

Degradation Studies on Benzoxazinoids.
Soil Degradation Dynamics of
2,4-Dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one
(DIMBOA) and Its Degradation Products, Phytotoxic
Allelochemicals from Gramineae

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Benzoxazinoids have been described as important allelochemicals from Gramineae as well as Acanthaceae, Rannunculaceae, and Scrophulariaceae plants. Several bioactivities have been described and evaluated for these compounds, including fungistatic, antifeedant, and phytotoxic. In ongoing studies about allelochemicals as natural herbicide models, the description of soil dynamics in phytotoxic agents has high importance, because the possible biotransformations developed by soil microorganisms could yield compounds with modified biological properties, affecting the overall allelopathic capability of the producer plant in a direct manner. Thus, a complete degradation study has been carried out for 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one (DIMBOA) and 6-methoxybenzoxazolin-2(3H)-one (MBOA) in two soils cultivated with *Triticum aestivum* L. varieties (cv. Astron and cv. Ritmo). The main purpose was to identify degradation products and to elucidate biotransformation dynamics. Results show DIMBOA to degrade rapidly, yielding MBOA in both studied soils at different doses ($t_{1/2} = 31 \pm 1$ h, $n = 12$) and reaching high conversions (80 ± 4 h, $n = 42$). MBOA, an intermediate in the degradation pathway from DIMBOA to 2-amino-7-methoxy-3H-phenoxazin-3-one (AMPO), was more resistant toward biodegradation ($t_{1/2} = 5 \pm 1$ days, $n = 6$). MBOA showed maximum conversions at a dose of 250 mg/kg of soil (36 ± 3 days, $n = 6$). Soil belonging to *T. aestivum* cv. Ritmo crops showed higher degradation capacity than cv. Astron soil. AMPO was the final degradation product observed for DIMBOA in the soils and experimental conditions selected. Consequences for activity and stability of these compounds in relation to allelopathy are discussed.

KEYWORDS: Allelopathy; benzoxazinoids; DIMBOA; MBOA; soil degradation; kinetics

INTRODUCTION

Cyclic hydroxamic acids with (2H)-1,4-benzoxazin-3(4H)-one skeletons are alkaloids produced by the Graminae family and other members of Acanthaceae, Rannunculaceae, and Scrophulariaceae (1, 2). These compounds, particularly in their aglucon form, have been related with plant defense against a wide variety of organisms, including fungi (3–6), bacteria (7), insects (8–11), and weeds (12, 13).

The objective of this study was to characterize the complete degradation sequence of 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one (DIMBOA) in wheat crop soil. In our

ongoing studies regarding allelopathy in higher plants, the fate and toxicity of chemicals released by them as a defense strategy have great importance. Biological effects of the released compounds could change if any biotransformation process for these chemicals takes place in soil, and this could affect the ecological role and chemical defense mechanism of the plant. This research is included in a wider project (14) in which insecticidal and fungicidal bioactivities as well as toxicity to humans and herbivores are also analyzed, in the search for wheat crop optimization. This research would lead to novel natural herbicide models and alternative crop practices.

The action of allelopathic plant–plant interaction agents needs their presence in the soil vicinity of the target plant (15). Degradation processes affecting these chemicals could enhance or decrease their phytotoxic effects, affecting the overall defense capability of the donor plant. Any degradation processes taking

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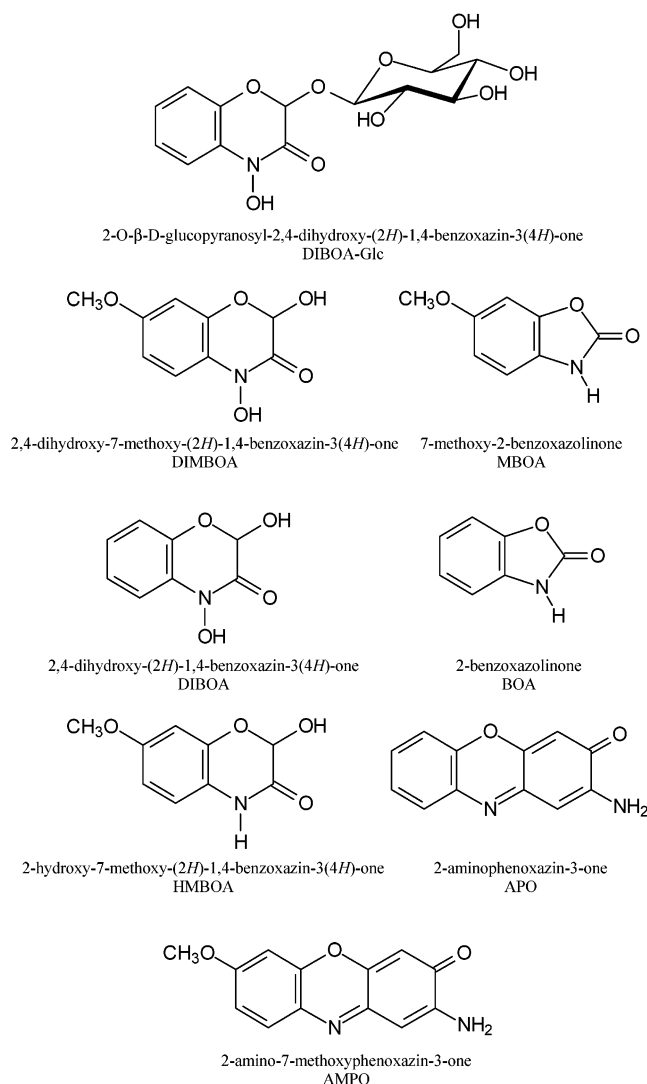


Figure 1. Structures for benzohydroxamic acids and their degradation derivatives.

place in the soil would be related with the biological environment of the plant present in it (16–18). These will define the allelopathic properties and the final destination of the allelochemicals released. Here, we present the degradation study in soil for DIMBOA (Figure 1) carried out with a methodology similar to that of the degradation study for the 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (DIBOA) chemical series (unpublished work).

There are no previous works about the degradation of DIMBOA in soil, because this compound decomposes to 6-methoxybenzoxazolin-2(3H)-one (MBOA; Figure 1) spontaneously in aqueous solution (20). A similar case occurs with the analogous DIBOA and its benzoxazin-2(3H)-one (BOA) (Figure 1). Thus, the degradation studies for both families of compounds have been focused on benzoxazolinones rather than their related hydroxamic acids. There are interesting reports about the degradation of these benzoxazolinones by endoparasitic fungi, root colonizing bacteria, and soil (5, 20–26). The studies have been carried out by combining soil samples from commercial wheat crops with stock solutions of the substrates to be degraded. The use of chromatographic analysis (HPLC-DAD) (27) for these solutions allowed us to record all of the data needed to elucidate degradation dynamics.

Effects of spontaneous degradation in water and side reactions catalyzed by soil elements (especially trivalent cations able to

form coordination complexes) have been eliminated in this study by the introduction of buffered water solutions and sterile soil solutions as control samples. We have studied the influence of the soil according to the cultivated wheat variety, the conversion according to the dose applied, and the competition for several substrates in one soil. We described the dynamics and persistence of DIBOA and its main degradation products: BOA and 2-amine-3H-phenoxazin-3-one (APO) (Figure 1). The glucosylated DIBOA (form in which the plant produces and stores the compound) was also studied (unpublished work). The results indicated that the half-life of DIBOA is not long enough (30 h) to understand the complete allelopathic phenomenon. In addition, BOA and especially APO must play a relevant role in the plant defense mechanism due to their phytotoxic bioactivity (28). Here, we report the corresponding study for DIMBOA and its derivative MBOA in two soils collected from wheat cultivars. The influence of degradation substrate mixtures and dose over biodegradation rate was studied, and the main degradation products were analyzed.

MATERIALS AND METHODS

Origin of DIMBOA and Its Derivatives. DIMBOA was isolated from corn (*Zea mays* L. cv. Apache) seedlings according to the method reported by Larsen and Christensen (29). A modification was introduced in this procedure, the addition of an extraction with ethyl acetate to the final aqueous extract, to ensure highest purity and better crystallization processes. MBOA was purchased from Lancaster Synthesis ($\geq 98\%$) and was used as received. 2-Amino-7-methoxy-3H-phenoxazin-3-one (AMPO) was synthesized from 5-methoxy-2-nitrophenol by a reductive dimerization reaction (30). The purity of the isolated standards and synthetic was determined by ^1H NMR and HPLC analyses and was found to be $>98\%$. ^1H and ^{13}C NMR spectra were recorded using $\text{MeOH}-d_4$ as solvent in a Varian INOVA spectrometer at 399.99 and 100.577 MHz, respectively. The resonance of residual chloroform for ^1H and ^{13}C was set to δ_{H} 3.30 and δ_{C} 49.00, respectively, and used as internal reference. HPLC PDA detector (diode array UV-vis system), column Phenomenex SYNERGI 4 micron Fusion RP-80 (250 \times 460 mm) Varian 1200L quadrupole MS/MS detector.

Contents of DIBOA and DIMBOA in Six *Triticum aestivum* Varieties. The contents of DIBOA and DIMBOA were determined in six wheat commercial varieties (*T. 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 of light/8 h of darkness 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 extracted again with acidified methanol. The two solutions were combined and the solvents eliminated by distillations in vacuo at 30 °C. The residue is 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/H₂O/HOAc, 60:40:1). The resulting solutions are combined, dried, and dissolved in 1 mL of MeOH/H₂O/HOAc (60:40:1) for their HPLC analysis.

Aqueous Lixiviates of Wheat Varieties. The aqueous solutions of the six wheat varieties were obtained from roots and leaves in their growing stage 21 (BBCH scale) (31). They were cultivated in experimental crop lands (see below). After harvest, they 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 (32). The resulting aqueous extract was filtered (<22 μm , 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.

Table 1. Physicochemical Analysis of Wheat Crop Soil

exchange capacity (mequiv/100 g)	33.91
exchangeable cations (mequiv 100 g ⁻¹)	
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 ₂ O: 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

Wheat seeds (*T. 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 section was removed and used for bioassay. All manipulations were performed under green safelight (33). 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 10 mL test tubes. The assay was made in duplicate. Phosphate–citrate buffer (2 mL) containing 2% sucrose (34) 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 automatically measured by generation of digital images of them that were processed by Photomed Equipment software (32). Data were statistically analyzed using Welch's test (35). Results are presented as cluster analysis.

T. aestivum Cultivars (Astron and Ritmo). The soil samples were collected in wheat crops of cv. Astron and Ritmo grown at the Institute of Agriculture and Development Center, Rancho de la Merced (Jerez de la Frontera, Spain), in November 2002. Natural fertilization procedures were used, without using herbicides and fungicides. The crop terrains were mechanically treated to eliminate weeds before cultivation. Each variety was sown in individual parcels (300 m², 12 plots of 25 m²). 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 m³).

The soil was sieved at 2 mm, placed in plastic bags, and preserved at -20 °C until its study. Before the degradation studies, dry vegetable 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 (36) and determined by colorimetry, according to the method of Murphy and Riley (37). Ca, Mg, Na, and K were determined by flame photometry (38).

Experiment Design. Soil samples (10 g) were placed at 50 mL vials, and test solutions (5 mL of sterilized Milli-Q water with the products to be degraded) were added over them. Fractions of the test solutions

were collected by using a glass syringe. Sample amounts, sampling periods, and treatments prior to the analysis are described below.

Degradation Substrates Dosage in Soil. For the dosage of the degradation substrates, they were diluted in distilled water and added to the soil. The resulting suspension was homogenized by using a vortex mixer (VMW International) at room temperature. For control samples, soil was sterilized by washing with methanol (30 mL per 5 g of soil, 12 h) and drying at 100 °C during three consecutive days (Gallenkamp Hotbox oven). An aqueous solution of the tested substance without soil was added as water control, to control spontaneous degradation of the substance. These samples were analyzed according to the same procedure and in the same times as the ones 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. The sampling intervals were determined by means of preliminary studies, in which degradation of each compound was achieved (5 min for DIMBOA and 24 h for MBOA).

For their HPLC analysis, solutions were centrifuged in a Selecta Microfiger BL 71379 apparatus at 13000 rpm during 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 during 5 min. This process was repeated using ethyl acetate (10 mL) as solvent. The soil aqueous solution and the methanol and ethyl acetate extracts were distilled at reduced pressure. The solid residues were dissolved in 2 mL of MeOH with 1% of acetic acid (HOAc) and filtered (<0.2 μm) before the injection.

Analysis of Hydroxamic Acids in Soil. All samples, including noninoculated soil, were analyzed on a Merck Hitachi HPLC equipped with a LaCrom 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 the analysis of hydroxamic derivatives were as follows: 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, 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 were obtained for each compound: DIMBOA, 10.69 min; MBOA, 16.56 min; HMBOA, 7.81 min; APO, 24.08 min; and AMPO, 19.74 min. The detection was carried out at the following wavelengths: 262, 230, 260, 228, and 235 nm for DIMBOA, MBOA, HMBOA, APO, and AMPO, 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 H₂O (1% HOAc) (70:30). These solutions were used to generate the external standard response calibration curves for subsequent measurements of quality parameters and concentration of the hydroxamic acids derivatives in soil at different times. All of the analytical procedures were validated by means of intercalibration laboratory study (27). Calibration curves for all tested compounds were compared in MeOH (1% v/v acetic acids glacial) 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 is used for this purpose. Calculations were performed on a Pentium IV 500 MHz computer running a Microsoft Excel 2000 spreadsheet and Microcal Origin v. 5.0 (Microcal Software Inc., Northampton, MA) for plotting and least-squares curve fitting.

Data Analysis. Degradation parameters require further treatment after laboratory study, in which the remaining allelochemical amount

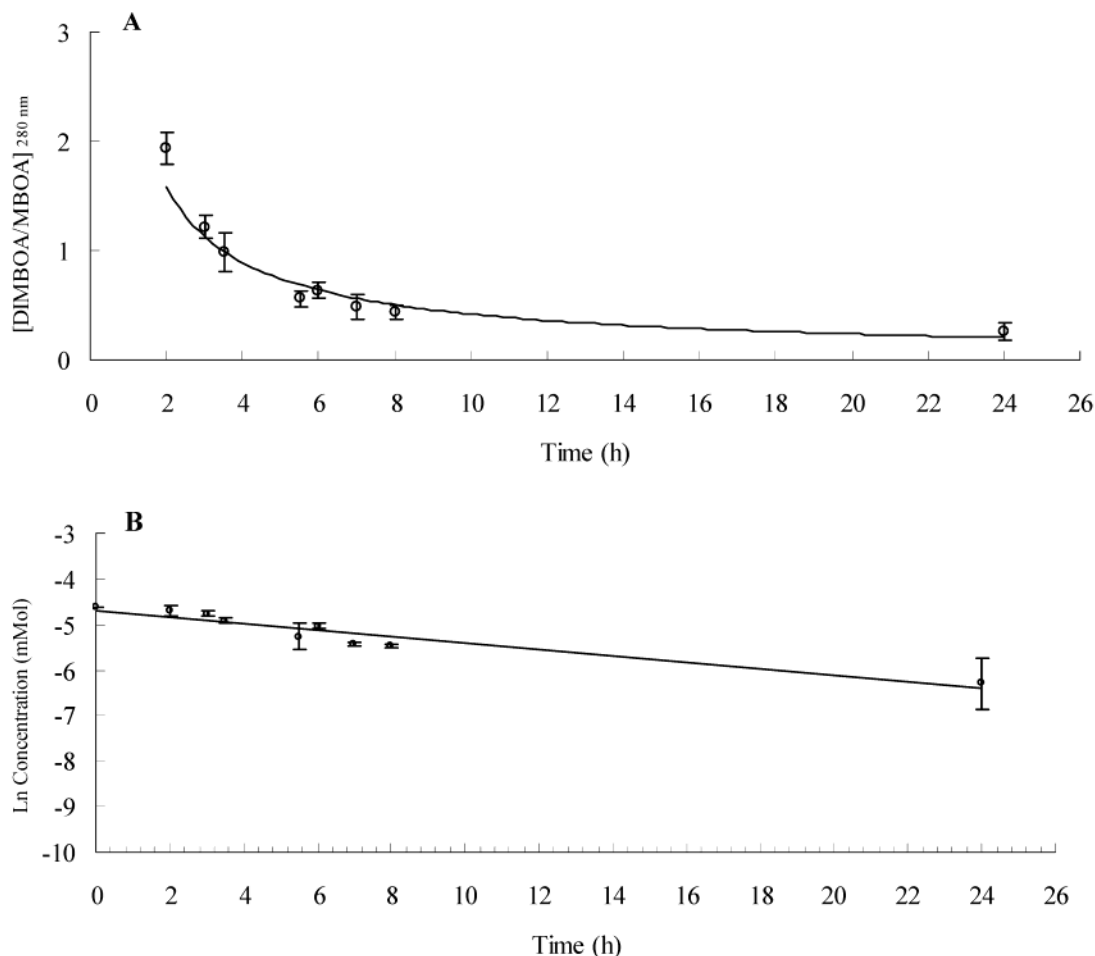


Figure 2. Stability of DIMBOA in buffered aqueous solution at 10^{-2} M citrate-phosphate buffer, pH 5.6: (A) concentration relationships ($[\text{DIMBOA}/\text{MBOA}]_{280\text{nm}}$); (B) first-order degradation kinetics.

in soil, studied under controlled conditions, is the experimental data to analyze. The decrease of allelochemical concentration through time is described according to first-order kinetics (monophasic or one-compartment kinetics), which is the most commonly used model to describe dissipation kinetics (39)

$$dC/dt = -KC \quad (1a)$$

where C is the concentration ($\text{mg}\cdot\text{kg}^{-1}$ of soil), t represents time (min), and K is the degradation rate (min^{-1}). The changing of allelochemical concentrations with time (dC/dt) is proportional to the concentration at that time. The integrated form of eq 1a gives

$$C_{(t)} = C_0 \exp(-Kt) \quad (1b)$$

where $C_{(t)}$ is the concentration at time t ($\text{mg}\cdot\text{kg}^{-1}$ of soil) and C_0 is the concentration measured immediately after application or the theoretically applied amount, at start time ($\text{mg}\cdot\text{kg}^{-1}$ of soil). A linear relationship is given for the logarithmic form of eq 1b:

$$\text{Ln } C_{(t)} = \text{Ln } C_0 - Kt \quad (2)$$

The time at which the concentration reaches the half of the initial is referred to as the half-life ($t_{1/2}$, time for 50% degradation of the initial amount of allelochemicals). Substitution into eq 2 gives

$$t_{1/2} = \text{Ln } 2/K \quad (3)$$

Under practical conditions (in the field), degradations cannot always be separated from other processes leading to allelochemical dissipation; in these cases, the term DT_{50} , time of dissipation of 50% of the initial concentration, is more appropriate. However, in this study, the dissipa-

tion of the allelochemicals for nonreversible adsorption and spontaneous degradation is considered and corrected for the total dissipation. Consequently, DT_{50} or $t_{1/2}$ could be used to describe compounds' stability.

Structure Determination. FTIR spectra were carried out on a Perkin-Elmer Spectrum BX FT-IR system. UV-vis spectra were recorded on a Merck Hitachi LaChrom L-7455 diode array detector.

AMPO: red powder; (MeOH) λ_{max} 235 nm; IR ν_{max} (film) cm^{-1} 3450, 3299, 1577, 847; ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) and ^{13}C NMR ($\text{DMSO}-d_6$, 100 MHz) see **Table 1**; EIMS (probe, 70 eV), m/z 243 $[\text{M} + 1]^+$.

RESULTS AND DISCUSSION

Stability of DIMBOA in Buffer Solution. DIMBOA has been described as a nonstable chemical in aqueous solution (20, 40). Thus, before the soil degradation study, several experiments were carried out to characterize the stability of this compound in buffered aqueous solution. The buffer chosen to maintain pH was the same one employed in general bioactivity evaluations of wheat leaf lixiviates (citric acid/ KH_2PO_4 , pH 5.6).

DIMBOA/MBOA concentration proportions and data treatment for a first-order kinetics in this buffer are shown in **Figure 2**. Starting DIMBOA concentration was 10^{-2} M. Half-life observed was 11 ± 3 h ($r^2 = 0.91$, $p < 0.0001$). Thus, in the wheat coleoptile bioassay, this allelochemical degrades to MBOA more rapidly than in nonbuffered aqueous solution, where a half-life of 24 h is currently admitted (19). This bioassay is usually employed for the characterization of bioactivity.

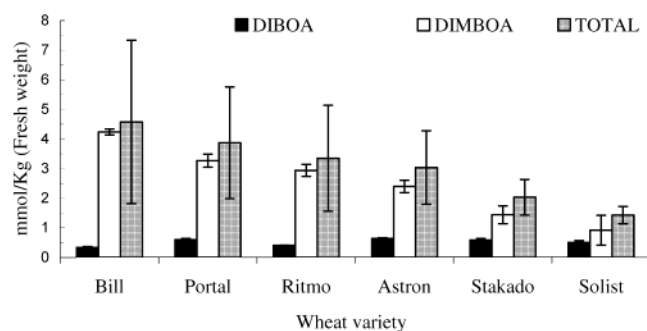


Figure 3. Levels of DIMBOA and DIBOA in six wheat varieties.

Therefore, toxicity levels observed for DIMBOA in this bioassay do not correspond to the action of a pure compound, because DIMBOA and its main degradation product MBOA are simultaneously present in the treatment solution. Previous reports of benzoxazinone degradation in buffered aqueous solutions and organic solvents establish a direct relationship between pH and degradability, because these degradation processes are related to the acid–base character of hydroxyls at C-2 and N-4 (19). Thus, degradation rates in water are very different from those observed in buffered aqueous solutions. On the other hand, acids or bases present in buffer preparations could chemically interact with solutes by mechanisms other than acid–base equilibrium, providing different effects over their stability.

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 exuded 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 cultivars: Astron, Bill, Portal, Ritmo, Solist, and Stakkado.

The results of these analyses are shown in Figure 3. We can observe DIMBOA as the major allelochemical in all of the

varieties, being the one that showed higher variation in its concentration in comparison to DIBOA. The cluster analysis obtained from the hydroxamic acids production analysis is shown in Figure 4. This cluster divided the varieties in two groups: Ritmo, Portal, and Astron (higher content of benzoxazinoids) and Bill, Solist, and Stakkado.

Because the microbiotic characteristics of soil in which wheat has been cultivated could vary by compounds lixiviated due to rain and/or dew, the second criterion for the selection was the general bioactivity shown by the lixiviation of the leaves. The activity was measured in samples from cultivars of the six varieties in Spain and Denmark, to record possible climate influences. Spanish samples showed higher activities, and Figure 5 shows the cluster analysis of their bioassay. The obtained profile is similar to the one obtained in the previous cluster study. Cultivars Astron, Ritmo, and Portal presented higher activities.

Additionally, lixiviates of Astron and Ritmo cultivars maintain their activities with dilution. Moreover, high capabilities for biotransformation of hydroxamic acids and derivatives have been reported for phytopathogenic fungi of the two cultivars (41, 42). Consequently, soil in which Astron and Ritmo were cropped was selected.

Degradation of DIMBOA in Soil. A degradation experiment was designed including two different soil samples, according to the previously reported selection process (19). DIMBOA was dosed to the soil solutions (1 and 5 mg of compound per gram of soil). Samples of soil solution were collected in a sampling period of 5 min and analyzed (HPLC-DAD). Control aqueous solutions of the compounds inoculated to soil (to eliminate spontaneous aqueous degradation) were used, and degradation experiments in sterile soil systems were recorded and employed to eliminate transformations induced by soil material and adsorption phenomena. Due to the rapid degradation observed for DIMBOA, we determined only the recovery at a contact time of 30 min ($73 \pm 3\%$ recovered at 5 mg/g of soil).

MBOA was detected as the main degradation product. Quantitative results are expressed as peak area relations between DIMBOA and MBOA (Figure 6). A reduction of the DIMBOA amount can be observed as MBOA concentration increases.

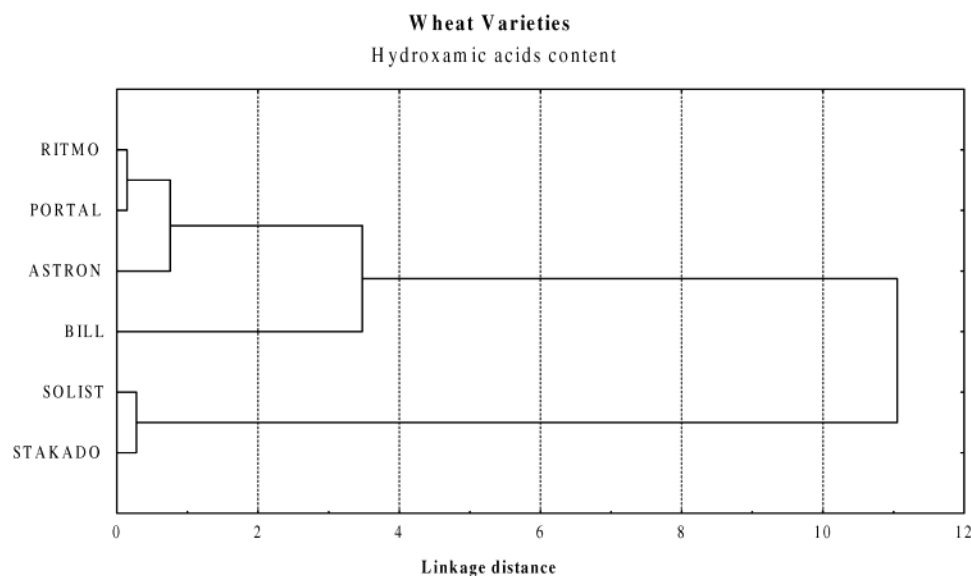


Figure 4. Cluster analysis for hydroxamic acids content in six cultivated wheat varieties.

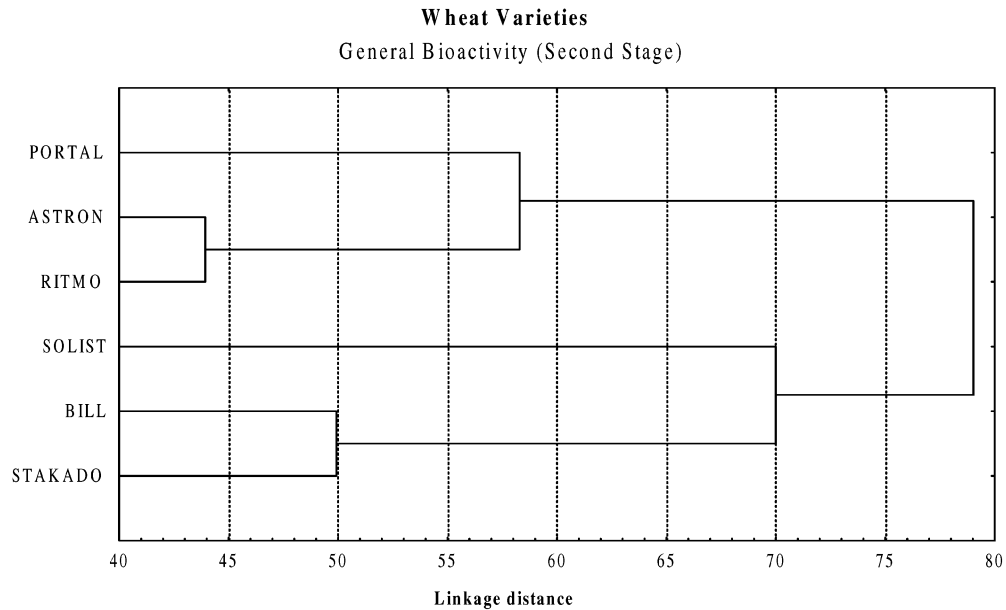


Figure 5. Cluster analysis of general bioactivity shown by aqueous extracts of six cultivated wheat varieties (growing stage, BBCH scale).

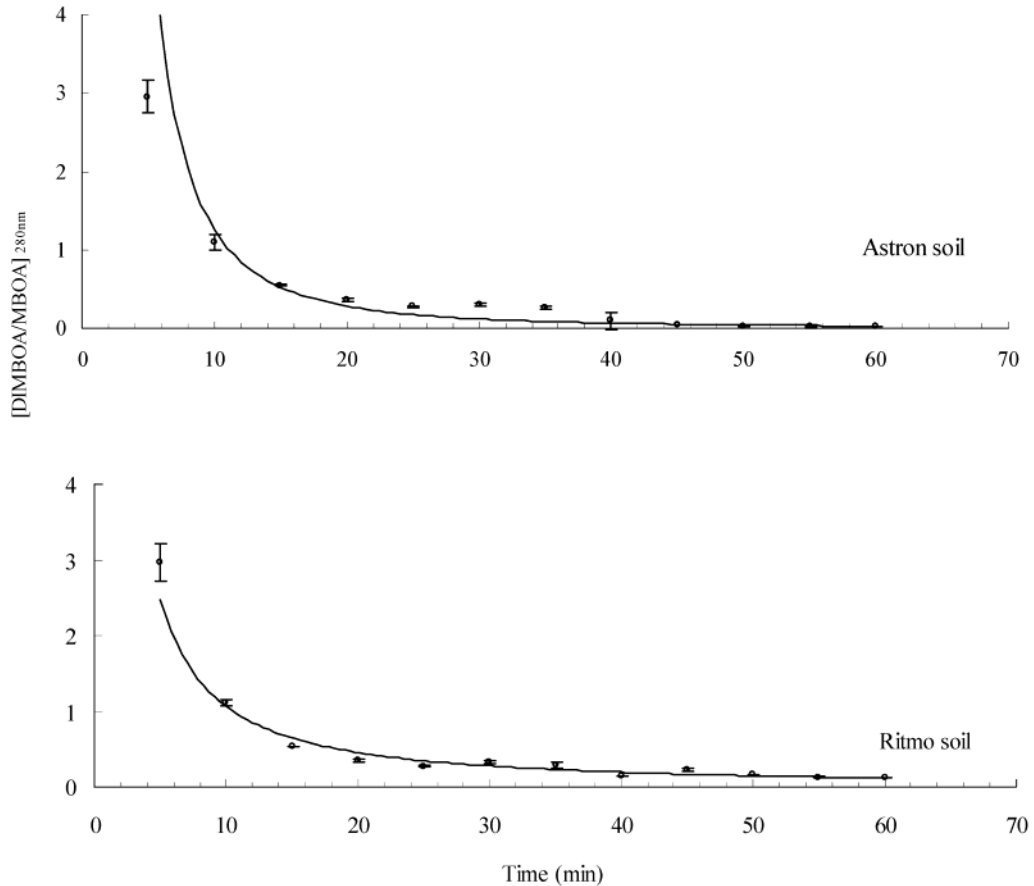


Figure 6. Variation of the concentration relationships $[\text{DIMBOA}]/[\text{MBOA}]$ in the two studied soils. Dose = $1 \text{ mg of DIMBOA} \cdot \text{g}^{-1}$ of soil. $n = 3$.

Total conversion was reached at 50 min (**Figure 7**). All analyses were repeated three times to ensure the accuracy of the methods employed. A second degradation product, 2-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (HMBOA), is detected at trace levels in the highest dose experiment (5000 mg kg^{-1} of soil, **Figure 8**). This experiment allows us to propose the degradation pathway for DIMBOA shown in (**Figure 9**). Nevertheless, more detailed studies to determine the final products and the possible different intermediates during the degradation process are needed.

The quantitative determination of the degradation rate was based on the first-order kinetics equation described above, as shown in **Figures 10** and **11** and summarized in **Table 2**. Although several mathematical treatments for analysis of the data have been developed to determine with better precision the destination and chemical agents concentration in soil (43, 44), the adjustment correlations obtained by the first-order kinetic treatment applied in this study ($r^2 > 0.97$) are reliable enough to estimate the parameters of organic compounds' persistence in soil (45).

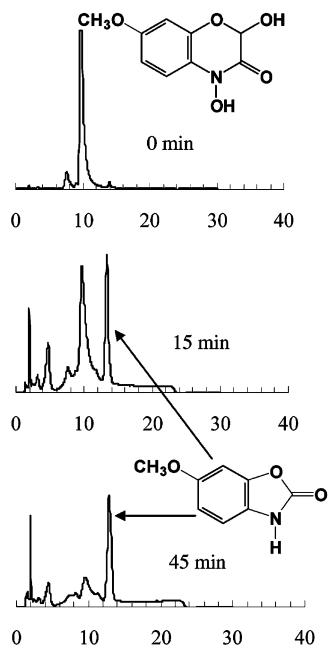


Figure 7. HPLC analysis (absorbance vs retention time) for crop soil solutions (*T. aestivum* L. cv. Astron) inoculated with DIMBOA ($1 \text{ mg} \cdot \text{g}^{-1}$ of soil) at different incubation times. Experimental conditions: 25°C , 14 h of light, 10 h of darkness.

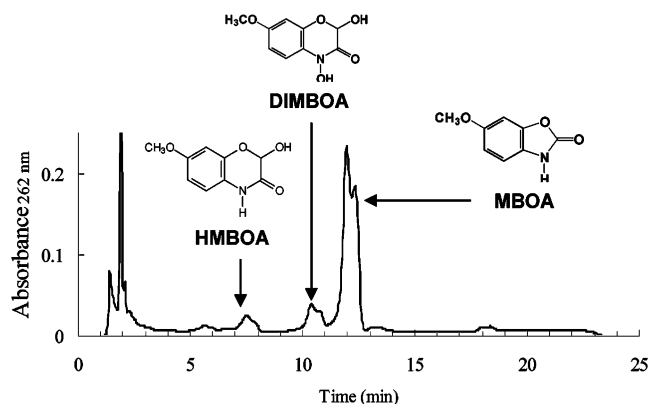


Figure 8. HPLC analysis (soil solution of *T. aestivum* L. cv. Ritmo). Dose = 5000 mg kg^{-1} . Incubation time = 50 min.

Statistical correlation data show the degradation process to be adequately described by the considered model. A similar half-

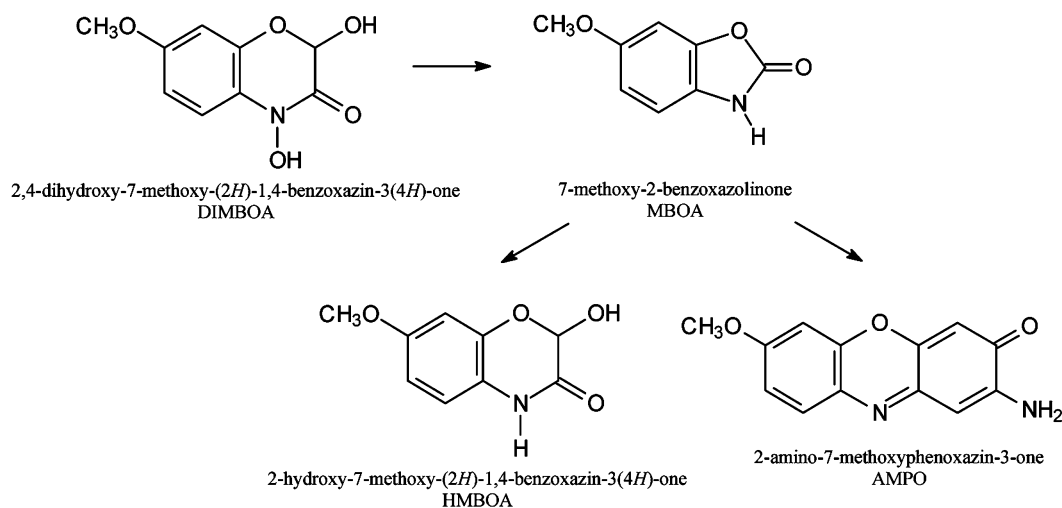


Figure 9. Degradation pathway found for DIMBOA in wheat crop soil.

life is observed in both soil types (31.5 ± 7.7 for Astron and 30.5 ± 2.3 for Ritmo). Both soils would have the same degradation capacity for DIMBOA (ANOVA, $\alpha = 0.05$, $F = 5$).

After this study, the influence of metabolite dose in soil over the degradation dynamics was examined, in the search for the maximum concentration tolerated for soil microbes. Thus, the degradation experiment described above was repeated at 250, 500, 1000, 2000, 3000, 4000, and 5000 mg of starting dose. This range was chosen to imitate amounts present in natural conditions. 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 (19). Moreover, these dynamic processes are strongly affected by biotic (growing stage, plant variety) and abiotic (environmental conditions) factors (15). 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 (46, 47). The sampling area used for soil collection in this study ($3 \pm 1 \text{ cm}$ around the plant lines) would contain $0.13\text{--}0.3 \text{ mg}$ of DIBOA considering this approximation. Other estimations provide values between 0.5 and 1 mg of DIBOA per gram of soil (48, 49). The doses employed in this study (from 0.5 to 5 mg of DIBOA/g of soil) screen a wide concentration range in the same magnitude order as these estimations describe. 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.

Conversion values for each dose are represented in **Figure 12**, together with their standard deviations for both soils. Each experiment was repeated three times.

High conversion rates of DIMBOA are observed independently from the type of soil and dose, $\sim 80\%$, because the maximum conversion capabilities of soil are difficult to reach in this conditions. Both soils have also the same degradative capacity for this compound.

Once the degradation process from DIMBOA to MBOA had been characterized, new experiments were performed to detect further transformations from MBOA to other metabolites. Thus, 5 days after the inoculation of DIMBOA, all soil solutions were extracted and afforded a red solid as major product. NMR experiments allowed it to be identified as 2-amino-7-methoxy-3H-phenoxazin-3-one (AMPO; **Figure 1**). ^1H NMR data matched exactly those presented by Kumar and collaborators

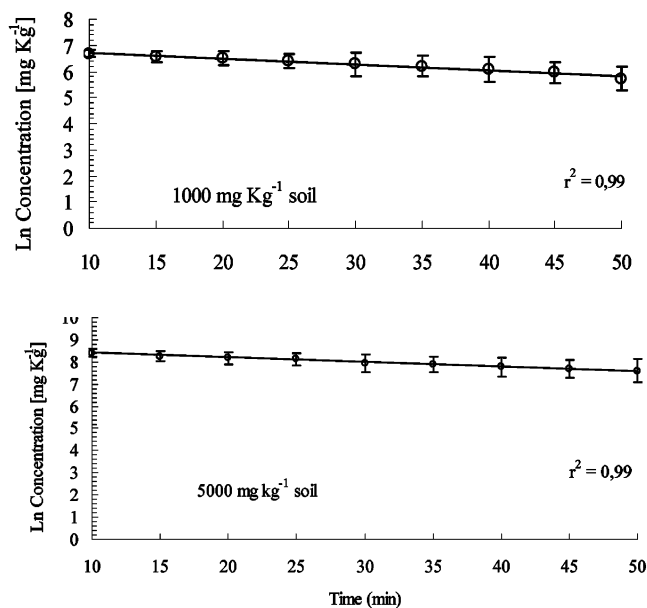


Figure 10. First-order degradation kinetics for DIMBOA in crop soil (*T. aestivum* L. cv. Astron). $n = 3$.

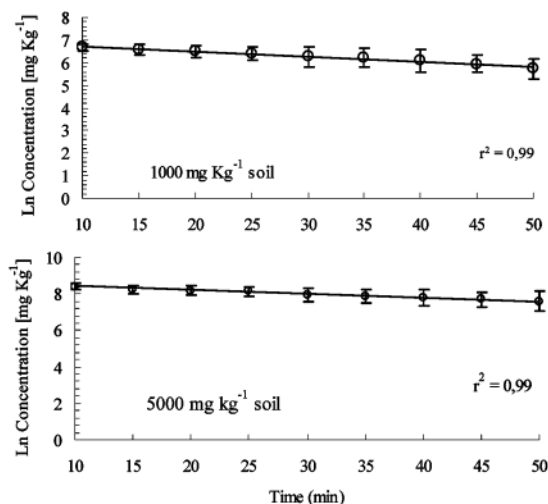


Figure 11. First-order degradation kinetics for DIMBOA in crop soil (*T. aestivum* L. cv. Ritmo). $n = 3$.

Table 2. Degradation Constant Rate, k , and Half-Life, $t_{1/2}$, for the Biodegradation of 2,4-Dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) to 6-Methoxybenzoxazolin-2(3*H*)-one (MBOA) According to Soil Type and Dose

soil according to wheat cultivation	dose (mg kg ⁻¹ of soil)	k (min ⁻¹)	$t_{1/2}$ (min)	r^2 ^a	p ^b
Astron	1000	0.024 ± 0.009	32 ± 12	0.98	<0.0001
Ritmo	1000	0.023 ± 0.003	31 ± 4	0.90	<0.0001
Astron	5000	0.022 ± 0.001	31 ± 1	0.87	<0.0001
Ritmo	5000	0.023 ± 0.001	30 ± 1	0.84	<0.0001

^a r^2 = coefficient of variance. ^b p = confidence limit.

in a previously reported degradation experiment for MBOA in soil (50). Here, we complete the structure characterization of this degradation product (Table 3).

Degradation Dynamics of MBOA. After the isolation of AMPO in soil inoculated with DIMBOA, a complete experiment was designed to characterize the stability and degradation dynamics of MBOA and its conversion to AMPO. Degradation experiments were carried out in the same soils and at the same

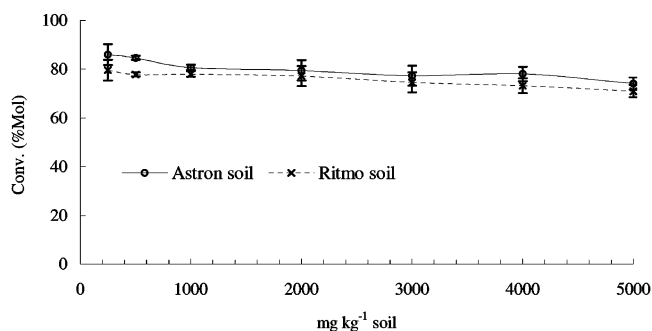


Figure 12. Conversion of DIMBOA to MBOA ($t = 1$ h) according to dose and soil type. Error bars are standards deviation from mean. $n = 3$.

Table 3. ¹H (400 MHz) and ¹³C NMR (100 MHz) Data of 2-Amino-7-methoxyphenoxazin-3-one (AMPO)

C	$\delta^{13}\text{C}$	H	$\delta^1\text{H}$	J (Hz)
1	98.8	1	6.35 s	
2	145.7			
3	179.9			
4	103.3	4	6.33 s	
4a	148.6			
5a	143.3			
6	100.1	6	7.11 d	2.7
7	160.0			
8	113.5	8	7.01 dd	9.0, 2.7
9	129.0	9	7.64 d	9.0
9a	128.4			
10a	146.6			
CH ₃ O-	56.1			

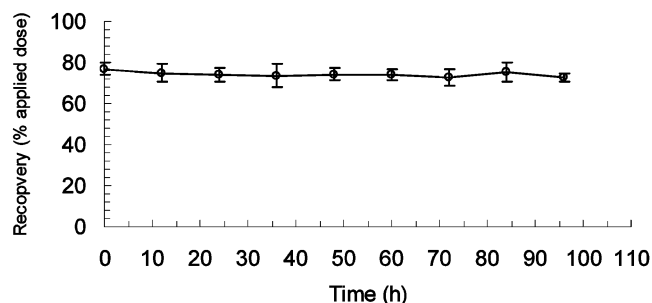


Figure 13. Extraction recovery of MBOA in sterilized soil over time. Soil was cultivated with *T. aestivum* cv. Astron. Test was carried out at a dose of 5 mg g⁻¹ of soil. Error bars are standard deviations of the mean. $n = 3$. One-way ANOVA, $\alpha = 0.05$, F critical = 4.6.

doses as for DIMBOA. Preliminary assays suggest MBOA to be much more stable than DIMBOA, so MBOA solutions were allowed to stand in contact with soil for 10 days. The MBOA recovery percentage remained constant through 4 days (no statistical difference was observed at the different sampling times) (Figure 13). The recovery value was 74 ± 3%.

Influence between dose and degradation rate was also studied for both types of soil, and the results were expressed as molar conversion percentage versus dose (Figure 14). Different behaviors for both soils were observed (ANOVA, $\alpha = 0.05$, $F = 4$), because Ritmo soil showed a higher conversion capacity than Astron soil (12 ± 3 and 4 ± 2% conversions, respectively). At a dose of 250 mg/kg (maximum conversion rate) there were no significant differences between the soils (Student's t test, $\alpha = 0.05$). In this case, there is a significant influence between dose and degradation in contrast to DIMBOA. Regarding degradation rate, a comparison between both types of soil at the dose for maximum conversion was carried out. For a first-order degradation kinetics, half-life times were 6.4 days (Ritmo)

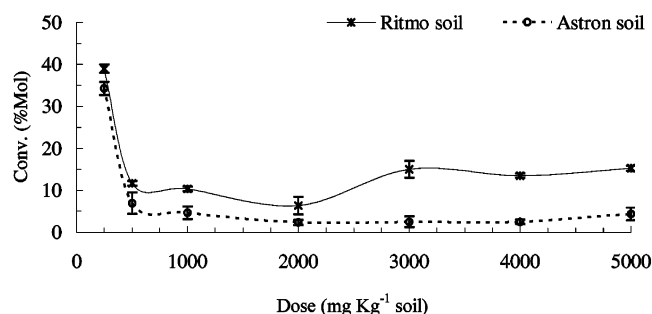


Figure 14. Conversion of MBOA for 10 days of incubation in the degradation soil according to dose and soil type. Error bars are standard deviations of the mean. $n = 3$.

Table 4. Degradation Constant Rate, k , and Half Life, $t_{1/2}$, for the Biodegradation of Benzoxazin-2(3*H*)-one (BOA) and Methoxybenzoxazolin-2(3*H*)-one (MBOA) According to Soil Type and Dose

soil according to wheat cultivation	dose (mg kg ⁻¹ of soil)	$t_{1/2}$	
		DIBOA (h)	DIMBOA (min)
Astron	1000	24 ± 5	32 ± 12
	5000	62 ± 7	31 ± 1
Ritmo	1000	28 ± 5	31 ± 4
	5000	57 ± 16	30 ± 1

and 4.3 days (Astron) (Table 4). Differences observed between both types of soil can be related to different microbial populations associated to each variety (51, 52). The amount and structure of the chemicals liberated by different varieties could be not equal and could influence the microbes or fungi populations associated with the radicular system, producing different degradation dynamics for other compounds released by the plant.

The soil that presents the higher conversion capacity is the one that degrades the tested compound at minor velocity. The degradation kinetics represent the response of the microorganism to a toxic substance (or carbon source) inoculated. This constitutes a dynamic response (kinetics), whereas conversions show the system behavior once equilibrium is reached (thermodynamics). For these systems, a complete biotransformation description involves the measurement of velocity (reaction kinetics) and thermodynamic conversions. The combination of both types of data ensures an accurate description of the system under study.

Degradation Study for AMPO. After the characterization of DIMBOA and MBOA soil degradation dynamics, similar degradation experiments were carried out to determine the degradability of AMPO by soil microbes. As happened to its analogous in DIBOA series, 2-amino-3*H*-phenoxazin-3-one (APO), no degradation occurred after 3 months of incubation at different doses. This fact allows us to propose aminophe-

noxazines APO and AMPO as the final degradation metabolites in the studied wheat crop soils for DIBOA and DIMBOA, respectively. They could have high importance in the ecological role previously assigned to benzoxazinones, due to their high persistence.

Comparison between DIMBOA and DIBOA Series. All of these degradation data allow us to make an interesting comparison between DIMBOA, DIBOA, and their derivatives regarding their behaviors in soil, according to our previously reported results for the DIBOA series. Half-life times for DIBOA and DIMBOA for both type of soils and two doses (1 and 5 mg of compound per gram of soil) are shown in Table 5. For DIBOA, times are expressed in hours and for DIMBOA, in minutes.

DIBOA presents a higher persistence to degradation than DIMBOA. The structural difference between them, a methoxy group at C-7, must be the key for the difference of reactivity in these compounds. This group could stabilize an intermediate generated in the degradation process. Some authors have suggested this group to be extremely important in the reactivity of DIMBOA with nucleophilic residues present in biomolecules (20). These reactions could be part of their degradation processes.

MBOA and BOA present higher resistance to degradation than their precursors in both types of soil (Table 5). MBOA is more stable than BOA, so in this case their structural difference (methoxy group at C-6) plays a different role than in the hydroxamic acids case. These differences in degradation dynamics suggest different stabilities for some degradation intermediates such as 2-amino-5-methoxyphenol and 2-amino-phenol. The stability of these intermediates could act in a positive manner for DIMBOA to MBOA transformation and inhibit degradation from MBOA to AMPO.

Effect of Allelochemical Mixtures on Their Degradation Rates. DIBOA and DIMBOA have been found together in wheat root exudates, so BOA and MBOA, their main degradation products, could be substrates for soil microbial degradation simultaneously. Thus, a complete laboratory simulation of this codegradation phenomenon has been achieved.

Equimolecular mixtures for BOA and MBOA were dosed to the soils, and the concentration versus time data were collected under the same conditions and with the same methodology as in the degradation studies described above.

In all cases the half-lives of the assayed compounds are in the same order. Nevertheless, these half-lives are higher than in their individual degradation experiments. Wheat, which produces both allelochemical series (53), could provoke a higher persistence of these defense metabolites in soil. This would increase the allelopathic potential of its lixivates and exudates. In any case, further studies are needed to achieve the combined effects of these allelochemicals. Benzoxazinones and aminophe-

Table 5. Degradation Constant Rate, k , and Half-Life, $t_{1/2}$, for the Biodegradation of 6-Methoxybenzoxazolin-2(3*H*)-one (MBOA) and Benzoxazin-2(3*H*)-one (BOA) According to Soil Type and Dose^a

soil	mg kg ⁻¹ of soil	MBOA				BOA			
		k (days ⁻¹)	$t_{1/2}$ (days)	r^2 ^b	p ^c	k (days ⁻¹)	$t_{1/2}$ (days)	r^2	p
Astron	2000	0.16 ± 0.02	4.3 ± 0.5a	0.98	0.0001	0.26 ± 0.04	2.7 ± 0.4a	0.71	<0.002
Ritmo	2000	0.12 ± 0.05	6.0 ± 2.0a	0.98	<0.0001	0.31	2.2 ± 0.3a	0.97	<0.0001
MBOA + BOA (1:1 mol)									
Astron		0.07 ± 0.02	9 ± 2 a	0.98	<0.0001	0.23 ± 0.02	3.0 ± 0.3a	0.97	<0.0001
Ritmo		0.15 ± 0.02	5 ± 1b	0.97	<0.0001	0.15 ± 0.07	5.0 ± 2.0a	0.97	<0.0001

^a The same letters indicate no significant difference. ANOVA, $\alpha = 0.05$. ^b r^2 = coefficient of variance. ^c p = confidence limit.

noxazines had to be included in these experiments to ensure an optimal simulation of natural conditions.

Conclusions. DIMBOA is degraded by soil microbial populations in a relatively short time. This fact forces us to reconsider its role in chemical defense mechanisms of plants with high production of this secondary metabolite and to take into account the possible effects of its degradation products. Depending on their potential phytotoxic effects, the defense capability of the plant could be enhanced or reduced in a significant manner.

DIMBOA and DIBOA have very different half-lives. The 7-methoxy group present in DIMBOA could have an important role in its high degradability. Some authors suggest this methoxy group could influence the reactivity of DIMBOA with nucleophiles (19). Those reactions could be part of degradation processes. Otherwise, DIBOA has a high persistence and could have a more important role in chemical defense mechanisms than DIMBOA in plants that produce large amounts of hydroxamic acids.

The 7-methoxy group present in DIMBOA has a determinant role in its high degradability, chemical reactivity, and biological activity. As electron density at the aromatic ring increases, the developing positive charge on nitrogen at the degradation transition state is more stabilized (19), providing faster degradation. This statement, valid for aqueous and organic solutions, is in agreement with the results observed for microbial degradation. More detailed studies about biodegradation mechanisms are needed to record the structure–degradability relationships of biological systems. The aromatic substitution patterns on benzohydroxamic acids play also a decisive role in their chemical reactivity, because they affect electron densities at reactive positions C-2 and N-4, provoking different chemical behaviors for both series (54, 55). There are also many authors that have compared the effects of methoxylated and non-methoxylated benzohydroxamic acids toward their bioactivities, finding highly significant differences (56). The higher persistence shown by DIBOA allows us to assign it a more important role in chemical defense mechanisms than DIMBOA in plants that produce large amounts of hydroxamic acids. When BOA and MBOA are simultaneously dosed to soil samples, degradation half-lives increase significantly. The presence of one of the products affects the degradation rate of the other one. There are two possible explanations for this result: both substrates compete for the microorganisms, affecting the degradation rate of one another. In addition to this, the concentration of each compound in the mixture experiments ($\chi = 0.5$ for each compound) is half the concentration at the individual experiment ($\chi = 1$), so the degradation rate (proportional to concentration) decreases.

The study of the fate and toxicity of products directly released by plants, including their degradation materials, will provide highly valuable and useful information in allelopathy research. Taking in account these degradation phenomena, the development of plant allelochemicals as natural herbicide models could be much more efficient. The fact that in most cases allelochemicals are released to the environment by means of different processes, root exudation being the most direct and dynamic, these compounds are subjected to different chemical modifications. These processes could take place due to the intrinsic thermodynamic properties of the compounds, physical interactions (adsorption and chemical reactions promoted by soil materials), and finally microbial degradation. All of these interactions will determine the structure and accumulation levels of the allelochemicals released, so their phytotoxic potential will depend on the compound diffusion through soil solution, final

fate, and soil persistence. The results presented here constitute a laboratory-level study, in which phenomena associated with crop conditions such as rain, volatilization, uptake by other plants present, photolysis, or lixiviation to deeper places of the soil are not present. Nevertheless, this work is a good approach to the evidence that classical allelochemicals associated with a great diversity of biological activities can be transformed by different processes and interactions once released. Thus, more detailed studies are necessary to understand how organic materials, mineral reactivity, ionic interchanging capacity, inorganic ions, and specific microbial and fungal activities could influence allelochemicals in the environment.

All of these phenomena closely depend on the type of soil under study. The assignment of a biological property to a compound or a family of them involves a more complex study, in which the behavior of releaser and target plants needs to be achieved (allelochemical levels and liberation capacity) together with soil dynamics of allelochemicals (diffusion through soil, chemical, and biochemical interactions) and their modes of action (chemical transformations and activity measured over target plant).

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