

Joint Action of Benzoxazinone Derivatives and Phenolic Acids

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The joint action of binary and ternary mixtures of benzoxazinone derivatives and phenolic acids was studied using the additive dose model (ADM) as reference model. The activity of fixed-ratio mixtures of phenolic acids [ferulic acid (FA), *p*-coumaric acid (CA), vanillic acid (VA), and *p*-hydroxybenzoic acid (HBA)] and benzoxazinone derivatives [2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), 6-methoxybenzoxazolin-2-one (MBOA), benzoxazolin-2-one (BOA), 2-aminophenol (AP), and *N*-(2-hydroxyphenyl)acetamide (HPAA)] on *Lolium perenne* and *Myosotis arvensis* root growth was assessed in Petri dishes. Root length was recorded 6 days after seeding, and EC₅₀ and EC₉₀ values were estimated using nonlinear regression analyses. The benzoxazinone derivatives were found to be more phytotoxic than the phenolic acids, particularly on *M. arvensis*. Binary mixtures of phenolic acids responded predominantly additively on both plant species. Deviations from additivity were species-specific with antagonistic responses on *L. perenne* and synergistic responses on *M. arvensis*. Similarly, binary mixtures of benzoxazinone derivatives also followed the ADM, although synergistic responses were observed for BOA + AP and BOA + HPAA. Binary and ternary mixtures of benzoxazinone derivatives and phenolic acids responded primarily antagonistically; however, a significant synergistic performance was observed with DIMBOA + FA and DIMBOA + VA on *L. perenne*. These results do not support the assumption that allelopathic effects of wheat can be attributed to synergistic effects of otherwise weakly active allelopathic compounds, and it is suggested that future research be directed toward identifying and studying the effects of other potential allelochemicals including the degradation products of the most abundant wheat allelochemicals.

KEYWORDS: Ferulic acid; *p*-coumaric acid; vanillic acid; *p*-hydroxybenzoic acid; DIMBOA; MBOA; BOA; AP; HPAA; binary mixtures; ternary mixtures

INTRODUCTION

Allelopathy among higher plants has been documented in both natural plant communities and agroecosystems (e.g., ref 1). In agroecosystems, evidence has accumulated that crop allelopathy has a potential for weed management either as the sole weed suppression measure or, more likely, as one of several control options in an integrated weed management strategy (2). In agroecosystems allelopathy can be exploited either by incorporating plant residues containing allelopathic compounds that release during decomposition or by growing crop varieties that are able to release phytotoxic compounds through root exudation, leaching, or volatilization (3).

The allelopathic potential of cover crops of wheat was demonstrated in field studies by Putnam et al. (4), and it has been found that aqueous extracts of wheat residues are allelopathic to several weed species (5, 6). Wheat seedling allelopathy has so far been demonstrated only in laboratory assays. Wu et

al. (7) screened a worldwide collection of 453 wheat varieties using an agar-based screening method and found significant differences in their ability to inhibit the growth of *Lolium perenne* L. (LOLPE). Belz and Hurlle (8) also found significant differences in wheat varieties in hydroponic culture with *Sinapis alba* L. (SINAL) as test plant.

Three main groups of allelochemicals have been identified in wheat: phenolic acids (9–11), hydroxamic acids (8, 10, 12–14), and short-chain fatty acids (15, 16). Whereas phenolic and hydroxamic acids are produced by living wheat plants and either exuded by the plants or released following degradation of incorporated plant material, short-chain fatty acids are secondary products produced as a result of anaerobic fermentation of polysaccharides.

The two hydroxamic acids found most abundantly in wheat, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), are rapidly transformed in soil and water to 6-methoxybenzoxazolin-2-one (MBOA) and benzoxazolin-2-one (BOA) and subsequently to a number of other compounds belonging to the aminophenoxazinones, malonamic acids, and acetamides (17–20). Any study

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on the activity of DIMBOA and DIBOA should therefore include transformation products to fully understand the allelopathic potential of this group of wheat allelochemicals.

Einhellig (3) has stated that nearly all allelopathic activity is due to mixtures of two or more compounds and that there is probably no case when effects can be explained by one allelochemical. Wheat seedlings produce both phenolic and hydroxamic acids, and these compounds may be exuded simultaneously into the growth medium (10, 21). Hence, it is very likely that wheat allelopathy could be attributed to the simultaneous action of phenolic acids and benzoxazinone derivatives and, consequently, it is imperative to examine and understand the joint action of wheat allelochemicals to assess the potential benefits of wheat allelopathy. Several studies have been dedicated to describing the effect of mixtures of wheat allelochemicals but with contrasting results. Reigosa et al. (22) reported additive effects of phenolic acids, whereas Inderjit et al. (23) found mostly antagonistic interactions between phenolic acids. In contrast, Einhellig and co-workers found synergistic effects with mixtures of phenolic acids (24–26). This discrepancy in the results can be largely attributed to the use of different statistical methods.

Essentially, two reference models exist to describe mixture effects of phytotoxic compounds: the multiplicative survival model (MSM) and the additive dose model (ADM) (27). MSM assumes that each compound in a mixture acts independently of the other compounds in the mixture and that the modes of action of the compounds are different. The efficacy of a mixture can be estimated by multiplying percent survivals of each compound, that is, assuming that the compounds act sequentially. ADM, in contrast, assumes additivity of doses, that is, one compound can be replaced, wholly or in part, by another compound at equivalent biological dose rates (28); hence, a fundamental difference between MSM and ADM is that MSM considers effects, whereas ADM considers concentrations or dose rates. MSM has been more widely applied because in its simplest form it requires only results with a single concentration of each compound and their mixture (29). It has been argued that ADM, from a conceptual point of view, has the advantage over MSM and that the latter should be applied only for mixtures of compounds assumed to work independently (30, 31).

The mode of action of wheat allelochemicals is not fully understood. Einhellig (32) and Blum et al. (33) suggested that phenolic acids depolarize cell membranes, affecting membrane ATPase activity and ion flux. Reigosa et al. (34) suggested that the effects of BOA, a transformation product of the hydroxamic acid DIBOA, were multiple, including membrane leakage, changes in ATPase activity, and oxidative stress. If the mode of action of BOA is representative for the mode of action of DIBOA and other benzoxazinone derivatives, then it seems justified to adopt ADM in joint action studies of wheat allelochemicals.

The objectives of the present study were to assess the joint action of benzoxazinone derivatives and phenolic acids, applying ADM as reference model, through a very extensive testing of binary and ternary fixed-ratio mixtures. The performance of mixtures of phenolic acids has been the subject of previous studies; however, the joint action of mixtures of benzoxazinone derivatives and of benzoxazinone derivatives and phenolic acids has not been studied previously.

MATERIALS AND METHODS

Germination Test. The following compounds were included in the study: ferulic acid (FA), *p*-coumaric acid (CA), vanillic acid (VA), *p*-hydroxybenzoic acid (HBA), DIMBOA, MBOA, BOA, 2-aminophe-

Table 1. Phenolic Acids and Benzoxazinone Derivatives Used in the Joint Action Bioassays

Name	Acronym	Chemical formula
Ferulic acid	FA	
<i>p</i> -Coumaric acid	CA	
Vanillic acid	VA	
<i>p</i> -Hydroxybenzoic acid	HBA	
2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one	DIMBOA	
6-Methoxybenzoxazolin-2-one	MBOA	
Benzoxazolin-2-one	BOA	
2-Aminophenol	AP	
<i>N</i> -(2-hydroxyphenyl)acetamide	HPAA	

Table 2. Mixtures and Ratios of Mixtures Included in the Study

mixture	mixture ratios			
CA + FA	25:75	50:50	75:25	
VA + FA	25:75	50:50	75:25	
FA + HBA	20:80	50:50	75:25	
VA + HBA	20:80	45:55	70:30	
DIMBOA + MBOA	25:75	50:50	75:25	
MBOA + BOA	20:80	40:60	70:30	
BOA + AP	25:75	50:50	75:25	
BOA + HPAA	15:85	33:67	60:40	
DIMBOA + FA	15:85	30:70	60:40	
DIMBOA + VA	10:90	30:70	60:40	
MBOA + FA	15:85	35:65	65:35	
MBOA + VA	15:85	35:65	60:40	
BOA + FA	20:80	45:55	70:30	
BOA + VA	20:80	35:65	65:35	
DIMBOA + FA + VA	12:38:50 ^a	6:19:75 ^a	6:66:28 ^a	30:30:40 ^a
	5:50:45 ^b	3:27:70 ^b	3:75:22 ^b	15:45:50 ^b
MBOA + FA + VA	14:43:43	5:25:70	5:70:25	30:35:35

^a Mixture ratios for *L. perenne*. ^b Mixture ratios for *M. arvensis*.

nol (AP), and *N*-(2-hydroxyphenyl)acetamide (HPAA) (Table 1). DIMBOA was isolated from maize and provided by Dr. Francisco Macias, Universidad de Cádiz, Spain, whereas the other compounds were purchased commercially. Stock solutions of individual compounds and fixed-ratio binary and ternary mixtures of the compounds were prepared in methanol. Three and four ratios were examined for each binary and ternary mixture, respectively. The ratio of the compounds in the mixtures varied between mixtures depending on the relative activity of the compounds (Table 2). Two milliliters of a concentration range of each compound and mixture was added to the Petri dishes (9 cm) containing two pieces of Whatman no. 1 filter paper. The methanol was allowed to evaporate, and 4.5 mL of deionized water was subsequently added to each Petri dish. Controls received only deionized water as preliminary experiments had shown that the methanol

application had no influence on the germination and growth of the test species. No attempt was made to control the pH of the solutions, and the pH values were not recorded. The phenolic compounds are all weak acids with pK_a values of ~ 4.5 , whereas the benzoxazinone derivatives are nonionized compounds, and it was assumed that the minor variation in pH would have no impact on germination and root growth of the test species. The stability of the compounds was not examined. The phenolic acids were assumed to be relatively stable in contrast to the benzoxazinone derivatives that have been found to decompose very rapidly (35).

Fifteen seeds of the monocotyledonous species *L. perenne* L. (LOLPE) and of the dicotyledonous species *M. arvensis* L. (MYOAR) were placed in each Petri dish and then covered and sealed with Parafilm. Seeds were allowed to germinate in the dark at 22–25 °C. The day of germination and the radicle length were recorded for each seed after 6 days. All treatments were replicated three times. Most seeds germinated on the same day, and the germination rate was generally very high (> 12 seeds). A comparison of germination rates did not reveal significant differences between treatments, and because lack of germination was not related to the treatments, nongerminated seeds were omitted from the statistical analyses of root growth. Root length of untreated *L. perenne* and *M. arvensis* varied between experiments from 30–40 mm to 25–30 mm, respectively.

Dose–Response Models. The response of biomass (U) on concentration (z) was described by a log–logistic four-parameter model (36)

$$U_{ij} = C + \frac{D - C}{1 + \exp[2b_i(\log(EC_{50i})) - \log(z_{ij})]} \quad (1)$$

where U_{ij} denotes biomass at the j th dose of the i th allelochemical preparation (z_{ij}), D and C denote the upper and lower asymptotes of biomass at zero and at infinite concentrations and were assumed to be similar for all dose–response curves within an experiment, EC_{50i} denotes the dose required of allelochemical i to reduce biomass by half between D and C , and b_i is proportional to the slope of the curve around EC_{50i} . By reparametrizing eq 1, the EC_{50} parameter can be replaced by any EC parameter, for example, EC_{90} :

$$U_{ij} = C + \frac{D - C}{1 + \exp[2b_i(\log(EC_{90i})) + 1.099/b_i - \log(z_{ij})]} \quad (2)$$

Within an experiment the nonlinear regression models were fitted simultaneously to the dose–response curves for the herbicides applied alone and the binary mixtures. A transform-both-sides method was applied to stabilize the variance (37). For each of the four parameters the nonlinear regression procedure produced an $\sim 95\%$ confidence interval. A test for lack-of-fit and graphical analyses of the distribution of residuals was used to test the assumption that the response of the herbicides and herbicide mixtures could be described by eqs 1 and 2 (36).

Joint-Action Model. Assuming that Z_A and Z_B are the doses of allelochemicals A and B producing, for example, a 50% effect, that is, the EC_{50} doses when applied singly, and z_A and z_B are the doses of A and B in a mixture producing the same biological response, the relative potency between the allelochemicals is

$$R = \frac{Z_A}{Z_B} \quad (3)$$

and the isobole defining additivity according to ADM can be described at any response level as

$$\frac{z_A}{Z_A} + \frac{z_B}{Z_B} = 1 \quad (4)$$

The relative potency between herbicides A and B expresses the “biological” exchange rate between the compounds when applied separately (38).

The predicted EC_{50} dose of the mixtures according to ADM (EC_{50mix}) can easily be calculated on the basis of the EC_{50} values of the

Table 3. Estimated EC_{50} Values for the Benzoxazinone Derivatives and Phenolic Acids Included in the Joint Action Studies

compound	no. of experiments	EC_{50} (min–max values, mM)	
		<i>L. perenne</i>	<i>M. arvensis</i>
FA	8	1.28–3.67	0.78–1.82
CA	1	1.03	0.94
VA	7	2.73–4.89	1.10–1.98
HBA	2	2.91–4.03	1.82–2.37
DIMBOA	4	0.93–1.44	0.08–0.11
MBOA	5	0.85–1.35	0.46–1.00
BOA	5	0.72–1.84	0.34–1.08
AP	1	0.22	0.12
HPAA	1	2.23	0.93

compounds applied singly and their ratio in the mixtures:

$$EC_{50mix} = \frac{EC_{50A}}{[\alpha + (1 - \alpha)R]} \quad (5)$$

where EC_{50A} is the EC_{50} of A, α is the ratio of A in the mixture, and R is the relative potency as defined in eq 3.

The ADM graphs were constructed by plotting the EC_{50} or EC_{90} values of each compound of the binary mixture on the x - and y -axes and drawing a straight line between them. The straight line represents the ADM isoboles of predicted responses, that is, the concentration of any mixture required to produce the same EC_{50} or EC_{90} response. To accommodate the results at EC_{50} and EC_{90} in the same plot, the x - and y -axes were standardized so that the EC_{50} or EC_{90} values of the allelochemicals applied separately were always fixed to 1. The mixture ratios of the compounds were selected so that the responses were evenly distributed along the isobole. The observed EC_{50} and EC_{90} values were plotted on the graph and compared to the ADM isoboles. Points above the isoboles indicate that the joint action of a mixture is lower than predicted by ADM, whereas points below the isoboles indicate a joint action higher than predicted by ADM. The concept of ADM can be extended to mixtures containing more than two compounds, and in this study we also examined ternary mixtures, for which ADM is defined by a response surface and not an isobole.

Significant deviations from ADM were identified by examining whether the predicted EC_{50} or EC_{90} values of the mixtures, calculated by using eq 5, were contained in the 95% confidence interval of the estimated EC_{50} or EC_{90} values derived from eqs 1 and 2. Significant deviations were termed antagonism if higher and synergism if lower than the corresponding estimated EC_{50} and EC_{90} values.

RESULTS

Phytotoxicity of Individual Compounds. In general, relatively minor differences were observed in the phytotoxicity of the examined compounds (Table 3). The EC_{50} values of the compounds on *L. perenne* ranged from around 1 to 4 mM with some variations between experiments. *M. arvensis* was more susceptible than *L. perenne*, and the differences in the inherent activity of the compounds were more pronounced, with EC_{50} values varying from 0.1 to 2 mM. On both plant species the benzoxazinone derivatives tended to be more phytotoxic than the phenolic acids.

Binary Mixtures of Phenolic Acids. Mixtures of CA and FA were not significantly different from ADM on *M. arvensis*, whereas an antagonistic interaction was observed with one and two mixtures at the ED_{50} and ED_{90} response levels on *L. perenne* (Figure 1A). In contrast, antagonistic, synergistic, and additive effects were found with mixtures of VA and FA with synergistic interactions being more frequent on *M. arvensis* than on *L. perenne* (Figure 1B). With mixtures of FA or VA with HBA more distinct differences were observed between the two plant

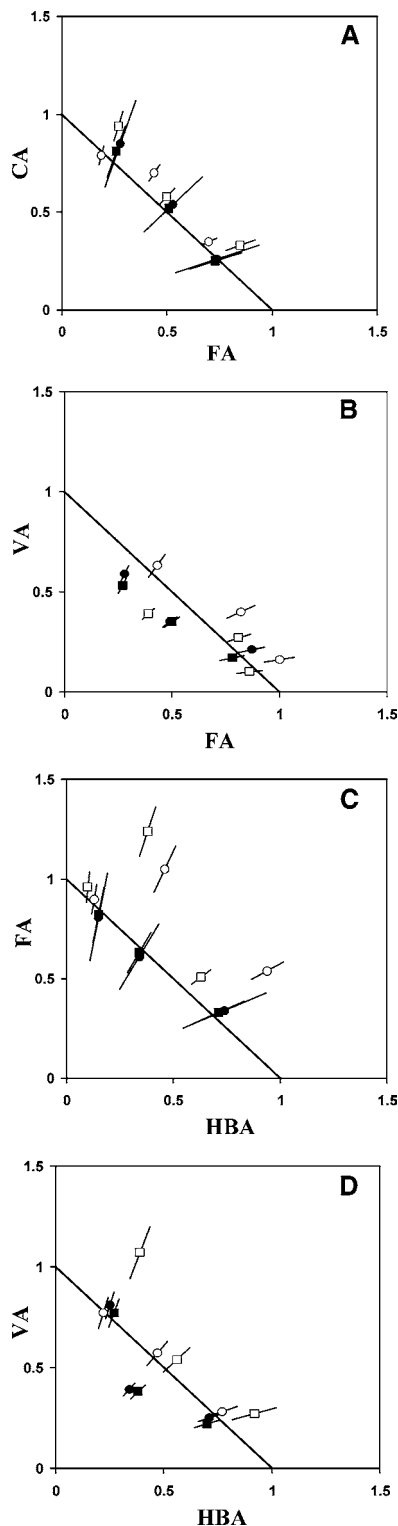


Figure 1. Isoboles and data for fixed-ratio binary mixtures of (A) CA + FA, (B) VA + FA, (C) FA + HBA, and (D) VA + HBA for *L. perenne* at the EC₅₀ (○) and EC₉₀ (□) response levels and for *M. arvensis* at the EC₅₀ (●) and EC₉₀ (■) response levels. Bars indicate 95% confidence intervals for the estimated EC₅₀ and EC₉₀ values. Doses were scaled so that the doses of the compounds applied individually were 1.0.

species. On *L. perenne* responses were either additive or antagonistic, whereas on *M. arvensis* additive or synergistic interactions were found (Figure 1C,D).

Binary Mixtures of Benzoxazinone Derivatives. Mixtures of DIMBOA and MBOA were mostly antagonistic (four of six observations for both plant species) or followed ADM (Figure

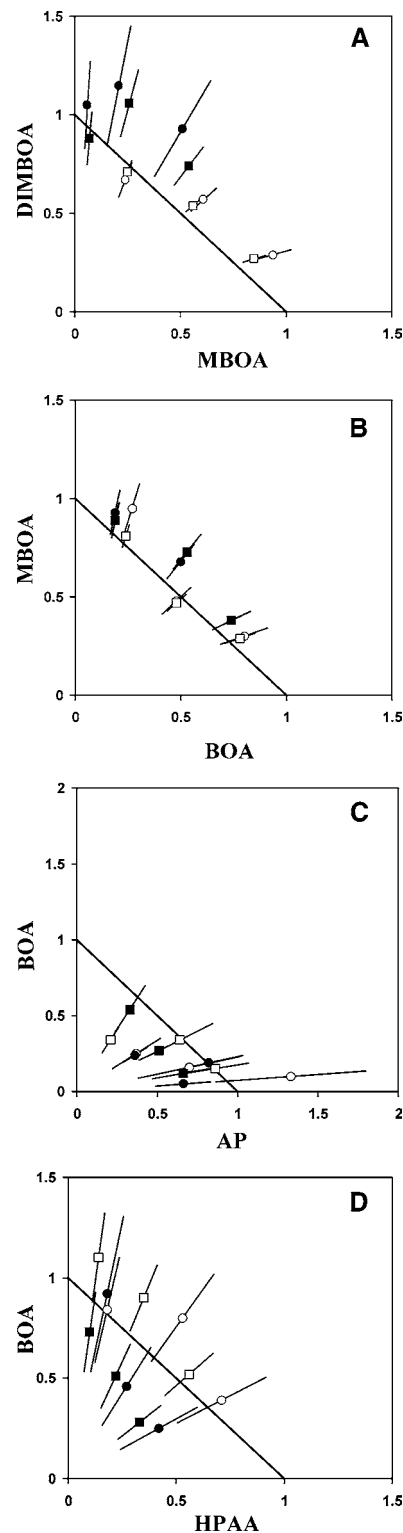


Figure 2. Isoboles and data for fixed-ratio binary mixtures of (A) DIMBOA + MBOA, (B) MBOA + BOA, (C) BOA + AP, and (D) BOA + HPAA for *L. perenne* at the EC₅₀ (○) and EC₉₀ (□) response levels and for *M. arvensis* at the EC₅₀ (●) and EC₉₀ (■) response levels. Bars indicate 95% confidence intervals for the estimated EC₅₀ and EC₉₀ values. Doses were scaled so that the doses of the compounds applied individually were 1.0.

2A). Mixtures MBOA and BOA tended to respond antagonistically, and no differences were observed between the two plant species (Figure 2B). Mixtures of BOA and AP tended to produce more than additive effects, but due to a high variation in this experiment, very few significant deviations from the

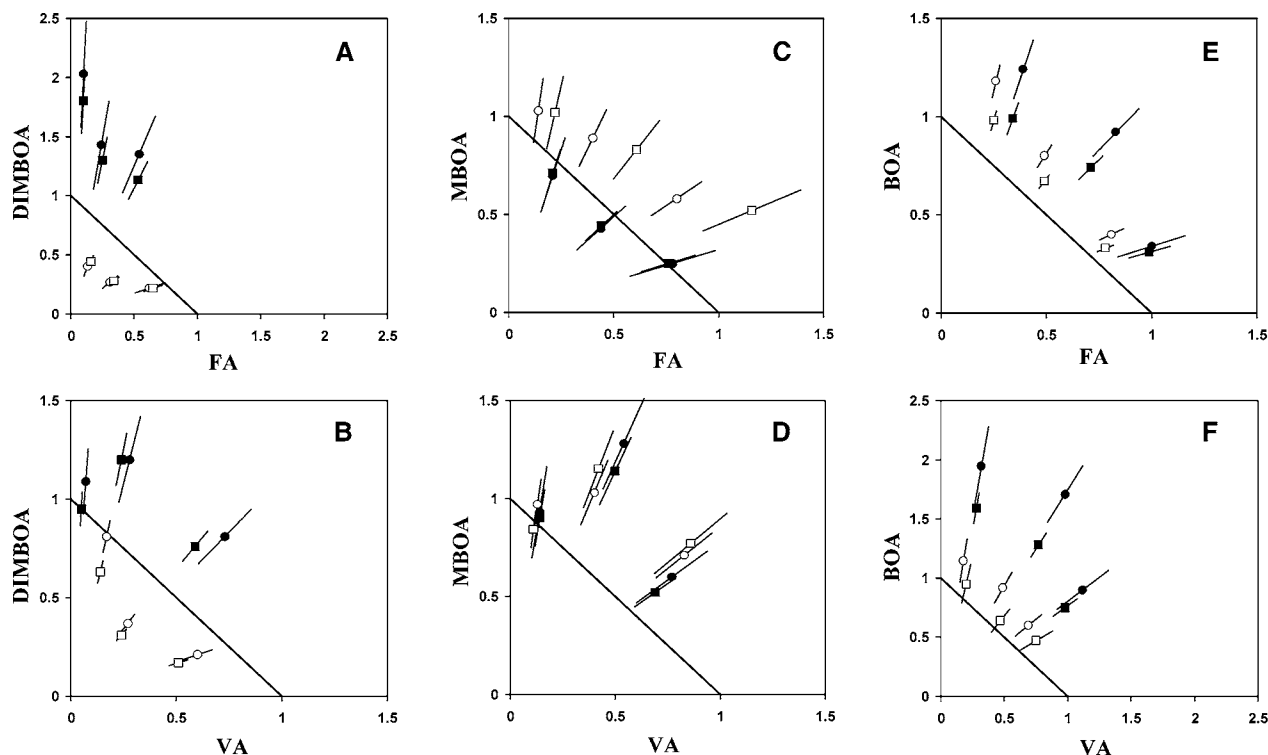


Figure 3. Isoboles and data for fixed-ratio binary mixtures of (A) DIMBOA + FA, (B) DIMBOA + VA, (C) MBOA + FA, (D) MBOA + VA, (E) BOA + FA, and (F) BOA + VA for *L. perenne* at the EC₅₀ (○) and EC₉₀ (□) response levels and for *M. arvensis* at the EC₅₀ (●) and EC₉₀ (■) response levels. Bars indicate 95% confidence intervals for the estimated EC₅₀ and EC₉₀ values. Doses were scaled so that the doses of the compounds applied individually were 1.0.

isobole were found (**Figure 2C**). More than additive responses were also observed for mixtures of BOA and HPAA on *M. arvensis*, but only three of six observations deviated significantly from the isobole. In contrast, *L. perenne* responded additively (**Figure 2D**).

Binary Mixtures of Benzoxazinone Derivatives and Phenolic Acids. Mixtures of DIMBOA and the phenolic acids FA and VA performed synergistically on *L. perenne* (**Figure 3A**), whereas a very pronounced antagonistic response was recorded on *M. arvensis* (**Figure 3B**). In contrast, mixtures of MBOA and FA responded antagonistically on *L. perenne* and performed additively on *M. arvensis* (**Figure 3C**). Mixtures of MBOA and VA, BOA, and FA as well as BOA and VA responded antagonistically on both plant species with only a few mixtures not deviating significantly from additivity (**Figure 3D–F**).

Ternary Mixtures of Benzoxazinone Derivatives and Phenolic Acids. Two ternary mixtures, DIMBOA + FA + VA and MBOA + FA + VA, were studied. The mixture of DIMBOA and the two phenolic acids was highly antagonistic with six and seven of the eight observations deviating significantly from additivity on *L. perenne* and *M. arvensis*, respectively (**Figure 4A,B**). With the ternary mixture containing MBOA, only two of the eight observations deviated from additivity on *L. perenne* (**Figure 5A**). On *M. arvensis* the MBOA ternary mixture apparently performed more antagonistically than on *L. perenne*; however, due to a larger variability in this experiment, again only two of the eight observations deviated significantly from the ADM response surface (**Figure 5B**).

DISCUSSION

Phytotoxicity of Individual Compounds. Among the phenolic acids only minor differences were observed in the activity

on *L. perenne* and *M. arvensis* (**Table 3**). Previous studies on the effects of phenolic acids on root growth have shown variable results. Einhellig and Rasmussen (24) and Rasmussen and Einhellig (25), using sorghum as test plant, found no pronounced differences in the activity of VA and HBA or of FA, CA, and VA, respectively. In contrast, Einhellig et al. (26) found a significantly better effect of CA than of FA on sorghum. Reigosa et al. (22) compared the activities of six phenolic compounds including CA, FA, VA, and HBA on six weed species and found no significant differences in the effects on root growth.

Only minor differences were observed between DIMBOA, MBOA, and BOA on *L. perenne*, whereas DIMBOA was significantly more effective on *M. arvensis* than MBOA and BOA (**Table 3**). DIMBOA is hydrolyzed very quickly to MBOA in aqueous solutions, with a half-life of <1 day (35); hence, in standard Petri dish tests lasting 6 days DIMBOA will have been transformed to MBOA and likely other metabolites during the test, if done under nonsterilized conditions (18, 19, 39, 40). It therefore seems reasonable to assume that the activities of DIMBOA and MBOA would be comparable; however, this was the case only on *L. perenne*. Macías et al. (41, 42) also reported higher activity of DIMBOA than of MBOA and BOA on *Allium cepa*, *Lepidium sativum*, *Lycopersicon esculentum*, *Lolium rigidum*, and *Avena fatua*, whereas no differences were observed on wheat. Mathiassen et al. (43) found a significantly higher activity of DIMBOA compared with MBOA and BOA on *Apera spica-venti* and *Amaranthus retroflexus* but not on *Echinochloa crus-galli* and *Setaria viridis*. The results of the present as well as previous studies have revealed that plant species do respond differently to DIMBOA, MBOA, and BOA and that the transformation of the parent compound DIMBOA to MBOA did not always result in a loss of activity.

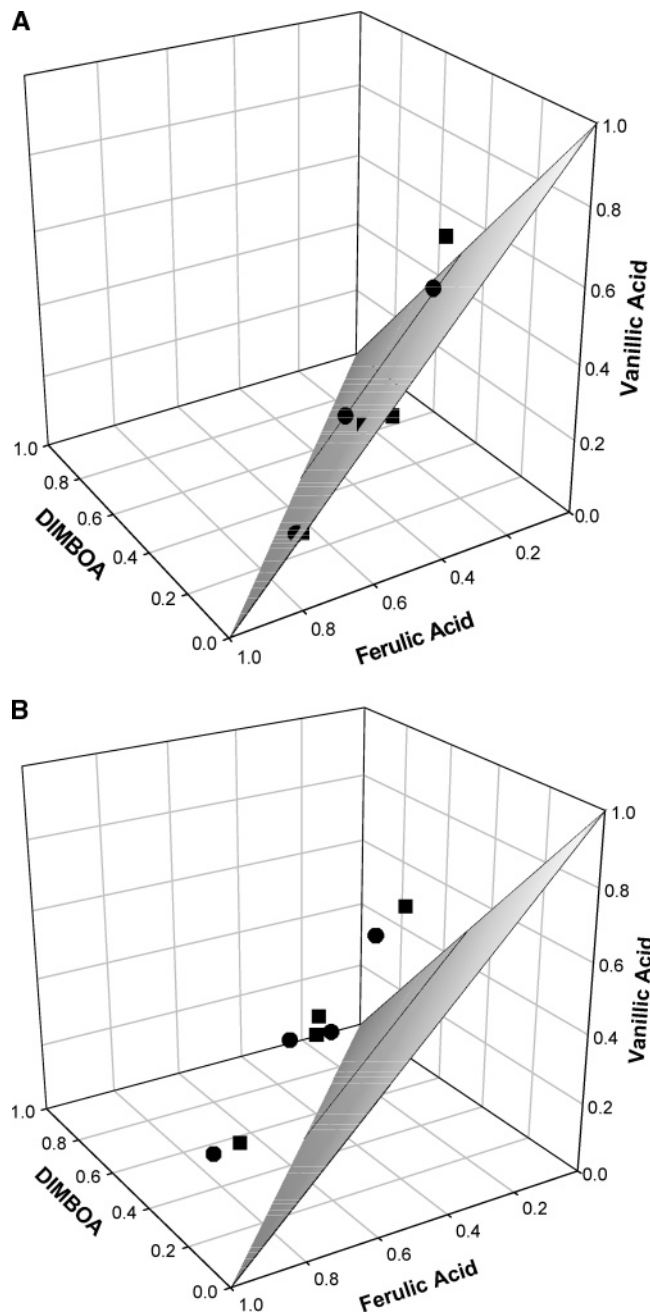


Figure 4. ADM response surface and data for fixed-ratio ternary mixtures of DIMBOA + FA + VA on (A) *L. perenne* and (B) *M. arvensis* at the EC_{50} (●) and EC_{90} (■) response levels. Doses were scaled so that the doses of the compounds applied individually were 1.0.

AP and HPAA are metabolites of BOA, and Macías et al. (41, 42) found a generally lower activity of AP than of BOA. This conclusion is not supported by the results from either this study or a study on *E. grus-calli*, *Poa annua*, *Matricaria tripleurospermum*, and *A. retroflexus* (unpublished data), suggesting that the response to BOA and AP seems to be plant-specific.

Binary Mixtures. Binary mixtures of phenolic acids responded variably depending on the composition of the mixture and the plant species (Figure 1A–D). FA and CA are classified as cinnamic acid derivatives, whereas VA and HBA are benzoic derivatives (33). Hence, the mixtures CA + FA and VA + HBA consisted of structurally more similar compounds than the mixtures VA + FA and FA + HBA, but there were no obvious differences between the two groupings of binary mixtures. A

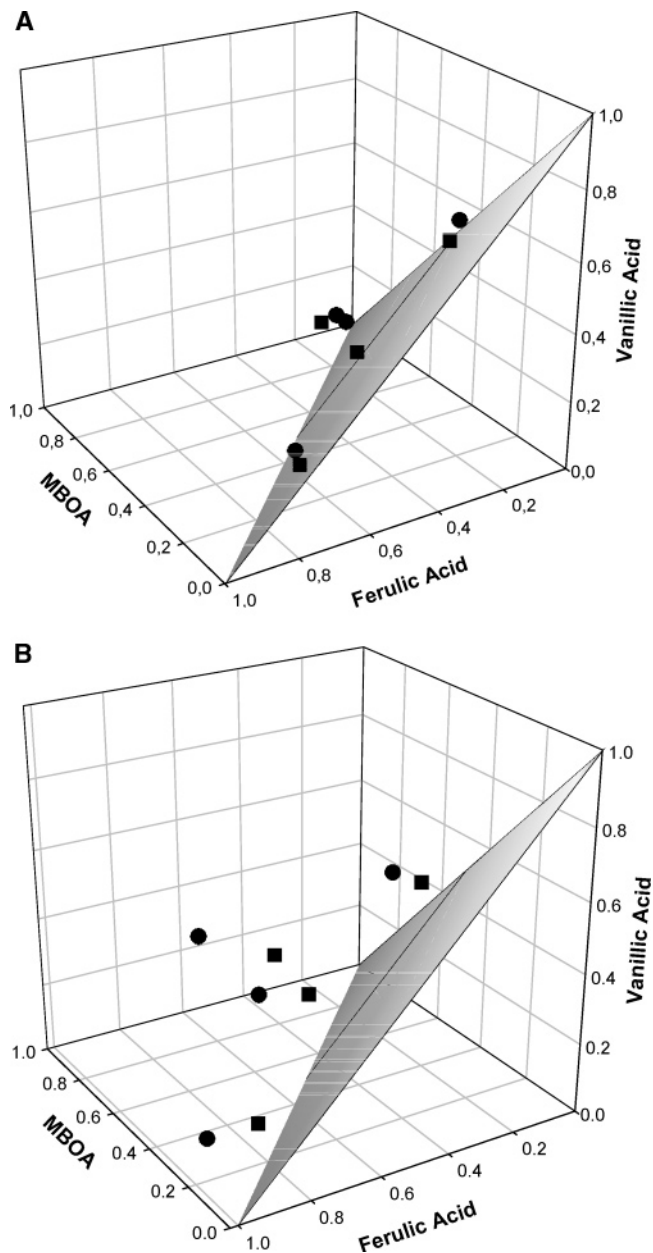


Figure 5. ADM response surface and data for fixed-ratio ternary mixtures of MBOA + FA + VA on (A) *L. perenne* and (B) *M. arvensis* at the EC_{50} (●) and EC_{90} (■) response levels. Doses were scaled so that the doses of the compounds applied individually were 1.0.

total of 48 observations are shown in Figure 1, and of these 11 responded antagonistically, 6 responded synergistically, and the remaining 31 did not deviate significantly from ADM. It should be noted, however, that all 11 antagonistic responses were observed on *L. perenne*, whereas 5 of 6 synergistic responses occurred on *M. arvensis*. Although additivity was the most commonly observed response and for both plant species, deviations from additivity were very different for the two plant species, emphasizing that generalizations concerning the joint action of phytotoxins should preferably be based on more than one plant species.

Einhellig and Rasmussen (24) and Einhellig et al. (26) reported synergistic effects on root growth of sorghum by studying mixtures of two, three, or four phenolic acids. Einhellig and Rasmussen (24) did not relate their results to a joint action reference model, whereas Einhellig et al. (26) used Colby's method (29). Reigosa et al. (22) concluded that they had found

evidence of additivity by comparing the activity of equimolar mixtures of six phenolic acids with the phenolic acids applied individually at the same dose as the mixture to six weed species. Similar to the findings of Einhellig and Rasmussen (24), no more than a fixed mixture had been used; hence, no general joint action model was tested. Inderjit et al. (23) studied binary mixtures of FA, CA, and HBA on *L. perenne* using ADM and concluded that antagonism was the most common response, although additivity and synergism were also observed. The results of Inderjit et al. (23) agreed with our results on *L. perenne*, for which antagonism and additivity were the most commonly observed responses.

Inderjit et al. (23) hypothesized that the joint action of phenolic compounds may depend on the concentration of the compounds in the mixture and that the mixture may perform additively at low concentrations and antagonistically at high concentrations. In the present study *M. arvensis* was more susceptible to all four phenolic acids than *L. perenne*. Hence, the different responses of the mixtures on the two plant species may reflect the fact that lower concentrations of the phenolic acids were used on *M. arvensis* than on *L. perenne*, rather than the existence of species-specific responses to binary mixtures.

Mixtures of benzoxazinone derivatives responded somewhat differently. Whereas DIMBOA + MBOA and MBOA + BOA responded primarily antagonistically, mixtures of BOA and the two BOA metabolites AP and HPAA responded predominantly additively, although synergistic responses were observed for BOA + HPAA on *M. arvensis*. We cannot provide an explanation for the differential responses of the four binary mixtures, but whereas the former two mixtures consisted of compounds with relatively similar structures, AP and HPAA are structurally very different from BOA, belonging to the chemical groups of aminophenols and acetamides (18). The joint action of hydroxamic acids and their metabolites has not been studied previously.

The results with mixtures of the benzoxazinone derivatives DIMBOA, MBOA, and BOA and the phenolic acids FA and VA supported antagonism as the most common response, although exceptions were found. Mixtures of DIMBOA and the phenolic acids performed synergistically on *L. perenne*, although the responses of the mixtures were clearly antagonistic on *M. arvensis*. In contrast, mixtures of MBOA and FA performed additively on *M. arvensis*, despite a clear antagonistic performance on *L. perenne*. A ternary mixture of DIMBOA, FA, and VA also performed antagonistically, and although the statistical analyses did not reveal any differences when the predicted EC₅₀ and EC₉₀ values were compared with the corresponding estimated values, a visual assessment of the results clearly indicated that antagonism was more pronounced on *M. arvensis*, which supported the findings with binary mixtures of DIMBOA and the two phenolic acids. Additivity rather than antagonism was the most commonly observed response with mixtures of MBOA, FA, and VA. However, similar to the DIMBOA three-way mixture, the response on *M. arvensis* tended to be more antagonistic than that on *L. perenne*, but this conclusion was not supported by the statistical analyses due to a higher variability in the *M. arvensis* experiment.

Lehmann and Blum (44) and Blum et al. (33) found that root contact, not uptake, was responsible for the inhibitory effects of phenolic acids. This can explain why the inhibitory effect of phenolic acids ceased and plants recovered when the phenolic acids were removed from the growth medium (45) and supports the hypothesis put forward by Einhellig (32) that the mode of action of phenolic acids was closely related to membrane

permeability and ion flux. Several authors have found good correlations between the content of allelochemicals in root exudates and the allelopathic potential of wheat varieties (21, 46, 47). Huang et al. (21) found that the content of seven phenolic acids and DIMBOA and DIBOA in the growth medium originating from experiments in which wheat seedlings were allowed to grow for 2–15 days could explain 91% of the variation in the growth inhibition of *L. rigidum* seeded immediately after the harvest of the wheat seedlings. Nevertheless, an artificial mixture mimicking the content of benzoxazinone derivatives and phenolic acids found in the growth medium from 6-day-old wheat seedlings, the time when the content of benzoxazinone derivatives was at a maximum, had no effect on *L. rigidum* root growth in a Petri dish assay, although the agar-based assay revealed an 85% effect on root growth. Huang et al. (21) speculated that the effect of phenolic acids on membrane permeability could open the way for an increased uptake of the more phytotoxic benzoxazinone derivatives such as DIMBOA and DIBOA and that this may lead to synergistic effects. Our study, providing information for the first time on the joint action of benzoxazinone derivatives and phenolic acids, does not support this hypothesis. On the contrary, additivity and antagonism were the predominant responses for mixtures of the two groups of putative wheat allelochemicals. Furthermore, the recent findings by Reigosa et al. (34) that the mode of action of BOA apparently was very similar to that of the phenolic acids and that plants, as reported for phenolic acids, recovered when BOA was not applied continuously also raise uncertainty about the hypothesis of Huang et al. (21).

It is widely accepted that allelopathic activity nearly always is due to the simultaneous presence of several compounds, but, as demonstrated by Huang et al. (21), even when the effects of the allelopathic compounds identified in the growth medium are added, they could not account for the observed allelopathic effects of wheat. The fact that allelopathy could be explained by adding the activities of the individual allelochemicals has led to the widely accepted hypothesis of wheat allelochemicals acting synergistically. Our results do not support this hypothesis, at least not for the two most abundant groups of putative wheat allelochemicals, the benzoxazinone derivatives and the phenolic acids. Hence, other, yet unidentified, compounds are probably involved in wheat allelopathy either through their inherent phytotoxicity or as synergists. These could be compounds belonging to other chemical groups, for example, flavonoids and coumarins, or these could be metabolites of phenolic acids or benzoxazinone derivatives. It has been shown that some metabolites of hydroxamic acids are not only more phytotoxic than the parent compound but also less prone to degradation by soil microorganisms, this being most evident for 2-amino-3H-phenoxazine-3-one, a degradation product of BOA, which was 5–50 times more potent than the parent compound (41, 42). It could therefore be suggested that more focus should be devoted to the potential role of the degradation products of the compounds already identified as being involved in wheat allelopathy.

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of January 24, 2006, contained incorrect equation numbering. These numbers have been corrected both for the individual equations and as cited in the text with the posting of February 2, 2006.

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