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Structure−Activity Relationship (SAR) Studies of Benzoxazinones, Their Degradation Products, and Analogues. Phytotoxicity on Problematic Weeds *Avena fatua* L. and *Lolium rigidum* Gaud.

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Avena fatua L. (wild oat) and *Lolium rigidum* Gaud. (rigid ryegrass) are highly problematic weeds affecting a wide variety of cereal crops worldwide. The fact that both of these weeds have developed resistance to several herbicide groups made them optimal candidates as target organisms for ongoing research about the potential application of allelochemicals and analogue compounds as natural herbicide models. Benzoxazinones, a family of natural allelochemicals present in corn, wheat, and rye, including 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one and 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one, together with some degradation products, found in crop soils as well as in other systems, and some synthetic analogues of them were tested on wild oat and rigid ryegrass seeds; the results were statistically treated, and some structure−activity relationships, useful in further development of natural herbicide models, were elucidated. The most active compounds were the synthetic benzoxazinone 2-acetoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one and the degradation product 2-aminophenoxazin-3-one, with highly significant inhibition on the development of both weeds. The ecological role of these compounds is discussed by considering both degradability and phytotoxicity. The bioactivity of aminophenoxazines has been correlated by their aqueous solubility−lipophilicity predicted by means of computational methods.

KEYWORDS: Allelopathy; SAR; benzoxazinones; aminophenoxazines; DIBOA; DIMBOA; wild oat; rigid ryegrass; bioassay; phytotoxicity

INTRODUCTION

Wild oat (*Avena fatua* L.) and rigid ryegrass (*Lolium rigidum* Gaud.) belong to the Poaceae family and are problematic weeds for cereal crops, affecting mainly barley and wheat. They are distributed worldwide, and many different cases of resistance to commercial herbicides with different modes of action have been reported to date. In the case of wild oat, resistance to ACCase inhibitors has been researched in great detail, and with regard to inheritance of resistance (*1*), resistant biotype detection (*2*) and cross-resistance (*3*) have been reported. Several rigid ryegrass biotypes have developed resistance to a wide variety of herbicides (*4*), including glyphosate (an inhibitor of EPSP synthase) (*5*) and substituted ureas (inhibitors of photosynthetic electron transport) (*6*). These facts direct the research for the management of these weeds to the search of alternative compounds with high phytotoxicity and alternative modes of action. We recently developed a complete structure−activity relationship (SAR) study with benzoxazinones and related compounds, directed toward the search for natural herbicide models based on allelochemical structures with potential use in weed management (*7*). Five plant species, selected as biological models for phytotoxicity evaluation according to a previously reported bioassay optimization procedure (*8*), were grown in contact with several natural benzoxazinones, synthetic analogues, and degradation products found in different systems. In this paper, we discuss the potential application of these compounds in the management of weeds *L. rigidum* and *A. fatua*, chosen as real problems that affect important crops such as wheat.

Benzoxazinones containing the hydroxamic acid moiety acquired high relevancy in phytochemistry research after the isolation of 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) (*9*) in 1959 and 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) in 1962 (*10*) (**Table 1A**). Interesting bioactivity was observed for both compounds, some of their degradation products, and also some synthetic analogues, antimicrobial (*11*), antifeedant, insecticidal (*12*), and phytotoxic

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Table 1. Structures, Functionalization, Names, and Acronyms of All Chemicals Employed in the SAR Study

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	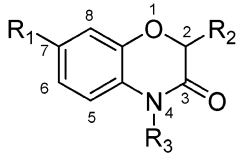	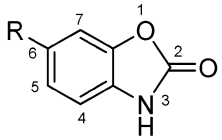	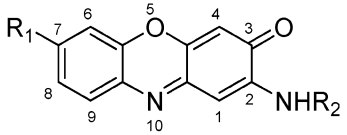	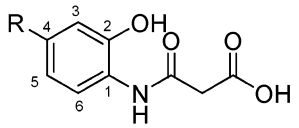	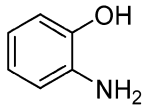
Natural allelochemicals	**R_1=H; R_2=O-β-D-glucose; R_3=OH:** 2-*O*-β-D-glucopyranosyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one **DIBOA-Glc** **R_1=H; R_2=OH; R_3=OH:** 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one **DIBOA** **R_1=OCH_3; R_2=OH; R_3=OH:** 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one **DIMBOA**	**R=H** Benzoxazolin-2(3*H*)-one **BOA** **R=OCH_3** 6-methoxybenzoxazolin-2(3*H*)-one **MBOA**	**R_1=H; R_2=H** 2-aminophenoxazin-3-one **APO** **R_1=OCH_3; R_2=H** 2-amino-7-methoxyphenoxazin-3-one **AMPO** **R_1=H; R_2=OAc** 2-acetamidophenoxazin-3-one **AAPO** **R_1=OCH_3; R_2=OAc** 2-acetamido-7-methoxy-phenoxazin-3-one **AAMPO** **R_1=OH; R_2=H** 2-amino-7-hydroxyphenoxazin-3-one **AHPO**	**R=H** *N*-[2-hydroxyphenyl]malonamic acid **HPMA** **R=OCH_3** *N*-[2-hydroxy-4 methoxyphenyl] malonamic acid **HMPMA**	2-aminophenol **APH**
Biosynthetic precursors/ degradation products	**R_1=H; R_2=OH; R_3=H:** 4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one **HBOA** **R1=OCH3; R2=OH; R3=H:** 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one **HMBOA**				
Synthetic analogues	**R_1=H; R_2=H; R_3=H:** (2*H*)-1,4-benzoxazin-3(4*H*)-one **D-HBOA** **R1=OCH_3; R2=H; R3=H:** 4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one **D-HMBOA** **R_1=H; R_2=H; R_3=OH:** 4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one **D-DIBOA** **R_1=OCH_3; R_2=H; R_3=OH:** 4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one **D-DIMBOA** **R_1=H; R_2=H; R_3=OAc:** 4-acetoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one **ABOA** **R_1=OCH_3; R_2=H; R_3=OAc:** 4-acetoxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one **AMBOA**				

behaviors (*13*, *14*) being widely described. The DIBOA natural glycoside [2-*O*-*β*-D-glucopyranosyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-Glc) (**Table 1A**)] is the form in which DIBOA is preserved inside the plant prior to its release (*15*). Benzoxazolin-2-one (BOA) and 6-methoxybenzoxazolin-2-one (MBOA) (**Table 1B**) are the first chemicals in the DIBOA and DIMBOA degradation series, respectively (*16*−*18*). There are interesting precedents regarding their bioactivity in different systems (*19*, *20*).

According to these previous evaluations, DIMBOA and DIBOA (**Table 1A**) were the most active compounds. Their degradation products MBOA and BOA (**Table 1B**) are much less active, and 2-deoxy analogues of DIMBOA and DIBOA (**Table 1A**) have different behaviors depending on the different species assayed.

In addition to this bioactivity research, the low stability of DIMBOA, DIBOA, and their related benzoxazolinones under various conditions, such as biotransformation by fungi (*21*) and degradation in crop soil (*22*, *23*) and in aqueous solution (*16*−*18*), has been investigated. After the characterization of conversion dynamics of these compounds in model wheat crop soils, 2-aminophenoxazin-3-one (APO) and 2-amino-7-methoxyphenoxazin-3-one (AMPO) (**Table 1C**) were the final products for DIBOA and DIMBOA degradation routes. Their *N*-acetyl derivatives, 2-acetamidophenoxazin-3-one (AAPO) and 2-acetamido-7-methoxyphenoxazin-3-one (AAMPO) (**Table 1C**), have been proposed as detoxification compounds produced by nonpathogenic organisms associated with Gramineae (*21*, *24*). They have also been found in degradation experiments of benzoxazolinones in wheat crop soil (*25*−*27*). APO has been already described as a potent phytotoxic agent for *Echinochloa crus-galli* (barnyardgrass) (*22*, *28*).

We recently reported a complete SAR study dealing with 21 chemicals, including natural benzoxazinones and a wide variety of synthetic analogues and degradation products belonging to four different structural types (*7*).

Our objectives in that study were to evaluate a complete phytotoxicity profile of these compounds, considering effects on standard target species (STS) germination and growth (*8*). The results were correlated with compound structures. Degradation products (benzoxazolinones, aminophenoxazines, and hydroxyphenylmalonamic acids) were included to evaluate their phytotoxic effect and to compare it with the chemicals from which they were transformed. This would allow us to establish their roles in Gramineae chemical defense. The relationship between phytotoxic effect and soil persistence of degradation products investigated by us allowed interesting conclusions about the ecological role of DIMBOA, DIBOA, and their main derivatives to be drawn.

This research included several synthetic compounds with a benzoxazinone skeleton, providing all possible combinations regarding functionalization of the C-2 and N-4 positions (2-hydroxy, 2,4-dihydroxy, 4-hydroxy, and 2-deoxy lactams, in addition to two 4-acetoxy derivatives, as shown in **Table 1A**). The phytotoxicity of these compounds, higher than that of their related natural products DIMBOA and DIBOA, showed the lack of a hydroxyl group at position C-2 to be an important requirement for phytotoxicity enhancement (*7*). The bioactivity profiles shown by benzoxazinones suggested 2-deoxy derivatives of natural allelochemicals DIBOA and DIMBOA (D-DIMBOA and D-DIBOA) (**Table 1A**) to be the best leads for new herbicides with this structural base. D-DIBOA matched all of the conditions that increase phytotoxicity on the species assayed: lack of a hydroxyl group at C-2, a hydroxyl group at the N-4 position, and absence of a methoxy group in the aromatic ring. The lower phytotoxicity levels shown by 2,4-dihydroxy compounds (DIMBOA and DIBOA) in some cases could be due to degradation phenomena leading to less active compounds such as BOA or MBOA, which are much more stable.

With regard to new herbicide model development, benzoxazinones were proposed to be the most interesting group because they are affordable by high-yield and easy-to-scale synthetic methods. In addition to this, their molecular structure and functionalization permit different modifications at the heterocycle and the aromatic ring, so a large number of candidates could be developed and tested in further research on this matter.

Aminophenoxazine APO was the most active degradation compound, with high phytotoxicity levels and also high maintenance of the effect at low concentrations. The lack of phytotoxic effect observed for its structurally related compounds such as AMPO and AHPO could be related to their lipophilicity. The effects observed for acetamidophenoxazines AAPO and AAMPO confirm them as detoxification derivatives. Taking into consideration reports from the literature (*25*−*27*, *29*) describing degradation for aminophenoxazines, which proceeds with detoxification, as phytotoxicity for AAPO and AAMPO data show, further research is needed to determine the final fate of aminophenoxazines in soil and other environments. Antifungal activity shown by APO (*29*) could affect its own degradation dynamics in soil, at least at the higher dose employed in its degradation experiments (*18*).

APO showed high phytotoxicity and also higher persistence in soil than its precursors BOA and DIBOA. Then, some of the allelopathic behaviors observed in plants that produce DIBOA could be due to its degradation compound rather than the compound originally released by the plant. The low phytotoxicity of AMPO points in the opposite direction with regard to the DIMBOA series, so a more accurate study about its interaction with soil biological population and inorganic materials will be needed to discover its ecological role. This fact, in addition to the higher persistence in soil shown by DIBOA degradation series compounds (*18*), allows us to conclude that DIBOA-producing species take more advantage of allelopathic defense strategies based on benzoxazinones than DIMBOA-producing ones.

2-Hydroxyphenylmalonamic acids (**Table 1D**) have been described as biotransformation products from benzoxazolinones, produced by endophytic fungi associated with several plants (*24*, *29*). These compounds have been tested over fungal colonies, and their influence over *Lepidium sativum* L. (cress) root length has been recorded, although comparison with commercial fungicides or herbicides was not made. Their activities have been compared with those for their precursors BOA and MBOA. The lack of phytotoxicity observed for these malonamic acids allows their proposal as detoxification products. No significant influence on STS (*7*) was recorded.

In this context, evaluation of these compounds on problematic weeds constitutes the next step for natural herbicide model development. Thus, the main objective of this work was to evaluate the phytotoxicity of benzoxazinones and related compounds on *A. fatua* and *L. rigidum*, in the search for new compounds able to be applied as part of strategies directed to weed control on cereal crops. Statistical treatment of the acquired data would allow discovering SAR useful in further development of highly phytotoxic and compounds based on the structures of these natural products.

MATERIALS AND METHODS

Isolation and Synthesis of Natural Allelochemicals, Degradation Products, and Their Derivatives. The studied chemicals belong to three different groups representing natural benzoxazinones, their degradation products, and several non-natural analogues.

*Natural Benzoxazinones (**Table 1A**).* DIBOA and DIMBOA were obtained from natural sources by means of previously reported isolation procedures (*30*) modified by us. The DIBOA natural glycoside 2-*O*-*β*-D-glucopyranosyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-Glc) was isolated from natural sources. Its isolation protocol, adapted from the literature, has been already described by us with regard to its degradation study in wheat crop soil (*18*).

Synthetic Benzoxazinones. These structures were obtained in our laboratory by adapting methods from the literature (*31*) as was previously described by us (*7*). They include two natural lactams (**Table 1A**): biosynthetic precursor 2-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (HBOA) and 2-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (HMBOA), a DIMBOA derivative found as a degradation product in wheat crop soil (*17*). Six non-natural benzoxazinones without a hydroxyl group at C-2 (**Table 1A**) were synthesized to study the influence of different functionalization patterns on phytotoxicity. They include hydroxamic acids 4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-DIBOA) and 4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-DIMBOA), the lactams (2*H*)-1,4-benzoxazin-3(4*H*)-one (D-HBOA) and 7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-HMBOA), and two 4-acetoxy derivatives: 4-acetoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (ABOA) and 4-acetoxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (AMBOA).

Degradation Products. These have been selected according to the precedents mentioned above, belonging to four different structural types.

*Benzoxazolinones (**Table 1B**):* both are commercial compounds. They were purchased from Fluka Chemika and Lancaster Synthesis, respectively, and used as received.

*Aminophenoxazin-3-ones (**Table 1C**)* were obtained in our laboratory by synthesis procedures described in our previous paper regarding the phytotoxicity evaluation and SAR studies of these compounds on STS (*7*).

*Malonamic acids (**Table 1D**)* were obtained by means of novel synthesis procedures as previously reported (*7*).

Additional Compounds Evaluated. To characterize the bioactivity and to discuss the phytotoxicity of DIBOA degradation products on wild oat and rigid ryegrass, 2-aminophenol (**Table 1E**) (purchased from Sigma-Aldrich Co., used as received) was evaluated.

We have also reported the detailed experimental procedures employed for the isolation and synthesis of all of these compounds (*32*).

Phytotoxicity Bioassays. *Methodology*. Bioassays used Petri dishes (90 mm diameter) with one sheet of Whatman no. 1 filter paper as substrate. Germination and growth were conducted in aqueous solutions at controlled pH by using 10^{-2} M 2-(*N*-morpholino)ethanesulfonic acid (MES) and 1 M NaOH (pH 6.0). Compounds to be assayed were dissolved in DMSO (0.2, 0.1, 0.02, 0.01, and 0.002 M), and these solutions were diluted with buffer (5 μL of DMSO solution/mL of buffer) so that test concentrations for each compound (10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5}, and 10^{-5} M) were reached. This procedure facilitated the solubility of the assayed compounds.

Target Species. L. rigidum and *A. fatua* seeds were purchased from Herbiseed Co. (Twyford, U.K.). They were used as received. The number of seeds in each Petri dish was 15 for *L. rigidum* and 10 for *A. fatua*, and 5 mL of treatment, control, or internal reference solution was added to each Petri dish. Four replicates (60 seeds) were used in the case of *L. rigidum* and 10 replicates (100 seeds) for *A. fatua*.

After the addition of seeds and aqueous solutions, the Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds were incubated at 25 °C in a Memmert ICE 700 controlled environment growth chamber, in the absence of light. The bioassays took 6 days in the case of *L. rigidum* and 7 days in the case of *A. fatua*. After growth, the plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process. This helped the handling of the plants and allowed a more accurate measurement of root and shoot lengths.

The commercial herbicide Logran, a combination of *N*-(1,1-dimethylethyl)-*N*-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (Terbutryn, 0.6%) and 2-(2-chloroethoxy)-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide (Triasulfuron, 59.4%), was used as internal reference, according to a comparison study previously reported (*8*). It was used at the same concentrations (10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5}, and 10^{-5} M) and in the same conditions as the compounds studied. Buffered aqueous solutions with DMSO and without any tested compound were used as control samples.

Bioassay Data Acquisition. Evaluated parameters (germination rate, root length, and shoot length) were recorded by using a Fitomed system (*32*) that allowed automatic data acquisition and statistical analysis by its associated software.

Statistical Analysis. Data were statistically analyzed using Welch's test, with significance fixed at 0.01 and 0.05. They are presented as percentage differences from control. Zero represents control, positive values represent stimulation of the studied parameter, and negative values represent inhibition (*8*, *33*).

Once the germination and growth data had been acquired, cluster analysis was used to group compounds with similar phytotoxicity behaviors and associate them with their molecular structure. Complete linkage was used as amalgamation rule, and the distance measurement was based on squared Euclidean distances (*34*), given by the equation

$$d(x,y) = \sum_i (x_i - y_i)^2$$

where $d(x,y)$ is the squared Euclidean distance (i-dimensional), i represents the number of variables, and x and y are the observed values.

The cluster was obtained by using Statistica v. 5.0 software. It was made on the basis of growth parameters for each weed individually and for both weeds together.

EC_{50} values were obtained after the adjustment of phytotoxicity data to concentration (logarithmic scale), to a sigmoidal dose−response curve, defined by the equation

$$Y = Y_{\min} + \frac{Y_{\max} - Y_{\min}}{1 + 10^{\log EC_{50} - X}}$$

where X indicates the logarithm of the concentration, Y indicates the response (phytotoxicity), and Y_{max} and Y_{min} are the maximum and minimum values of the response, respectively. Goodness of fit is described by determination coefficient (r^2). The adjustment and the r^2 were obtained by using Graph Pad Prism software v. 4.00. All phytotoxicity data were normalized to compare compounds' effects.

Lipophilicity Calculations. Lipophilicity, expressed as log*P* (water/*n*-octanol partition coefficient) value, was obtained by computational methods according to the Ghose, Pritchett, and Crippen methodology (*35*). This algorithm is implemented in Hyperchem v. 7.0 software.

RESULTS AND DISCUSSION

General Bioactivity Profiles. Effects on *L. rigidum* and *A. fatua* roots are shown in **Figures 1** and **2**, respectively. All compounds, when active, showed inhibitory profiles. In general terms, the assayed compounds provoked significant effects at the three most concentrated treatments (10^{-3}, 5×10^{-4}, and 10^{-4} M). The compounds showing more intense effects at lower dilutions were the degradation compound APO (**Table 1C**) ($EC_{50} = 2.4 \times 10^{-5}$ M, $r^2 = 0.99$, root length, *A. fatua*) and the synthetic benzoxazinones ABOA and D-DIBOA (**Table 1A**), with EC_{50} values of $\sim 10^{-4}$ M for the same parameter (2.6×10^{-4} M, $r^2 = 0.99$; and 1.6×10^{-4} M, $r^2 = 0.98$, respectively). The same is observed on *L. rigidum* roots, for which EC_{50} values for these compounds were 2.3×10^{-5}, 2.4×10^{-4}, and 3.6×10^{-4} M, respectively, with good adjustment to a sigmoidal dose−response model ($r^2 = 0.96$, 0.98, and 0.99). *A. fatua* was more affected by the assayed compounds than *L. rigidum*, these three active compounds being similar to the commercial herbicide Logran ($EC_{50} = 1.4 \times 10^{-4}$ M, $r^2 = 0.99$) in their effects.

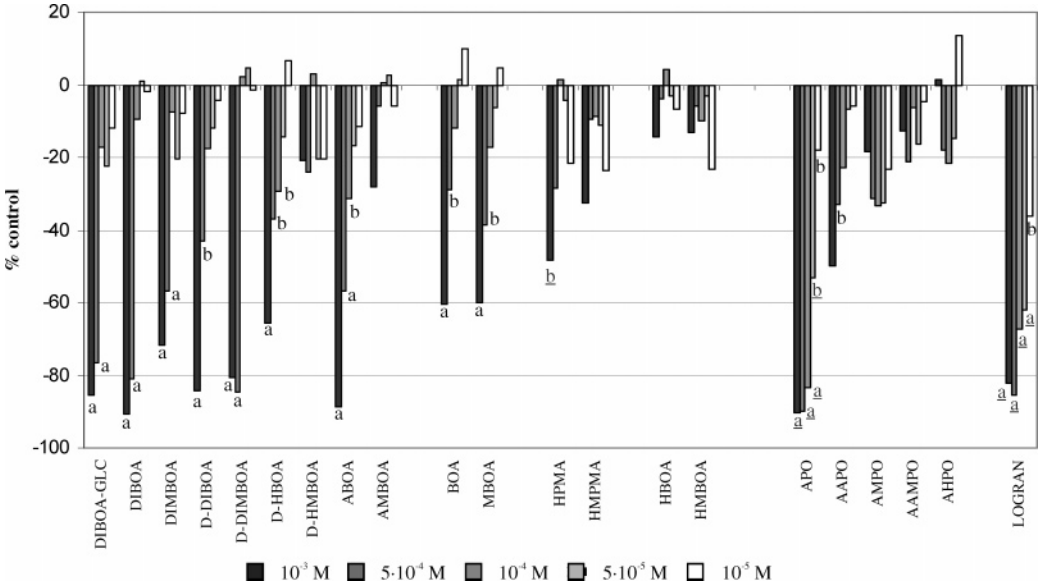

Figure 1. Phytotoxicity bioassay results (root length, percent from control) for rigid ryegrass (*L. rigidum* Gaud). If it is not indicated, $P > 0.05$ for Welch's test: (a) values significantly different at $P < 0.01$; (b) values significantly different at $0.01 < P < 0.05$.

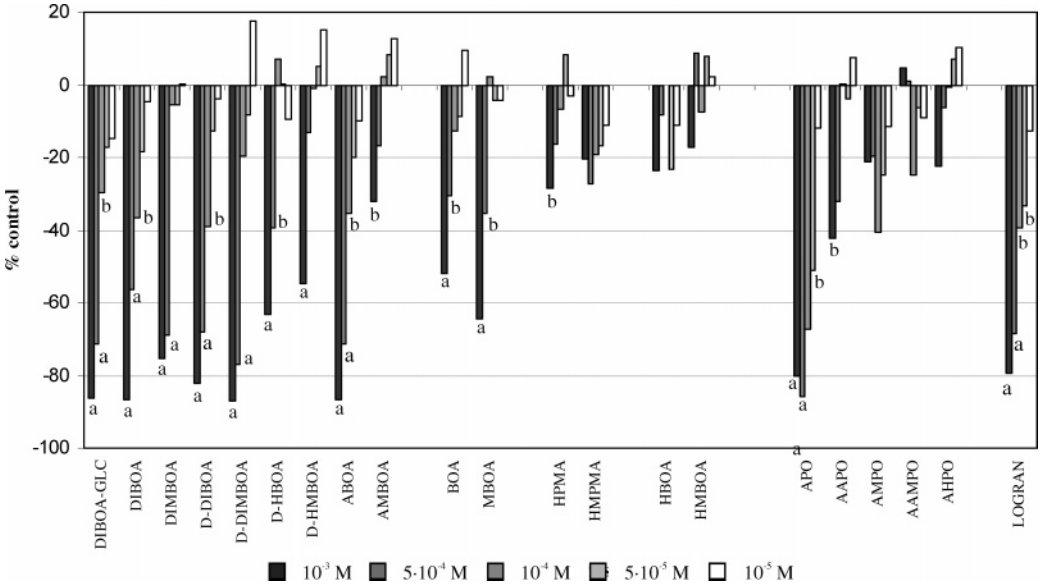

Figure 2. Phytotoxicity bioassay results (root length, percent from control) for wild oat (*A. fatua* L.). If it is not indicated, $P > 0.05$ for Welch's test: (a) values significantly different at $P < 0.01$; (b) values significantly different at $0.01 < P < 0.05$.

Shoot length was slightly inhibited on both species. The relative activities of the compounds were similar than in the roots, but with lower effects. There were no significant effects on germination rate for almost all compounds assayed, except for D-DIMBOA (**Table 1A**), which exhibited stronger effects than the internal reference, as shown in **Figure 3**. This behavior is observed for both species, *A. fatua* being the most affected at the lower concentration treatments.

APO was the only degradation product to have effects of this order. Benzoxazolinones BOA and MBOA had significant activity on both species, the inhibition values being ~60% at 10^{-3} M. Malonamic acids (**Table 1D**), lactams HBOA and HMBOA (**Table 1A**), and aminophenoxazines AAPO, AMPO, AAMPO, and AHPO (**Table 1C**) did not affect the species in a significant manner.

Structure−Activity Relationships. The fact that both weeds affect the same crops led us to consider their effects globally for the SAR study. Cluster analysis was made on the basis of root length for both species at all concentrations, and it is shown

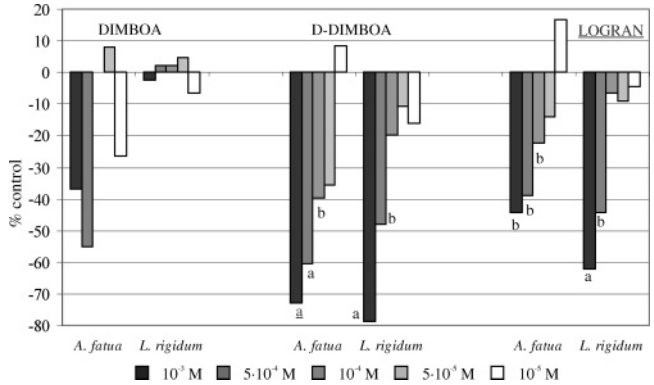

Figure 3. Phytotoxicity of D-DIMBOA and DIMBOA (germination rate, percent from control) for wild oat (*A. fatua* L.) and rigid ryegrass (*L. rigidum* Gaud.). If it is not indicated, $P > 0.05$ for Welch's test: (a) values significantly differ with $P < 0.01$; (b) values significantly differ with $0.01 < P < 0.05$.

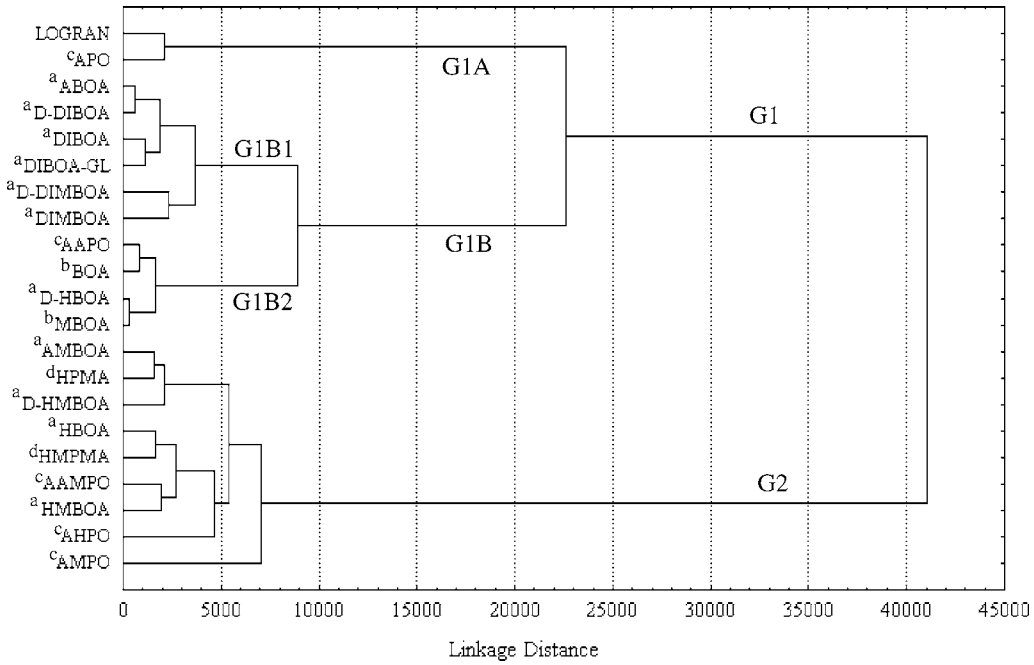

Figure 4. Cluster analysis for *L. rigidum* Gaud. and *A. fatua* L. growth inhibition (effects on root length): (a) benzoxazinones; (b) benzoxazolinones; (c) aminophenoxazines; (d) malonamic acids. Compounds with similar bioactivity profiles form a group. The closer is the linkage distance between clusters, the closer in average phytotoxicity are the compounds.

in **Figure 4**. According to their phytotoxicity profiles, the assayed compounds could be divided into two groups: G1, which includes the compounds with the higher phytotoxic effects, and G2, in which compounds with moderate or null effects are grouped. Group G1 is divided into two subgroups: G1A, formed by the synthetic herbicide internal standard and the aminophenoxazine APO **(Table 1C**), and G1B, formed mainly by benzoxazinones in addition to the degradation product AAPO (**Table 1C**) and the benzoxazolinones BOA and MBOA (**Table 1B**). This group is subdivided in two: G1B1, formed by benzoxazinones, and G1B2, with degradation products and the synthetic lactam D-HBOA (**Table 1A**).

Benzoxazinones. The synthetic benzoxazinones have been selected to study the influence of the different functional group combinations at C-7, C-2, and N-4. The 7-methoxybenzoxazinones can be structurally correlated with DIMBOA and HMBOA, whereas the lack of functional group at C-7 corresponds to the DIBOA and HBOA series. Both of these groups have compounds with a 2-hydroxy moiety (D-HMBOA and D-HBOA, respectively), a 4-hydroxy moiety (D-DIMBOA and D-DIBOA), and a 2-acetoxy moiety (AMBOA and ABOA).

Benzoxazinones, overall, are the most active structures of all compounds assayed, because they are the compounds mainly present in group G1. It should be noted that the compounds with this backbone belonging to this group contain the hydroxamic acid moiety. On the other hand, lactams (oxidized or not at C-2) belong to group G2.

Group G1B1 highlights clearly the optimal requirements for phytotoxicity at the benzoxazinone skeleton. The presence of the 7-methoxy group in the aromatic ring decreases phytotoxicity, as the cluster formed by D-DIMBOA and DIMBOA located below the group formed by nonmethoxylated benzoxazinones illustrates. The effect of the 7-methoxy group is the same considering natural or synthetic lactams. Considering both series separately (methoxylated and nonmethoxylated), the influence of the hydroxyl group at position C-2 is also displayed clearly, because nonhydroxylated compounds ABOA, D-DIBOA, and D-DIMBOA are more active than their analogues oxidized at C-2. The same happens in the case of lactams, HBOA and HMBOA being less active than their 2-deoxy synthetic analogues D-HBOA and D-HMBOA (**Table 1A**).

The effect of the acyl group at position N-4 is different depending on the series considered. For nonmethoxylated benzoxazinones, the presence of the acyl group increases phytotoxicity, as shown for ABOA (**Table 1A**), the most active benzoxazinone. Some authors have suggested that the presence of this acetyl leaving group at N-4 position enhances the toxicity of these 2-deoxy benzoxazinones, acting as a leaving group and provoking the formation of a highly stable electrophilic intermediate (*36*). For methoxylated benzoxazinones, the effect is the opposite, AMBOA (**Table 1A**) being outside group G1.

Degradation Products. As was observed in our previous study regarding the effects of benzoxazinones and related compounds on STS (*7*), 2-aminophenoxazin-3-one (APO; **Table 1C**) is the only degradation compound belonging to the most active group. Any other assayed functionalization present in the aminophenoxazine skeleton leads to a loss of phytotoxicity.

2-Acetamidophenoxazines (AAPO and AAMPO; **Table 1C**) have been proposed to be detoxification products of the amines APO and AMPO (*21*, *25*−*27*, *29*). This hypothesis can be accepted in the case of APO and AAPO due to the moderate effect observed for the latter one. The phytotoxicity for AMPO is much lower than expected in comparison to that of its nonmethoxylated analogue APO. Taking into account the similarity of APO and AMPO structures, their big differences in activity could be due to other phenomena, such as aqueous and lipid solubility and their consequences regarding the chemicals' diffusion through the cell membrane.

According to the Lipinski (*37*) and Tice (*38*) models for bioactivity in pharmaceuticals and agrochemicals, solubility in lipids or aqueous media is a very important parameter in the search for bioactive compounds. These solubilities can be quantified by using log *P* (water/*n*-octanol partition coefficient). Previous studies regarding the bioactivity of sesquiterpene lactones allowed establishing correlations between log *P* and EC_{50} for several bioactivity parameters (*39*). In our previous

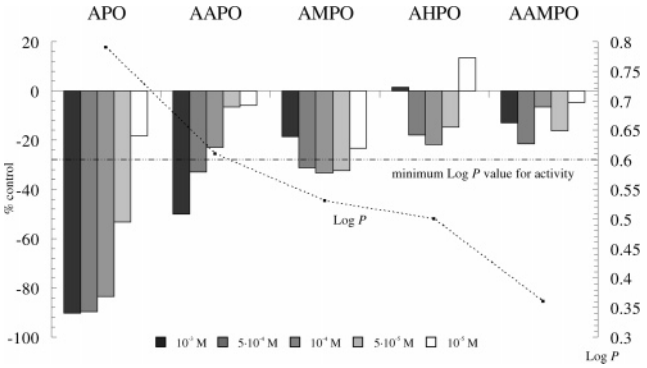

Figure 5. Phytotoxicity (*L. rigidum* Gaud., root length) (bars) and lipophilicity (log *P*) (line) for aminophenoxazines.

paper regarding effects on STS for these chemicals, a good correlation between phytotoxicity and solubility was observed for aminophenoxazines (*7*). Thus, log *P* was theoretically calculated for all assayed aminophenoxazines by computational methods (*34*). A correlation between phytotoxicity values (root length of *L. rigidum*) and log *P* can be observed in **Figure 5**. When log $P > 0.6$ (APO and AAPO), a significant activity value, with a suitable adjustment to a dose−response profile, can be observed. For lower log *P*, this regular profile is lost, and those compounds are considered to be nonactive.

Benzoxazolinones BOA and MBOA (**Table 1B**), placed in group G1B2, had significant effects just at the highest concentration assayed as mentioned above. The degradation process in which benzoxazinones DIMBOA and DIBOA are transformed into BOA and MBOA, respectively, which takes place in aqueous solution, wheat crop soil, and other environments (*17*, *18*, *29*), leads to a loss of phytotoxic effect.

Malonamic acids HPMA and HMPMA (**Table 1D**) and natural lactams HBOA and HMBOA (**Table 1A**) are placed in group G2. They did not show significant effects on any parameter assayed.

Ecological Role of Benzoxazinones and Their Degradation Products: Phytotoxicity−Stability Relationships. Once the allelochemicals DIBOA and DIMBOA are released to soil, they participate in several degradation mechanisms depending on the soil type and the microorganisms present in it (*17*, *18*, *29*). Thus, the allelopathic effect shown by a plant depends on the phytotoxic potential of the chemical combination present in the soil. The more phytotoxic and persistent the chemicals are, the higher the effects shown on competitive organisms should be. DIBOA-Glc is progressively transformed in DIBOA, BOA, APH, APO, and AAPO in a complete degradation series that we found to occur in wheat crop soil (*18*). A correlation between phytotoxicity (effects on root length) for both species, and stabilities, expressed as half-lives, for the DIBOA degradation series in wheat crop soil can be made. APO (**Table 1C**) is the most active compound of the degradation series, and it is also much more persistent than benzoxazinones (DIBOA-Glc, DIBOA) (**Table 1A**) or benzoxazolinone BOA (**Table 1B**) (*18*). Taking into consideration that *L. rigidum* and *A. fatua* constitute real agronomic problems for Gramineae crops such as wheat or barley, which produce benzoxazinones (*29*), the data shown allow us to propose APO as the main chemical responsible for defense interaction based on these compounds.

These results constitute a good illustration of the complexity of allelopathic interactions. In a real ecosystem, not only the plant that produces allelopathic agents but also the target organisms have to be taken into account to describe a chemical defense interaction. Microorganisms associated with the rhizosphere of the producer plant will transform those compounds in several ways. The products of those biotransformations could be more or less stable and more or less phytotoxic than the original compounds, and the responses of the target plants can be radically different.

The results shown above constitute the next step in the research on the potential use of benzoxazinones as leads for new herbicide models after the evaluation of those compounds on STS (*7*). Wild oat and rigid ryegrass development in crops are real agronomic problems, and the effects shown by the most active compounds confirm our hypothesis regarding their utility on weed management. These results are in good accordance with those obtained in our previous evaluation of these compounds on STS (*7*).

Benzoxazinones (**Table 1A**) constitute a group of compounds with high interest in new herbicide model research. Their phytotoxicities shown on *L. rigidum* and *A. fatua* allow us to propose benzoxazinones 4-acetoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (ABOA), 4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-DIBOA), and 4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4H)-one (D-DIMBOA) (**Table 1A**) as the best leads for new herbicide model development, in good accordance with their previous evaluation (*7*). The optimal functionalization for the benzoxazinone skeleton can be summarized as lack of a hydroxyl group at C-2 (2-deoxybenzoxazinones), a hydroxyl group at N-4 (hydroxamic acid moiety), and absence of a methoxy group at the aromatic ring (DIBOA-related compounds). The effects shown by ABOA suggest the modification of N-4 functionalization to be the key to phytotoxic candidates for herbicide models development.

D-DIMBOA is the only compound belonging to the DIMBOA series that showed interesting bioactivity, especially dealing with its effects on the *A. fatua* germination rate ($EC_{50} = 4.7 \times 10^{-5}$ M, $r^2 = 0.97$), as expressed above. This is the case in which the lack of functionalization at C-2 provides a more radical bioactivity modification. Further research involving phytotoxicity evaluation at several target plant growth stages and the combination of compounds with different effects would provide useful tools for weed management based on allelochemical-like structures.

Phytotoxic effects of benzoxazinones decrease when they are degraded to benzoxazolinones, lactams, or malonamic acids. 2-Aminophenoxazin-3-one (APO) (**Table 1C**) is the only degradation compound in which an increase of the phytotoxicity from natural benzoxazinones is observed. Then, aminophenoxazines constitute the most interesting group of degradation compounds. Their effects could be correlated with their solubility, as the relationships between log *P* and growth parameters suggest. Further modifications in the search for optimization of their structures to Lipinski (*37*) and Tice (*38*) models for bioactivity will be needed in the search for phytotoxic agents based on that structure. The low biodegradability of APO does not allow it to be considered as a practical herbicide model at this research stage. Further investigations about its behavior in different ecological systems will be needed, because some authors suggested this compound to be degraded in different conditions (*29*). Nevertheless, the correlation between phytotoxic effects and stabilities for the DIBOA degradation series compounds, especially APO, offered a more accurate description of the ecological interactions between benzoxazinone-producing plants and common weeds.

The SAR found for these chemicals on weeds are similar to those found on STS (*7*). The main groups are formed by the same compounds in both cases, and just some chemicals have

different interactions with weeds and STS. The most significant difference of interaction can be found by comparing results on wheat (which was not inhibited or inhibited in only a moderate manner) (*7*) with the strong inhibition observed on these Gramineae weeds, which affect wheat crops among others.

These studies, in which phytotoxic effects are correlated with structure, stability, and molecular properties of the chemicals assayed, provide very useful information for new herbicide model development and constitute an example of the importance of chemical ecology research for weed management tools optimization.

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