechanically treated to eliminate weeds prior to planting. After approximately one month

Table 1. Physicochemical Analysis of Wheat Crop Soil

exchange capacity (mequiv/100 g)	33.91
exchangeable cations (mequiv/100 g)	
Ca^{2+}	28.96
Mg^{2+}	3.08
Na^{+}	0.29
K^{+}	1.58
carbonates (% w/w)	18.25
active limestone (% w/w)	8.81
assimilable phosphorus (Olsen, ppm)	8.7
total organic content (ppm)	1.35
organic nitrogen (% w/w)	0.08
pH (H_2O:1/2.5)	8.33
pH (KCl)	7.31
assimilable potassium (ppm)	635
clay (% w/w)	52.6
sand (% w/w)	15.4
slime (% W/W)	32.0
textural classification	clay

of growth (growing stage 21-BBCH scale), samples of each variety were sown in individual parcels (300 m^2, 12 plots of 25 m^2). Wheat samples were collected and stored at −20 °C prior to their analysis.

Soil Collection. The selected parcels for the cultivation contained similar physicochemical characteristics at the moment of their cultivation (**Table 1**) and did not contain any other plant species in growth. After harvest, samples of soil from the vicinities of the plants were taken to get the maximum density of the microbial population associated with the plant underground parts. The samples were taken at the 15−20-cm depth, in a radius of 3 cm around the plant lines. One hundred random soil samples were collected for each crop (0.014 m^3). The soil was sieved at 2 mm, placed in plastic bags, and preserved at −20 °C until its study. Before the degradation studies, dry plant material was removed from the samples as well as calcareous stones by a sieve that allows a distribution of soil particles smaller than 1 mm.

Soil pH was measured in a 1:2.5 (v/v) aqueous extract and in KCl solution. Total organic nitrogen (TON) was determined according to the Kjeldhal method, and total organic carbon (TOC) was determined by oxidation with potassium dichromate. Available phosphorus was extracted with sodium bicarbonate (*23*) and determined by colorimetry, according to the method of Murphy and Riley (*24*). Ca, Mg, Na, and K were determinated by flame photometry (*25*).

Experiment Design. Soil samples (10 g) were placed in 50-mL vials and test solutions (5 mL of sterilized Milli-Q water with 10 mg of DIBOA-Glc or DIBOA, and 2.5−50 mg of BOA) were added to them. Samples of these solutions were collected using a glass syringe. Sample amounts, sampling periods, and treatments prior to the analysis are described below. The resulting suspension is homogenized by using a vortex mixer (VWR International) at room temperature. For control samples, soil was sterilized by washing with methanol (30 mL/5 g soil, 12 h) and drying at 100 °C over a period of three consecutive days (Gallenkamp Hotbox Oven). An aqueous solution of the tested substance without soil was added as water control to evaluate spontaneous degradation of the substance. These samples were analyzed by the same procedure and at the same times as those containing nonsterilized soil samples. Recovery extractions in water and sterile soil control samples were used to correct the determinations in the degradation experiments.

Sampling and Processing of Soil. Samples (0.5 mL) of incubation solution were taken from the soil at different time periods after homogenization of the soil−water suspension. Samples were preserved at −20 °C after the addition of methanol (1 mL) to avoid degradation between the end of the experiment and the moment of the analysis. Samples were preserved at −20 °C after the addition of methanol (1 mL) to avoid degradation between the end of the experiment and the moment of the analysis. The sampling intervals were determined by means of preliminary studies, in which degradation of each compound was achieved (12 h for DIBOA-Glc, 12 h DIBOA and 24 h for BOA).

For HPLC analysis, solutions were centrifuged in a Selecta Microfiger BL 71379 apparatus at 13.000 rpm for 10 min and then filtered (<44 μm). The resulting solid residue was extracted with methanol (10 mL) by means of an ultrasound bath (15 min, 5 °C). The extract was centrifuged again for 10 min and the process was repeated three more times. This process was repeated using ethyl acetate (10 mL) as solvent. The soil aqueous solution, the methanol, and the ethyl acetate extracts were distilled at reduced pressure. The solid residues were dissolved in 2 mL MeOH with 1% of acetic acid and filtrated (<0.2 μm) before the injection.

Analysis of Hydroxamic Acids in Soil. All samples were analyzed on a Merck HITACHI HPLC equipped with a LaChrom L-7100 quaternary gradient pump, an L-7455 LaChrom diode array detector, and an L-7200 LaChrom autoinjector. Data were collected and processed by using an HPLC data system Merck HITACHI D7000. Instrumental conditions for analysis of hydroxamic derivatives were Lichrospher 100 RP-18 (250 × 4.0 mm, 5 μm) reversed-phase column at 25 °C. Mobile phases were water: 1% AcOH (A) and methanol: 1% AcOH (B) at a flow rate of 1 mL min^{-1}. Injection volume was 50 μL. The following gradient was used for separation: at 0 min, 30% B; 2 min, 30% B; 19 min, 60% B; 21 min, 100% B. Under these conditions, the following retention times are obtained for each compound: DIBOA-Glc 5 min; DIBOA 9.01 min; DIMBOA 10.69 min; BOA 12.56 min, and APO 24.03 min. The detection was carried out at the following wavelengths: 254, 253, 263, 271, and 280 nm for DIBOA-Glc, DIBOA, DIMBOA, BOA, and APO, respectively. For quantitative analysis, stock solutions (1 mg/mL) of each individual standard were prepared by dissolving accurate amounts of pure standard in acidified MeOH (1% AcOH). Working standard solutions were obtained by further dilution of stock solutions with MeOH:acidified H_2O (1% AcOH) (70:30). These solutions were used to generate the external standard response calibration curves for subsequent measurement of quality parameters and concentration of the hydroxamic acid derivatives in soil at different times. All the analytical procedures were validated by means of interlaboratory calibration study (*26*). Calibration curves for all tested compounds were prepared in MeOH:1% AcOH and also in soil solution (filtered at <0.22 μm.). Optical densities and UV spectra were identical for both conditions (identical molar extinction coefficients), ensuring an identical instrumental response.

Derivation of Rate Constants. Rates constants were fitted in models using least squares, which assumed first-order kinetics. The remaining concentration of the substance in soil after the incubation period was used for this purpose. Calculations were performed using Microsoft Excel 2000 Spreadsheet and Microcal Origin v. 5.0 (Microcal Software Inc., Northampton, MA) for plotting and least-squares curve fitting.

RESULTS AND DISCUSSION

Selection of Wheat Varieties and Soil for the Degradation Study of the Allelochemicals Released. Any degradation process taking place in the soil will be affected by the biological environment of the plants present in it. These processes will define the allelopathic properties and the final destination of the allelochemicals released. Before the degradation studies of the different allelochemicals exudated by wheat roots, we carried out a quantitative analysis of the major benzoxazinoids, DIMBOA and DIBOA, in six commercial wheat varieties. The results were used as one of the criteria in the selection of the wheat varieties to be used for further studies. The levels of benzoxazinoids were analyzed in the six wheat c.v.: Astron, Bill, Portal, Ritmo, Solist, and Stakkado.

We observe DIMBOA as the major allelochemical in all the varieties being the one that showed higher variation in its concentration in comparison with DIBOA. The cluster analysis obtained from the hydroxamic acids production analysis is shown in **Figure 2**. This cluster divided the varieties in two groups: Ritmo, Portal, and Astron (higher content of benzoxazinoids) and Bill, Solist, and Stakkado.

Since microbiotic characteristics of soil where wheat has been cultivated could vary owing to compounds lixiviated because

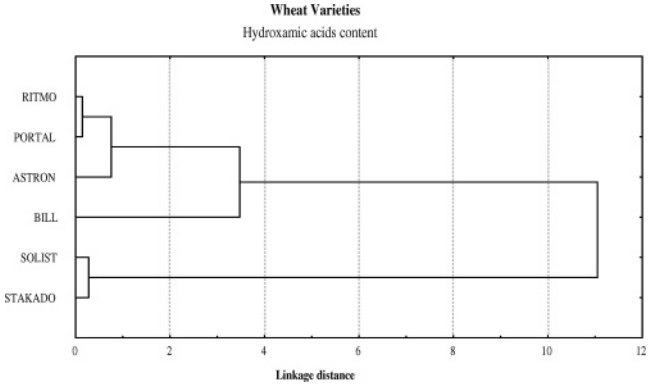

Figure 2. Cluster analysis for hydroxamic acids content in six cultivated wheat varieties.

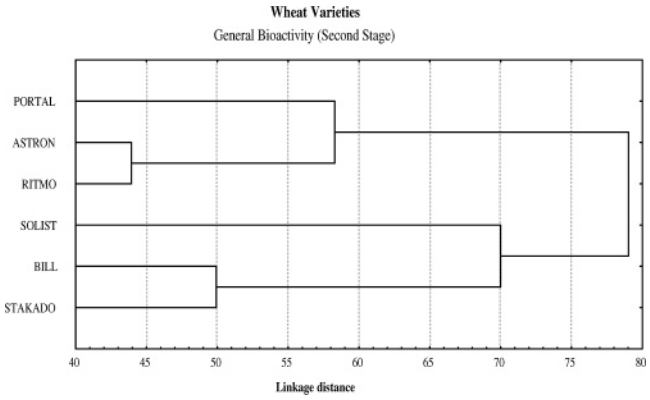

Figure 3. Cluster analysis of general bioactivity shown by aqueous extracts of six cultivated wheat varieties (growing stage 21-BBCH scale).

of rain or dew, the second criterion for the selection was the general bioactivity shown by the lixiviated of the leaves. The activity was measured in samples from cultivars of the six wheat varieties in Spain and Denmark, to differentiate possible climate influences. Spanish samples showed higher activities, and **Figure 3** shows the cluster analysis of their bioassay. The profile is similar to the one obtained in the previous cluster study. Varieties Astron, Ritmo, and Portal presented higher activities.

Additionally, lixiviates of Astron and Ritmo varieties maintain their activities with dilution. Moreover, high capabilities for biotransformation of hydroxamic acids and derivatives have been reported for phytopathogenics fungi associated with these two varieties (*27, 28*). Consequently, soils where Astron and Ritmo varieties cropped were selected.

Microbial Transformation of 2*R*-2-*O*-*β*-D-Glucopyranosyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-Glc). Benzoxazinoids are present in plants as glucosides. The plant only releases them after the action of the specific enzyme *β*-glucosidase, generating the aglycone, which is the commonly detected product. It is interesting to study the degradation of DIBOA-Glc since it could be possible that it could be released to soil from crop residues during decomposition after harvest.

Preliminary studies show that the soil in which wheat varieties were cultivated is able to transform the allelochemicals studied. Under sterile conditions, the allelochemicals are recovered without finding degradation products or transformation of the compounds. The analysis of the nonsterile soil solutions yielded the degradation product 2-aminophenoxazin-3-one (APO). It was isolated as the major degradation product for DIBOA and BOA.

The results of the recovery tests for the extraction of the three allelochemicals during the times of study are shown in **Figure 4**. This experiment was necessary to establish if the allelochemical residue extraction procedure is effective throughout the periods of study. The recovery average during the study was of 81.5 ± 2.4 (DIMBOA-Glc), 73.4 ± 2.9 (DIBOA), and 70.4 ± 2.7 (BOA).

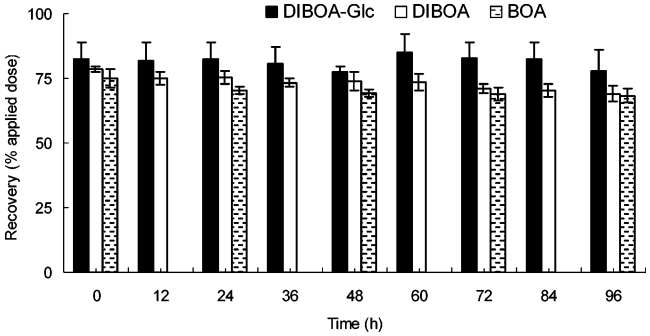

Figure 4. Extraction recovery of allelochemicals studied in sterilized soil over the time. Cultivated soil with *Triticum aestivum* cv. Astron. Test carried out at a dose of 5 mg of allelochemicals/g of soil. Error bars are standards deviation of the mean, $n = 3$. One-way ANOVA, $\alpha = 0.05$, F critical = 2.5 DIBOA-Glc, 0.4 DIBOA, 4.4 BOA.

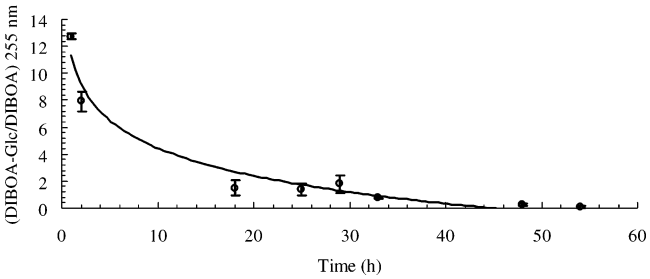

Figure 5. Variation of the concentration relationships (DIBOA-Glc/DIBOA) at 1 mg DIBOA-Glc/g of *T. aestivum* cv. Ritmo soil, $n = 3$. Incubation conditions: 25 °C, 14 h light, 10 h darkness.

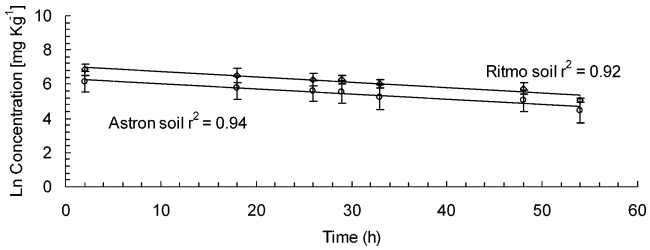

Figure 6. First-order degradation kinetics for DIBOA-Glc in crop soil (*T. aestivum* cv. Astron and Ritmo). A 1 mg/g soil $n = 3$.

These recoveries are not statistically different with time for DIBOA-Glc. This is not the case of DIBOA and BOA. These facts allowed us to conclude that this research represents a real study of transformation of the allelochemicals because of degradation/transformation processes. So, in any other degradation studies, it could be necessary to determine the recovery ratios over the same time periods.

The ratio of DIBOA-Glc/DIBOA decreased significantly in the first 21 h. After this, it remained the same until the 31st hour (**Figure 5**). The half-life of DIBOA-Glc in the soil was calculated by a first-order kinetic model, affording the following result: 23 h ($r^2 = 0.94$; $p = <0.0001$) in Astron soil and 25 h in Ritmo soil ($r^2 = 0.92$, $p < 0.002$). Half-lives did not differ significantly (**Figure 6**; **Table 2**).

Microbial Transformation of 2,4-Dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) and 2-Benzoxazolinone (BOA). Samples of soil cultivated with wheat (cv. Astron and Ritmo) were inoculated with DIBOA (1 mg/g soil). After 24 h

Table 2. Degradation Constant Rate, *K*, and Time of Half-Life, $t_{1/2}$, for the Biodegradation of DIBOA-Glc, DIBOA, and BOA, According to Soil Type and Dose. Means and Standard Deviation for $n = 3$[a]

soil	dose (g/kg soil)	K (h^{-1})	$t_{1/2}$ (h)	r^2 [b]	p[c]
		DIBOA-Glc			
Ritmo	1	0.028 ± 0.051^a	25.0 ± 4.5	0.92	<0.002
Astron	1	0.030 ± 0.003^a	23.3 ± 2.5	0.94	<0.0001
		DIBOA			
Ritmo	1	0.025 ± 0.004^a	28.3 ± 4.7	0.91	<0.002
Astron	1	0.030 ± 0.006^a	24.0 ± 4.6	0.95	<0.0001
Ritmo	5	0.013 ± 0.004^b	57.3 ± 6.0	0.98	<0.001
Astron	5	0.011 ± 0.002^b	62.3 ± 7.0	0.95	<0.04

BOA

soil	dose (g/kg soil)	*K* ($days^{-1}$)	$t_{1/2}$ (days)	r^2 [d]	p[e]
Ritmo	2	0.318 ± 0.041^c	2.20 ± 0.26	0.71	<0.002
Astron	2	0.260 ± 0.038^c	2.71 ± 0.40	0.97	<0.0001

[a] Same letters indicate not significant difference Student's *t* test, $\alpha = 0.05$. [b] r^2 = coefficient of variance. [c] *p* = confidence limit.

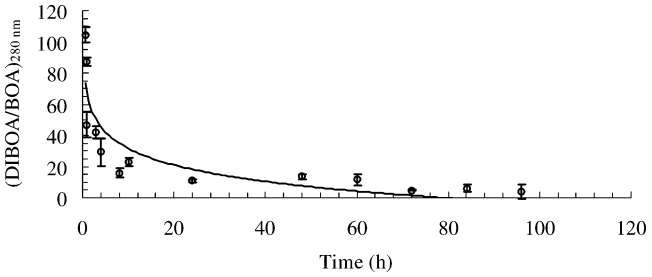

Figure 7. Variation of the concentration relationships (DIBOA/BOA) at 1 mg DIBOA/g of soil *T. aestivum* cv. Ritmo soil, $n = 3$. Incubation conditions: 25 °C, 14 h light, 10 h darkness.

of incubation, a significant amount of a degradation product could be observed. This compound was identified as 2-benzoxazolinone (BOA) by comparison with a BOA standard, using HPLC-DAD methods. Amounts of DIBOA could be detected even after 3.5 days.

The DIBOA/BOA ratio at different incubation times showed the conversion of the DIBOA to be essentially completed between 2 and 10 days (**Figure 7**). The ratio showed a pronounced fall in the first 10 h, but it stabilized during the following days. After 10 days, a second degradation product was detected. This compound could be isolated and identified as 2-aminophenoxazin-3-one (APO) by comparison of its spectroscopic data with those previously reported (*17*) and with synthetic samples of the compound.

Optimal Dose Determination. It is difficult to estimate the real amounts of these compounds that could be released to soil, because the liberation modes are diverse. They include root exudation, lixiviates of aerial parts or decomposition of them in soil, and others (*29*). Moreover, these dynamic processes are strongly affected by biotic (growing stage, plant variety) and abiotic (environmental conditions) factors (*30*). Decomposition of aerial plant parts of rye (*Secale cereale* L.) could produce 0.04 mg of DIBOA/cm^2 considering a biomass of 820 g/m^2 (*31*, *32*). The sampling area used for soil collection in this study (3 ± 1 cm around the plant lines) would contain 0.13−0.3 mg of DIBOA considering this approximation. Other estimations provide values between 0.5 and 1 mg of DIBOA per gram of soil (*33*, *34*). Nevertheless, these estimations could increase, considering other factors, the possibility of genetic manipulation

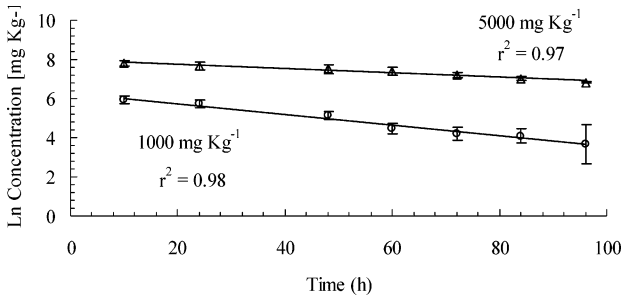

Figure 8. First-order degradation kinetics for DIBOA in crop soil (*T. aestivum* cv. Astron), $n = 3$.

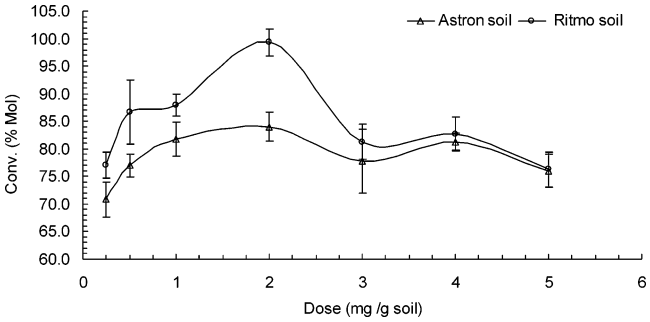

Figure 9. Conversions of BOA after 10 days of incubation, according to dose and soil type. Incubation conditions: 25 °C, 14 h light/10 h dark. Error bars are standards deviation of the mean, $n = 3$. Only at dose 2 mg/g soil are statistical differences found. Student's t-test, $\alpha = 0.05$.

to produce larger amounts of DIBOA (*35*−*37*). The doses employed in this study (0.25−5 mg of compound/g of soil) screen a wide concentration range in the same magnitude order as these estimations described. Although there are no precise data for wheat and corn crops, the doses employed here are similar to those estimated for rye, and they are adequate to contrast the stability of these compounds in two different soils at the same doses.

For the determination of DIBOA half-life, 1- and 5-mg doses of DIBOA per gram of soil were inoculated, three times for each soil where the two selected varieties of wheat were grown. Total experiment time was 100 h with a sampling every 12 h. The residual quantity of DIBOA as a function of time is presented in **Figure 8** in crop soil *T. aestivum* cv. Astron. The degradation of DIBOA in both soils was fitted to a first-order reaction affording different values, depending on the initial concentration. Thus, when the applied dose was 1 mg/g of soil, the mean half-life was 26 h, whereas 60 h was the value obtained for the 5-mg dose (**Table 2**). Although the values of half-life applying first-order kinetics are independent of the initial concentrations of substrate, dependence between them and the starting concentrations is observed here. Some reports have suggested dependence among the prediction values based on first-order kinetics models and the substrate concentration (*38*−*40*). Here, we apply this model trying to find parameters that could indicate the resistance of the different allelochemicals to biodegradation, knowing the limitations of this model. This is the reason two different concentrations have been tested.

Our results suggest that in these soils, DIBOA degrades to BOA, followed by conversion of BOA to APO. To confirm this hypothesis and study persistence, different amounts of BOA (0.25, 0.5, 1, 2, 3, 4, and 5 mg/g of soil) were added in both types of soils for 10 days (**Figure 9**). Although biotransformation of BOA to APO has been previously reported (*15*−*17*), this is the first time that the whole sequence of biodegradations of DIBOA-Glc to APO in soil is demonstrated.

BOA degraded rapidly in both soils, with a loss of 70−99% after 10 days. However, the soil cultivated with wheat variety Ritmo had higher activity at some initial doses of BOA. Maximum conversion in both soils occurred at a dose of 2 g/Kg, the conversion being significantly different at this dose in both soils. At higher doses, conversion did not increase despite a more substrate available amount. Those concentrations could saturate the soils or their maximum biotransformation capacities. The half-life of BOA was 2.20 and 2.71 days for the biotransformation in cultivated soil with varieties Ritmo and Astron, respectively; these are not statistically significant different. The comparison of these half-lives with those obtained for DIBOA in the two soils for a dose of 1 mg/g of soil (24 and 28 h) allowed us to conclude that BOA is more resistant to biotransformation than its precursor DIBOA.

The differences observed in the two soil types can be related with the population and type of microorganisms associated with the variety grown in it (*41*−*43*). An explanation of this fact may be that the compounds released from the plant depend on the wheat variety, and its chemicals can influence the micro- and macroflora associated to the root system, particularly fungi, and especially if there are competing species with different capacities to metabolize these compounds. The population would increase for those microorganisms that are able to metabolize the toxic compounds liberated by the plant or use them as a carbon source. The fact that APO is the major degradation product of DIBOA-Glc suggests that during the biodegradation process the concentration can reach toxic levels for the associated microorganisms. APO has toxic effects over 20 bacteria and two fungi species (*44*). Considering that even its precursor 2-aminophenol (AP) has also shown toxicity on bacterias present in the environment (*45*−*48*), it is possible that the biotransformation could stop when concentrations of AP and APO become toxic. Consequently, no further transformations could be observed.

Several experiments were carried out to study APO stability in both soil types. Biotransformation was not observed over a period of 90 days. Thus, APO would be the final product of the microbial degradation of DIBOA-Glc in these soils, with DIBOA and BOA being intermediate products of this transformation, at least for this time period. A scale of time of residence in the biotransformation shows that it is evident that the biological activities registered in bioassays of these degradable products can be due to a mixture of them with their degradation metabolites, which have also been described in aqueous solution and cultivation media (*41*−*43*). For instance, bioassays carried out with DIBOA with incubation times of 3 days or more would be also measuring activity of BOA and APO.

Bioassays with these incubation times have been developed in the search for active principles that would give a good reason for allelopathic effects shown by rye, corn, and wheat cultivations wastes, when they are used as rotation crops (*49*).

Our studies regarding degradation behavior of benzohydroxamic acids and their derivatives are included in a much wider research project, which includes optimization of their analysis methodology, bioactivity evaluations (fungicidal, phytotoxic, insecticidal), ecotoxicity research, structure−activity relationships studies (SAR), and development of new herbicide models and crop protection methodologies based on these compounds and their degradation derivatives (*50*). The overall objective of this project is to perform an environmental and human risk assessment of exploiting the allelopathic properties of winter wheat in conventional and organic farming and to develop a framework for future assessments of allelopathic crops.

The two studied soils, coming from two wheat cultivars (Astron and Ritmo), possessed a high capacity for DIBOA-Glc, DIBOA, and BOA degradation, while APO was stable over a period of three months. There are not significant differences between the two soils toward the biotransformation at the different doses studied. This fact suggests a similar microbial population in the soil belonging to both wheat varieties, although it has to be studied with higher detail. Moreover, these microbial populations could vary into crop periods, so it is necessary to test them during different cultivation cycles. In any case, DIBOA-Glc is the most susceptible chemical to the biodegradation, while APO is not transformed during three months of incubation; these differences can be related to their different chemical structures.

Degradation dynamics depended on the inoculated dose of allelochemical. Higher concentration experiments increased degradation times. So, the level at which these allelochemicals are released influences their persistence in soil.

The studies about DIBOA allelopathic potential should take into account the persistence of the degradated allelochemicals in soil. DIBOA half-life is too short to allow accuracy in the study without considering its degradation products, mainly BOA and APO. Both compounds are present in the soil after a relatively short time, once DIBOA is released by the plant. Thus, any allelopathic interaction involving DIBOA should also be influenced by BOA and APO. The results of this report indicate that APO should be the most important compound in allelopathy of wheat. (*51*−*53*).

Allelopathic role of DIBOA has been widely researched in recent years without taking into consideration the possible effects of its degradation metabolites. These degradation processes take place in soil as well as in bioactivity evaluation treatment solutions, so the results obtained in those experiments have to be carefully reanalyzed. New experiments should be designed to assign the real ecological role to each compound present in the biotransformation route.

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(50) The FATEALLCHEM project (Fate and Toxicity of Allelochemicals in Relation with the Environment and Consumer) is financially supported by the European Commission under the program "Quality of life and management of living resources (1998 to 2002)", Contract QLRT-2000-01967.

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Received for review August 2, 2004. Revised manuscript received November 2, 2004. Accepted November 3, 2004. This work was financially supported by the program "Quality of life and management of living Resources (1998 to 2002)" of the European Union, FATEALLCHEM Contract QLRT-2000-01967. Fellowships from the Universidad de Los Andes-Venezuela (A.O.B.) and from the European Commission (European Union) and Junta de Andalucía-Spain (D.M.) are also gratefully acknowledged.

JF048702L