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Combined Strategy for Phytotoxicity Enhancement of Benzoxazinones

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Fifteen new derivatives of D-DIBOA, including aromatic ring modifications and the addition of side chains in positions C-2 and N-4, were synthesized and their phytotoxicity, selectivity, and structure−activity relationships evaluated. The most active compounds among the derivatives at the C-2 position were 6-Cl-2-Et-D-DIBOA and 6-F-2-Et-D-DIBOA. Of the derivatives at N-4, the most active compounds were 6-Cl-4-Pr-D-DIBOA and 6-Cl-4-Val-D-DIBOA. These four compounds showed high levels of inhibition in root length at very low concentrations in all species. The most remarkable result is the 70% inhibition observed for the root length of cress at 100 nM caused by the latter two compounds. These results support our previous research and conclusions regarding the steric, electronic, and solubility requirements to achieve the maximum phytotoxic activity.

KEYWORDS: Benzoxazinoids; phytotoxicity; QSAR; herbicides; SAR

INTRODUCTION

Natural products based on the (2*H*)-1,4-benzoxazin-3(4*H*)-one system have been prominent in the field of phytochemistry research for decades, since the first isolation of 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) from maize (*1*) (*Zea mays* L.) and barley (*2*) (*Secale cereale* L.), respectively. Since these early days, the interesting bioactivity of these compounds has encouraged research into natural products and modified derivatives as potential biopesticides (*3*) among other applications. The development of pesticides from natural products is believed to provide new modes of action and a more specific interaction with the pest and to be more environmentally friendly (*4*). The technical and economic optimization of bioactive chemical production has encouraged traditional research on natural bioactive agents with the aim of simplifying the natural structure. In most cases, however, this leads to loss of the bioactivity. The current trend of using a natural product structure as a template to obtain new leads in the discovery of bioactive molecules is greatly exemplified by the research on phytotoxic benzoxazinones.

Among the studies on benzoxazinones performed to date, some concern the elucidation of structure−activity relationships of several modified benzoxazinones. The primary objective of these studies was to optimize the molecular functionalization to achieve the maximum phytotoxicity, as detailed in the recent reports on the phytotoxicity of benzoxazinones and their direct degradation products on various plant species, including common crops (*5*) and problematic weeds (*6*, *7*). The results of this research show that the relative activity to the crop or weed is maintained for each of the benzoxazinone modification patterns studied. The synthetic benzoxazinones 4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-DIBOA) and 4-acetoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (ABOA) were found to be the most phytotoxic benzoxazinones of those with the closest analogy to the natural products (**Figure 1**).

On the basis of these results, structural modifications were planned, and a structure−activity relationship study of several modified benzoxazinones was carried out. On the one hand, the systematic esterification of D-DIBOA at the N-4 position resulted in a new generation of esters with increased lipophilicity. These compounds showed more marked phytotoxic effects and higher selectivity in comparison to natural benzoxazinones (*8*, *9*). In addition, the absence of a hydroxyl group at position C-2 was found to have a crucial influence on phytotoxicity, in contrast to other previously evaluated bioactivities, including algicide and fungistatic properties (*10*). On the other hand, the addition of alkyl groups at position C-2 also gave rise to an increase in bioactivity. In both cases the improvements were attributed to a modification of the transport phenomena necessary for the phytotoxic action of the tested chemicals in the context of the Tice model for agrochemical design (*11*).

Moreover, an improvement in the benzoxazinone skeleton through modification of steric and electronic features was developed (*12*, *13*). These modifications included the addition of halogen substituents to the aromatic ring. Optimal ranges for molecular volume, dipole moment, and polarizability of the benzoxazinones for the maximum phytotoxic activity were characterized. The synthetic routes used to obtain these new derivatives are easier to develop and scale-up than those used for natural benzoxazinones substituted at C-2. Benzoxazinones are therefore a rare example in which the simplification of a natural structure leads to increased bioactivity.

As a final part of these studies, and considering the promising results obtained for some of the derivatives, the synthesis and

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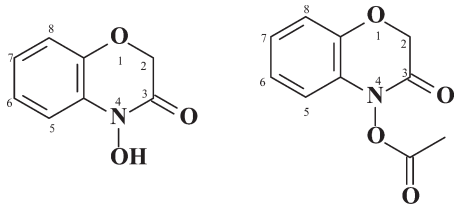


D-DIBOA ABOA

Figure 1. Structures of 4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-DIBOA) and 4-acetoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (ABOA).

bioactivity evaluation of models including more than one kind of structural modification were planned. The main objective of this research was to achieve the maximum phytotoxic effects in different standard target species. As a first approach, the bioactivity of these novel leads on common crops selected as standard target species is reported herein.

Fifteen derivatives were synthesized, and these included aromatic ring modifications plus the addition of side chains in positions C-2 and N-4 (**Tables 1** and **2**). These compounds were evaluated in terms of the phytotoxicity results, selectivity, and structure−activity relationships, and the results are summarized below.

MATERIALS AND METHODS

General Experimental Procedures. The purities of the compounds to be tested were determined by ^{1}H NMR and HPLC analyses and were found to be >98%. ^{1}H and ^{13}C NMR spectra were recorded using $CDCl_3$ as solvent on a Varian INOVA spectrometer at 399.99 and 100.577 MHz, respectively. The resonance of residual chloroform was set to δ 7.25. The solvent peak for ^{13}C was set to δ 77.00 (chloroform), and this was used as the internal reference. UV−vis spectra were obtained using a Varian Cary 50 BIO spectrophotometer with chloroform as the solvent. Mass spectra (EIMS) were recorded using a Voyager Thermoquest spectrometer. FTIR spectra were obtained on a Perkin-Elmer Spectrum BX FTIR system. Frequency values are given in cm^{-1}.

Preparation of Derivatives. The chemicals were obtained according to the previously mentioned methodology, by a sequence of nucleophilic substitution (side chain linkage), reductive cyclization (benzoxazinone ring formation), and esterification for the N-4 derivatives. The starting materials employed were 2-nitrophenols substituted at the appropriate positions: 4-fluoro-2-nitrophenol, 4-chloro-2-nitrophenol, and 2-chloro-2-nitrophenol. These compounds were purchased from Sigma-Aldrich Co.

General Procedure for Nucleophilic Substitution. The starting halo-2-nitrophenol was dissolved in a 0.1 M solution of KOH in absolute ethanol (1 mol equiv of KOH). After 1 h, the solvent was removed under reduced pressure. The resulting alkoxide was redissolved in DMF (50 mL/g of starting material), and 1.2 mol equiv of the appropriate 2-halo ester was added. The reaction mixture was stirred under argon for 24 h. After this time, ethyl acetate (50 mL/g of starting material) was added, and the resulting organic solutions were washed with five portions of distilled water. The organic layers were combined and dried over anhydrous sodium sulfate, and the solvent was distilled under reduced pressure. The crude product was purified by column chromatography (CC; SiO_2, ethyl acetate/hexane, 20:80) to give ethyl 2-(2′-nitrophenoxy)acetates in quantitative yield (**Figure 2**).

General Procedure for Reductive Cyclization. Pd/C (10% Pd, 10% w/w with respect to starting nitrophenoxyacetate) was suspended in an aqueous solution of 1,4-dioxane (1:1) (100 mL/g of starting material). Sodium borohydride (2 mol equiv) was added, and the solution was vigorously stirred. A solution of the nitrophenoxyacetate in 1,4-dioxane (0.5 g/mL) was added dropwise to this stirred suspension. The progress of the reaction was monitored by TLC. Once the reaction was complete, the suspension was vacuum filtered through Celite to remove the catalyst, and the filtrate was treated with 10% HCl until pH = 2 was reached. This solution was further extracted with ethyl acetate (×3). The organic layers were combined and dried over anhydrous sodium sulfate, and the solvent was distilled under reduced pressure. The resulting residue was purified by column chromatography (CC; SiO_2, ethyl acetate/hexane, increasing polarity) to give the corresponding benzoxazinone (**Figure 2**).

General Procedure for Esterification. The appropriate D-DIBOA halo derivative (100 mg) was dissolved in dry pyridine (25 mL), and 1.2 mol equiv of the appropriate acyl chloride (commercial) was added dropwise at 0 °C (ice bath) under a dry argon atmosphere. The reaction mixture was allowed to warmp to room temperature; 12 h later, EtOAc (25 mL) was added, and the mixture was transferred to an extraction funnel and washed with 0.1 N HCl (3 × 50 mL) and 0.1 N $NaHSO_4$ (3 × 50 mL). The organic phases were further combined and dried over anhydrous magnesium sulfate. The solvent was removed (rotatory evaporator), and the residue was chromatographed (CC; SiO_2, EtOAc/hexane, 1:10) to give the benzoxazinone ester derivatives (**Figure 3**).

Calculation of IC_{50} and Log*P*. The phytotoxicity data were fitted to a sigmoidal dose−response model (constant slope) by employing the GraphPad Prism v.4.00 software package (GraphPad Software Inc.). cLog*P* values were estimated using the OSIRIS property explorer (ChemExper Inc.). This software uses the Chou and Jurs algorithm, which is based on computed atom contributions (*16*).

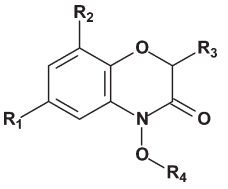
Molecular Modeling and QSAR Calculations. Three-dimensional models of the tested chemicals were obtained from AM1 calculations performed by Hyperchem 7.01 software (Hypercube Inc.). Dipole moments, partial charges, polarizabilities, and molecular volumes were obtained by employing the algorithms implemented in this software. Molecular parameter/activity correlations were performed using Microsoft Office Excel 2007 spreadsheets (Microsoft Corp.).

Phytotoxicity Bioassays. *Target Plants*. The selection of target plants was based on an optimization process carried out by us in the search for a standard phytotoxicity evaluation bioassay (*19*). This process led to several standard target species (STS) being proposed, including monocot *Allium cepa* L. (onion) and dicots *Lycopersicon esculentum* Will. (tomato), *Lepidium sativum* L. (cress), and *Lactuca sativa* L. (lettuce), which were assayed for this study.

Methodology. Bioassays were carried out using Petri dishes (90 mm diameter) with one sheet of Whatman No. 1 filter paper as the substrate. Germination and growth were conducted in aqueous solutions at controlled pH using 10^{-2} M 2-(*N*-morpholino)ethanesulfonic acid (MES) and the addition of 1 M NaOH to give a pH of 6.0. Solutions (0.2, 0.1, 0.02, 0.01, and 0.002 M) of the compounds to be assayed were prepared in DMSO and then diluted with buffer (5 μL of DMSO/mL of buffer) to give the test concentrations for each compound (1, 0.5, 0.1, 0.05, and 0.01 mM). This procedure facilitated the solubility of the assayed compounds. The number of seeds was 25 in each Petri dish. The treatment control (or internal reference solution) (5 mL) was added to each Petri dish. Four replicates were used for each species.

After the addition of seeds and aqueous solutions, Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds were further incubated at 25 °C in a Memmert ICE 700 controlled-environment growth chamber in the absence of light.

Table 1. Derivatives Prepared with Functionalization at C-2



R_1	R_2	R_3	R_4	compound
F	H	$-CH_2CH_3$	H	2-ethyl-6-fluoro-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
F	H	$-CH_2CH_2CH_3$	H	6-fluoro-4-hydroxy-2-propyl-(2*H*)-1,4-benzoxazin-3(4*H*)-one
F	H	C_6H_5	H	2-phenyl-6-fluoro-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
F	H	EtOCO	H	2-ethoxycarbonyl-6-fluoro-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
Cl	H	$-CH_2CH_3$	H	2-ethyl-6-chloro-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
Cl	H	$-CH_2CH_2CH_3$	H	6-chloro-4-hydroxy-2-propyl-(2*H*)-1,4-benzoxazin-3(4*H*)-one
Cl	H	C_6H_5	H	6-chloro-2-phenyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
H	Cl	$-CH_2CH_3$	H	8-chloro-2-ethyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
H	Cl	$-CH_2CH_2CH_3$	H	8-chloro-4-hydroxy-2-propyl-(2*H*)-1,4-benzoxazin-3(4*H*)-one
H	Cl	C_6H_5	H	8-chloro-2-phenyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
H	Cl	EtOCO	H	8-chloro-2-ethoxycarbonyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one

Table 2. Derivatives Prepared with Functionalization at N-4

R_1	R_2	R_3	R_4	compound
F	H	H	$-COCH_2CH_3$	6-fluoro-4-propionyloxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
F	H	H	$-CO(CH_2)_3CH_3$	6-fluoro-4-valeroyloxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
Cl	H	H	$-COCH_2CH_3$	6-chloro-4-propionyloxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
Cl	H	H	$-CO(CH_2)_3CH_3$	6-chloro-4-valeroyloxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
H	Cl	H	$-COCH_2CH_3$	8-chloro-4-propionyloxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
H	Cl	H	$-CO(CH_2)_3CH_3$	8-chloro-4-valeroyloxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one

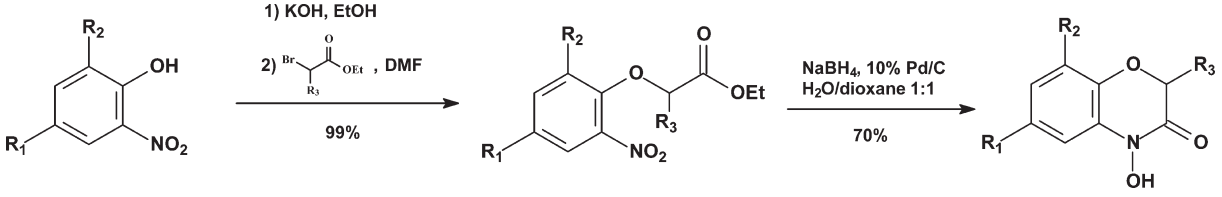


Figure 2. General procedure for the synthesis of tested chemical functionalized at C-2.

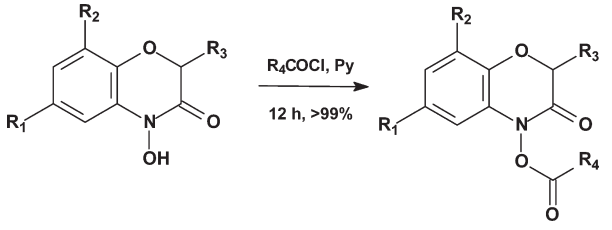


Figure 3. General procedure for the synthesis of tested chemical functionalized at N-4.

Bioassays took 4 days for cress, 5 days for lettuce and tomato, and 7 days for onion. After growth, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process. This helped in 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, 59.4%) and 2-(2-chloroethoxy)-*N*-{[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl}benzenesulfonamide (triasulfuron, 0.6%), was used as the internal reference in accordance with a comparison study reported previously (*15*).

Bioassay Data Acquisition. Evaluated parameters (germination rate, root length, and shoot length) were recorded using a Fitomed system (*16*), which 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. Results are represented in bar charts in which the null value represents control, negative values represent inhibition, and positive values represent stimulation of the studied parameter (*15*). Phytotoxic activities expressed in this way can be found in the Supporting Information for all chemicals and species. Once the germination and growth data had been acquired, cluster analysis was used to group compounds with similar phytotoxicity behavior and associate them with their molecular structure. Complete linkage was used as an amalgamation rule, and the distance measurement was based on squared Euclidean distances, 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

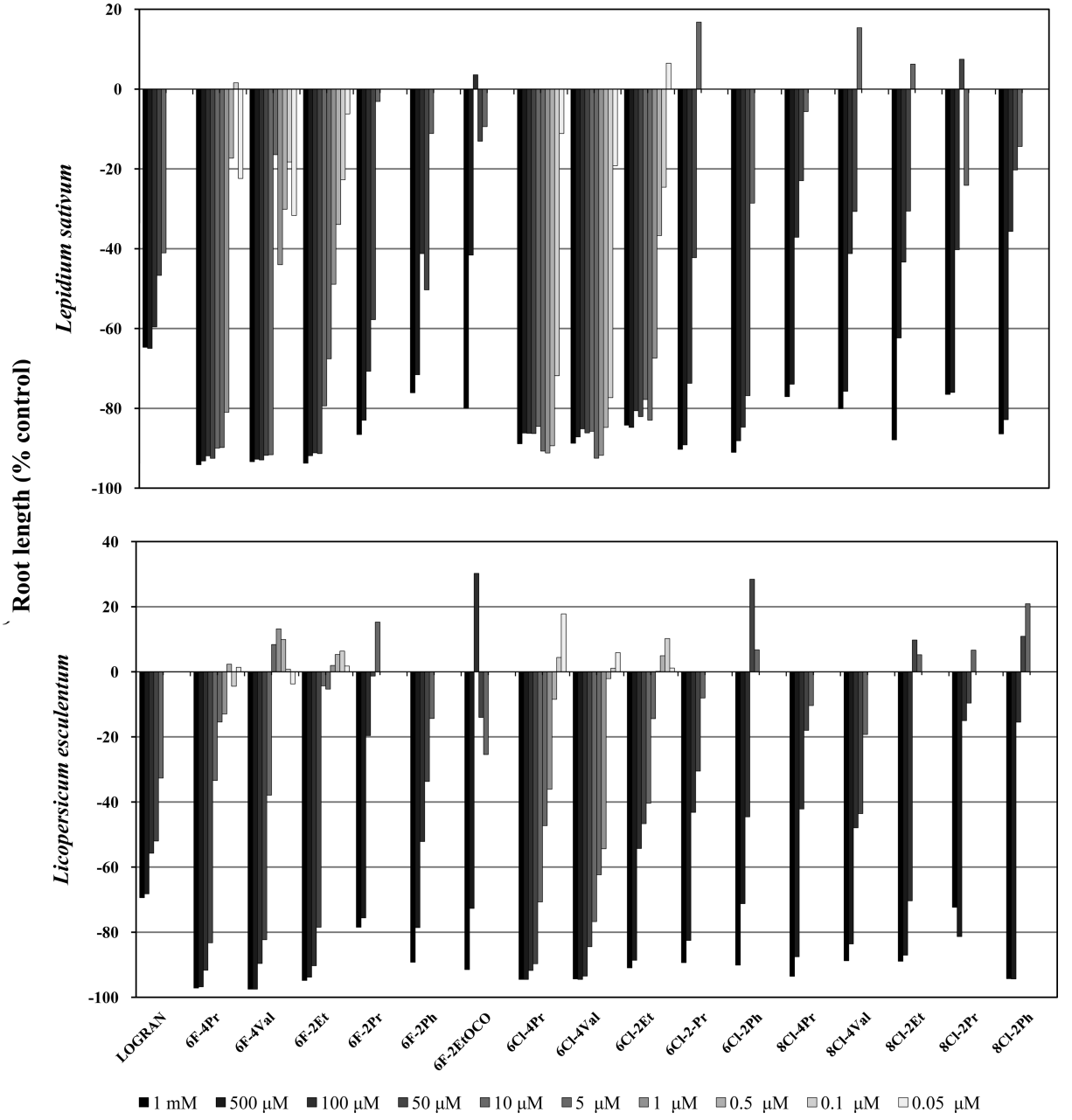


Figure 4. Phytotoxicity effects of tested compounds on the root length of the species *L. sativum* L. and *L. esculentum* Will. According with Welch's test, inhibition values higher than −40% are significantly different at $P < 0.01$, inhibition values between −40% and −20% are significantly different at $0.01 < P < 0.05$, and values between −20% and +30% present $P > 0.05$.

values. The cluster was obtained using Statistica v.5.0 software (Statsoft Inc.). Germination rate, shoot length, and root length effects for all tested species were included in the analysis to provide an overall view of the phytotoxicity and its relationship with chemical structure. EC_{50} values were obtained after the phytotoxicity data had been adjusted to concentration (logarithmic scale), to a constant slope 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 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 the determination coefficient (r^2). The adjustment and r^2 were obtained using GraphPad Prism software v.4.00 (GraphPad Software Inc.).

RESULTS AND DISCUSSION

General Bioactivity Profiles. All assayed compounds were active and showed inhibitor profiles in the growth parameters evaluated. In all cases the most affected parameter was root length (**Figures 4** and **5**). The phytotoxic activity levels of these derivatives were species dependent. Thus, in the case of cress the most active compounds were 6-Cl-4-Val-D-DIBOA, 6-Cl-2-Et-D-DIBOA, and 6-F-4-Pr-D-DIBOA, all of which showed activity even at concentrations below 1 μM. In the cases of onion, tomato, and lettuce the most active compounds were 6-Cl-4-Pr-D-DIBOA and 6-Cl-4-Val-D-DIBOA, and these showed high activities at very low concentrations (1 and 5 μM).

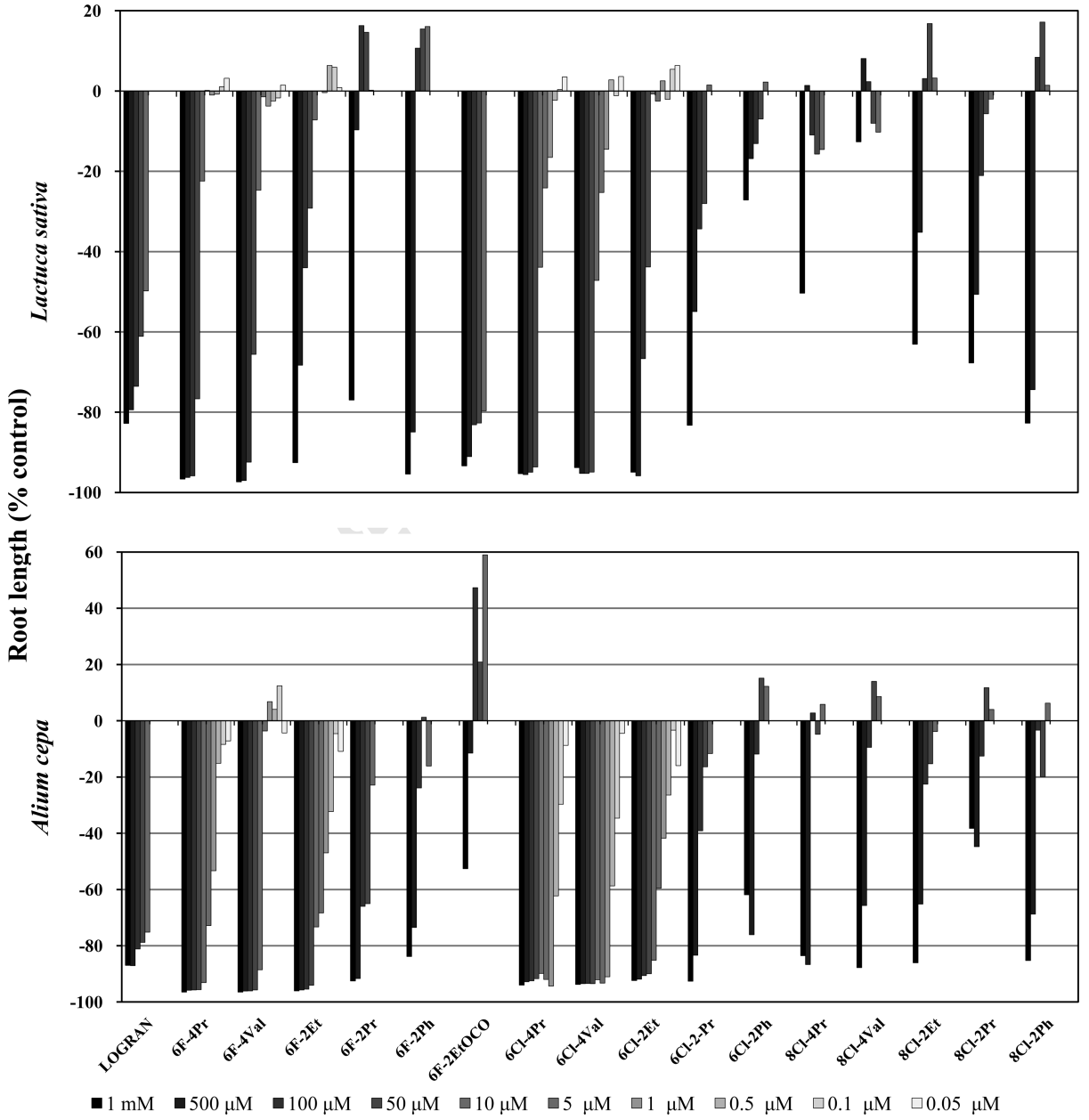


Figure 5. Phytotoxicity effects of tested compounds on the root length of the species *A. cepa* L. and *L. sativa* L. According with Welch's test, inhibition values higher than −40% are significantly different at $P < 0.01$, inhibition values between −40% and −20% are significantly different at $0.01 < P < 0.05$, and values between −20% and +30% present $P > 0.05$.

Derivatives at C-2. The most active compounds of this series were 6-Cl-2-Et-D-DIBOA and 6-F-2-Et-D-DIBOA. The first compound showed inhibition values of about 60−70% for root length of onion and cress at a concentration 1 μM. The second compound showed values of 50% at the same concentration.

Derivatives at N-4. The most active compounds of this series were 6-Cl-4-Pr-D-DIBOA and 6-Cl-4-Val-D-DIBOA. Both compounds showed high levels of inhibition in root length at very low concentrations in all species. For example, the inhibition of root length for tomato and lettuce was 50% at 1 and 5 μM, whereas the inhibition in the case of onion was closer to 90% at the same concentration. The most remarkable result is the 70% inhibition observed for root length of cress at 100 nM.

Quantitative Structure−Activity Relationship (QSAR). A cluster analysis for growth results in the four species for all compounds is shown in **Figure 6**. Activities can be divided in two main groups (G1 for the highest activities and G2 for weaker effects). G2 is divided into two subgroups (G2a for high effects and G2b for low effects). The most active group (G1) is formed by the derivatives 6-Cl-4Pr, 6-Cl-4-Val, 6-F-4-Pr, 6F-4-Val, 6-Cl-2-Et, and 6-F-2-Et-D-DIBOA. It is worth pointing out that all compounds were more active than the commercial herbicide Logran, including those in group G2. Most compounds included in G2 have good activity profiles, with the exception of 6-F-2-EtCO, which is included as the only member of subgroup G2b.

On the basis of the cluster analysis we can analyze the data according to the position of the functionalization. Regarding the aromatic part of the molecule, compounds halogenated at the C-6 position are generally more active than the corresponding compounds halogenated at C-8, except for derivatives with a phenyl group at C-2, for which there is little difference between them. A steric effect caused by the aromatic moiety could be the cause of

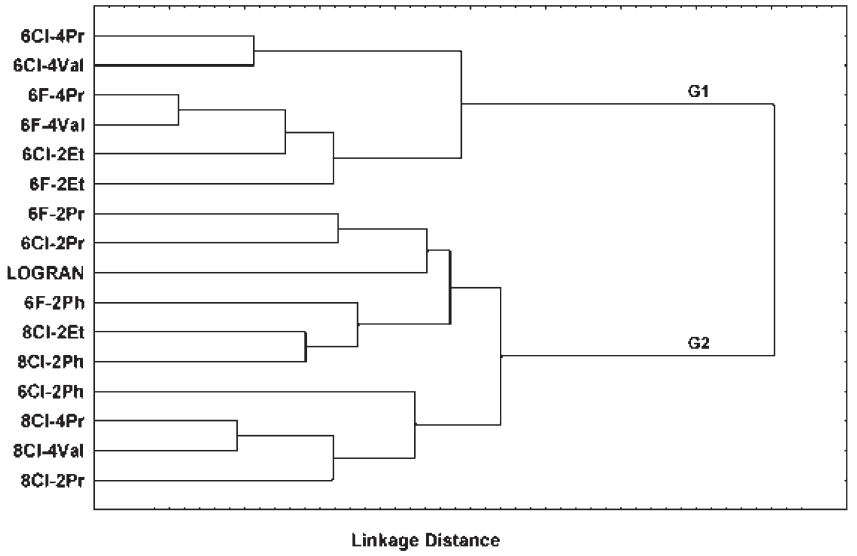


Figure 6. Cluster analysis (phytotoxicity data, germination, and growth parameters, all tested species) of the tested chemicals.

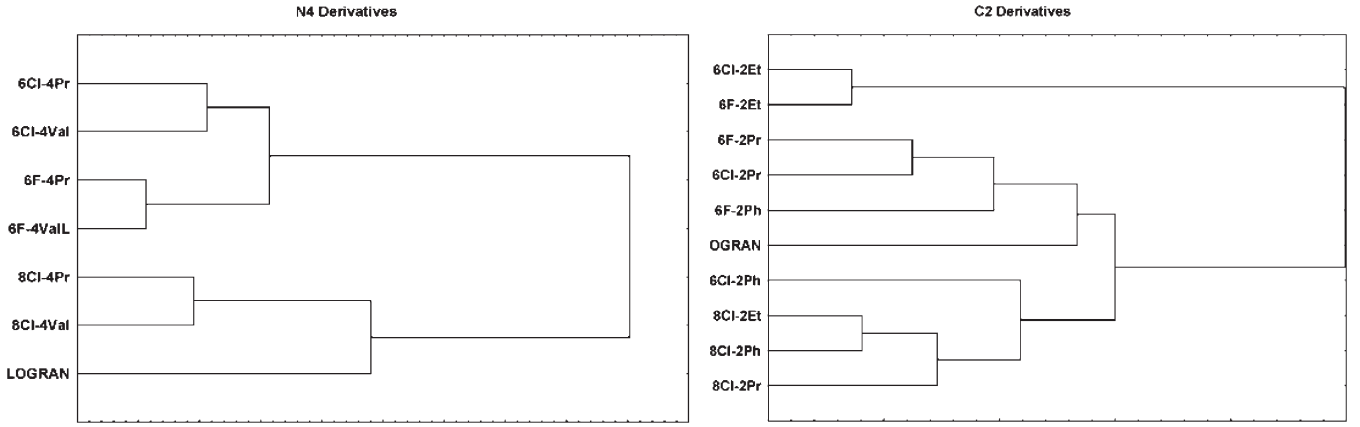


Figure 7. Cluster analysis (phytotoxicity data, germination, and growth parameters, all tested species) of the N-4 and C-2 derivatives.

this result. If we analyze separately the two types of functionalization (**Figure 7**), it can be seen from the clusters that the behavior is maintained for both families, with the most active compounds being halogenated at C-6. For C-2 derivatives, the most active compounds are clearly 6-Cl-2-Et-D-DIBOA and 6-F-2-Et-D-DIBOA. Both compounds showed highly significant inhibition levels. For example, the derivative 6-Cl-2-Et-D-DIBOA showed an IC_{50} of 400 nM (r^2 = 0.9614) for the root of cress as well as values of 2.3 μM (r^2 = 0.9528) and 1.6 μM (r^2 = 0.9732) for the root of tomato and onion, respectively. Compound 6-F-2-Et-D-DIBOA exhibited an IC_{50} at 1 μM (r^2 = 0.9838) and 1.3 μM (r^2 = 0.9791) for the roots of cress and onion, respectively.

The most active N-4 derivatives were 6-Cl-4-Pr-D-DIBOA and 6-Cl-4-Val-D-DIBOA, both of which showed IC_{50} values of 60 nM (r^2 = 0.8957 and r^2 = 0.9567) for cress and 200 nM (r^2 = 0.9808 and r^2 = 0.9743) for onion.

6-F-D-DIBOA, 6-Cl-D-DIBOA and 8-Cl-D-DIBOA can be considered as reference compounds in our study (*12*), and it can be seen by comparing the values of IC_{50} for each species (**Figure 8**) that activity varies depending on the type of substitution. In general, the modifications carried out on the N-4 position lead to an increase in the activity levels with respect to reference compounds, and this increase is greater than that generated by changes at the C-2 position. Analysis of each of the species individually shows that in the case of cress (*L. sativum*) and onion (*A. cepa*) the modifications introduced do not cause a substantial improvement in the activity. In the case of lettuce (*L. sativa*), the derivative 6-F-4-Pr-D-DIBOA has enhanced activity by almost one logarithmic unit, but the clearest effect is observed for 8-Cl-2-Ph-D-DIBOA. Finally, for tomato (*L. esculentum*) the most marked improvement in activity is observed with the derivative 6-Cl-4-Val-D-DIBOA.

The effects of the halogen substituents are clearly position-related, as demonstrated in our previous research (*8*, *17*). The high phytotoxic effects provoked by halogenated benzoxazinones cannot therefore be attributed to the intrinsic toxicity of the halogen itself but rather to the marked electronic transformation of the molecule. Once the optimal functionalization for maximum phytotoxicity is reached, further research to assess environmental and nontarget organism toxicity is needed.

The bioactivity data for these chemicals match completely with those previously recorded for the lead halo compounds: the choice of position for functionalization and the nature of the optimal functional group, as well as a combination of these two factors, produce a synergistic effect on the activity values. This supports our previous research and conclusions regarding the steric, electronic, and solubility requirements to achieve the maximum phytotoxic activity. These novel compounds should also be assayed in particularly problematical weeds and the results compared to those for strategic crops such as wheat or rice (*7*, *8*, *12*, *17*).

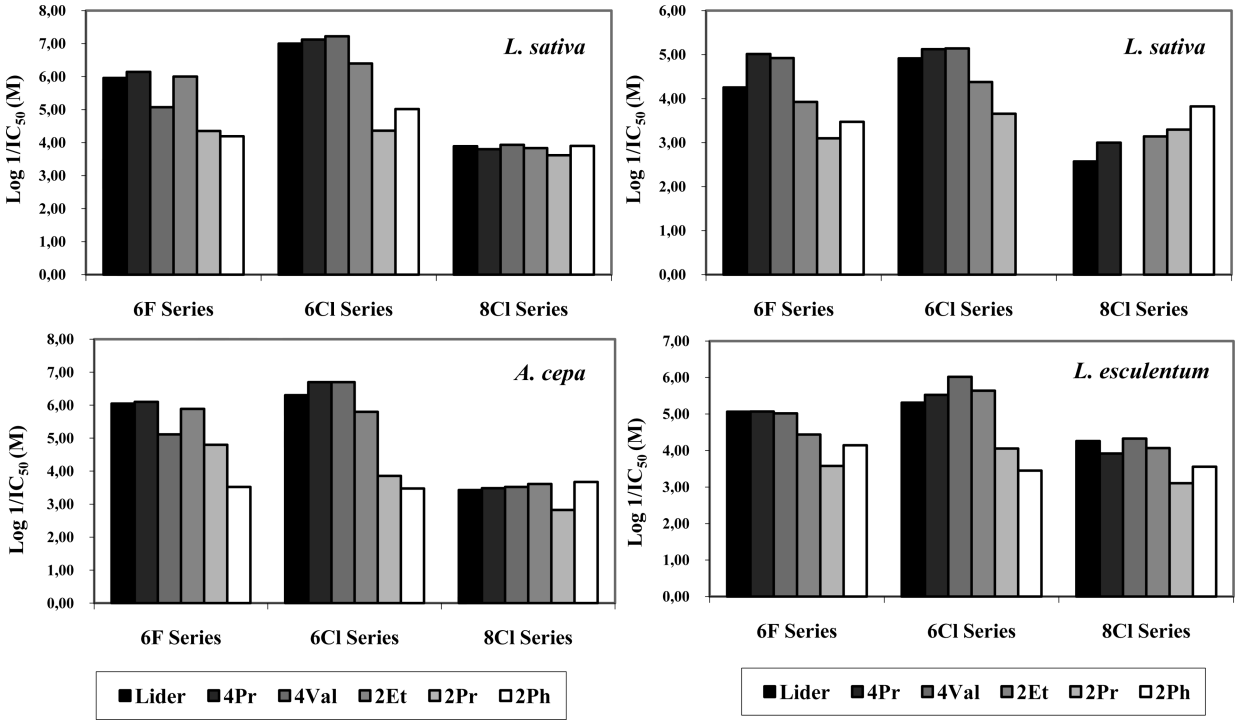


Figure 8. Comparison of the IC_{50} values (root length) of tested compounds grouped by the aromatic substitution for each species.

Supporting Information Available: (1) Complete spectroscopic data of synthesized compounds and (2) bioassay data on *L. sativum* L., *L. sativa* L., *L. esculentum* L., and *A. cepa* L. This material is available free of charge via the Internet at http://pubs.acs.org.

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