JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Benzoxazinoid Allelochemicals in Wheat: Distribution among Foliage, Roots, and Seeds

MARTA VILLAGRASA,† MIRIAM GUILLAMÓN,† ANA LABANDEIRA,† ANDREU TABERNER,§ ETHEL ELJARRAT,*,† AND DAMIÀ BARCELÓ†

Department of Environmental Chemistry, IIQAB, CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain, and Servicio de Sanidad Vegetal, Malherbología, University of Lleida, c/Rovira Roure 191, 25198 Lleida, Spain

In this study, the distribution of eight allelochemicals among the foliage, roots, and seeds of different wheat varieties is reported for two different sampling campaigns, corresponding to two consecutive years. The determination of benzoxazinoid derivatives was performed by combining pressurized liquid extraction−solid-phase extraction followed by liquid chromatography−electrospray mass spectrometry. To the authors' knowledge, there are no previous works about the content of allelochemicals in seed tissue of germinated wheat seedlings. Allelochemicals found in seeds were detected at levels similar to those found in foliage and roots. The results showed that the type of metabolites detected depends strongly on the working up procedure of the plant material, as well as of plant growth stage. A general decrease of the total amount of allelochemical content in the plants was observed with plant age. There was a significant difference in the total amount of benzoxazinoid derivatives in the different wheat varieties analyzed.

KEYWORDS: Allelochemicals; hydroxamic acids; wheat varieties; foliage; roots; seeds

INTRODUCTION

Plants produce a wide variety of secondary metabolites that play important roles in complex interactions among living organisms in the natural environment. Allelopathy has been defined as "any process involving secondary metabolites produced by plants, microorganisms, viruses, and fungi that influence the growth and development of agricultural and biological systems (excluding animals), including positive and negatives effects" (*1*). The term allelopathy has mostly been used by plant ecologists who have focused on allelopathic effects between agricultural crops and weeds. There is an enormous diversity of allelochemicals in nature. About 10 000 compounds are known, whereas another 400 000 are estimated to exist (*2*). Poaceae such as wheat, rye, and maize contain secondary metabolites at different concentrations, depending on varieties and environmental conditions. One group of allelochemicals that has been heavily in focus during the past decade is the benzoxazinoid derivatives. The group of chemical compounds named benzoxazinoid derivatives is subdivided into hydroxamic acids (Hx), lactams, benzoxazolinones, and methyl derivatives of the hydroxamic acids. Benzoxazinoids and particularly their aglycone forms have been reported to be involved in the defense of the plant against a wide variety of organisms, including fungi, bacteria, and a range of insects. The concentration of these compounds in plants is influenced by a range of factors, such as light intensity (*3*), temperature (*4*), and water availability (*5*). The latter authors suggested that these compounds could be stress metabolites.

The cyclic Hx and lactams are naturally present in the seedling of several Poaceae as 2-*O*-β-D-glucosides. The glucosides are enzymatically converted into aglycone forms by the action of β-glucosidases after the plant tissue is crushed (*6*). Aglycones are unstable and are easily converted to benzoxazolinones. The main structures are shown in **Figure 1**. Previous studies showed that maximal concentrations of hydroxamic acids in roots and aerial parts of cereal seedlings occurred between 4 and 6 days after germination (*7*, *8*). Despite the subsequent decline in concentrations of Hx, the total amount within the whole seedling continued to increase, albeit at a slower rate. This increase continued up to 8 days after germination (*9*). Hx has not been detected on nongerminated seeds (*9*−*12*). Copaja et al. (*13*) analyzed germinated seeds of wheat and detected no benzoxazinoids. Several authors present the levels of some allelochemicals in wheat seedlings. The concentration of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) found in the shoots of wheat seedlings ranged from 1.4 to 10.9 mmol/kg of fresh weight in a collection of Chilean cultivars (*8*), from 0.99 to 8.07 mmol/kg of fresh weight in a worldwide collection (*7*), and from 0.21 to 16.0 mmol/kg of fresh weight in a collection of wheat progenitors (*14*).

The aim of our study was to determine the distribution of the eight allelochemicals [2-β-D-glucopyranosyloxy-4-hydroxy-

* Corresponding author (telephone +34 93 400 6100; fax +34 93 204 59 04; e-mail eeeqam@cid.csic.es).
† IIQAB, CSIC.
§ University of Lleida.

10.1021/jf050898h CCC: $33.50
Published on Web 01/24/2006

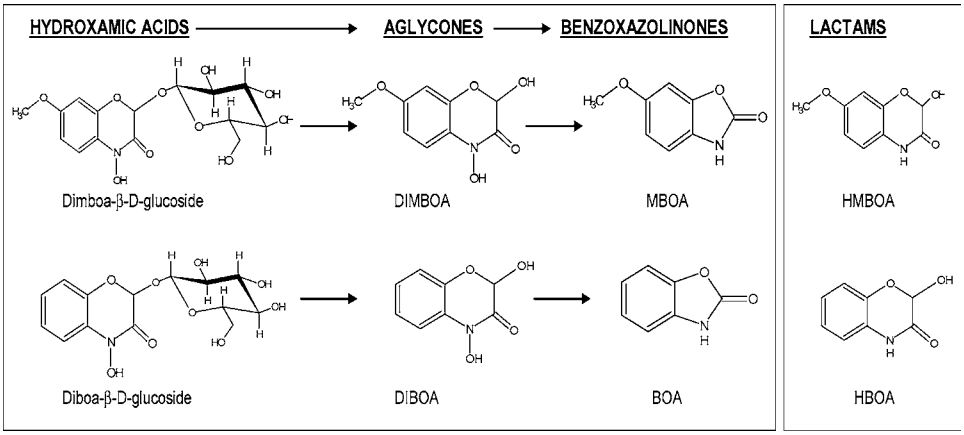

Figure 1. Structures of main benzoxazinoid derivatives.

1,4-benzoxazin-3-one (DIBOA-β-D-glucoside), 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-β-D-glucoside), 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), DIMBOA, 2-hydroxy-1,4-benzoxazin-3-one (HBOA), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA), benzoxazolin-2-one (BOA), and 6-methoxybenzoxazolin-2-one (MBOA)]among the foliage, root, and seed of different wheat seedlings cultivated under different cultivation conditions. To our knowledge, there are no previous works about the content of allelochemicals in seed tissue of germinated wheat seedlings.

MATERIALS AND METHODS

Chemicals and Materials. The benzoxazinoid standards were obtained from commercial and private sources as available: DIBOA-β-D-glucoside and DIMBOA-β-D-glucoside, from Prof. Dr. Hajime Iwamura (Kyoto University), Prof. Dr. Lisbeth Jonsson (Södertörn University College), and Dr. F. Macías (University of Cadiz); DIMBOA from Dr. Scott Chilton, University of North Carolina; HBOA, HMBOA, DIMBOA, MBOA, and BOA, from Dr. F. Macías (University of Cadiz); DIBOA and the non-naturally occurring synthetic derivative 2-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (2-MeO-HBOA) from Dr. Sicker (University of Leipzig).

HPLC-grade solvents water (H_2O), methanol (MeOH), and 98% pure acetic acid (HOAc) were purchased from Merck (Darmstadt, Germany). Diatomaceous earth was obtained from Varian Inc. LiChrolut RP C_{18} (500 mg) solid-phase extraction (SPE) cartridges were purchased from Merck.

Sample Collection. The distribution of the allelochemicals among foliage, roots, and seeds was analyzed in two different sampling campaigns, corresponding to two consecutive years. The wheat plants were grown in Lleida (Spain). In the first sampling campaign (autumn 2001), four different wheat varieties, Astron (As), Ritmo (Ri), Bill (Bi), and Solist (So), were grown in conventional cultivation conditions; the samples were collected and were then frozen in the laboratory and stored at −20 °C until further manipulation. In the second sampling campaign (autumn 2002), three wheat varieties, As, Ri, and Stakado (Sa), were grown in conventional and organic cultivation. Immediately after the harvest, the samples were frozen and stored at −20 °C. In each sampling campaign, 10 plants of each wheat seedling were collected at the Zadocks stages 10 and 12 days, corresponding to two different stages. The stages were defined by BBCH scale (a system for uniform coding of phenologically similar growth stages of all mono- and dicotyledonous plant species).

Sample Preparation Procedures. The water in wheat samples was removed by lyophilization until weight loss was no longer observed. The samples were divided into foliage, roots, and seeds; roots and seeds were cut finely, whereas foliage was ground with a pestle in a mortar. Pooled samples were obtained by combining the 10 plants collected at each Zadocks stages. A 0.1 g subsample was extracted by pressurized liquid extraction (PLE) using an ASE 200 (Dionex, Idstein, Germany) apparatus, equipped with 11 mL stainless steel extraction cells. Diatomaceous earth was used to fill the extraction cells, with the matrix and sample thoroughly mixed to ensure good dispersion of the sample. The diatomaceous earth was cleaned by ultrasonication with the same solvent of the extraction and dried at 70 °C prior to use. Conditions of pressure (1500 psi), static times (3), cell purge (60 s), flush volume (60%), solvent composition [100% acidified MeOH (1% HOAc)], and temperature (150 °C) were used. A purification of the samples prior to instrumental analysis is recommended because in the raw extracts of plants the broad variety of substances (salts, lipids, glycosides, phosphates, peptides, macromolecules, and chlorophyll) can influence the quantification. The organic extracts were concentrated to dryness by rotary evaporation and redissolved in 2 mL of acidified water (1% HOAc) prior to the cleanup step. The reconstituted extracts in acidified H_2O resulted in a turbid solution; a filtration step prior to purification was therefore required to prevent clogging of the cartridge. Filtration was performed using 1 μm 25 mm syringe-driven filter units. Purification was performed via LiChrolut RP C_{18} SPE cartridges activated and preconditioned with 5 mL of acidified MeOH (1% HOAc) followed by 5 mL of acidified H_2O (1% HOAc). The concentrated and filtered extracts were then applied to the purification step. A two-step elution procedure was used: first, with 6 mL of acidified H_2O (1% HOAc) and second with 5 mL of acidified MeOH/H_2O (1% HOAc) (60:40). Benzoxazinoid derivatives were well recovered in the second fraction (52−99%), with the exception of the most polar compounds (DIBOA-β-D-glucoside, HBOA, and DIBOA), which eluted in the first fraction (56−69%) (*15*). Both fractions were then analyzed by LC-ESI-MS.

Chromatographic Conditions. Analyses were performed on a HP 1100 LC-MS. A Synergi Max-RP 80A (C-12 TMS) LC column (250 × 4.6 mm Phenomenex) with a solvent flow rate of 1 mL/min was used. The sample injection volume was set at 50 μL. Acidified H_2O (0.05% HOAc) and MeOH were used as the elution solvents A and B, respectively. The solvent gradient adopted was as follows: 0−2 min, 100−70% A; 2−19 min, 70−40% A; 19−21 min, 40−5% A; 21−23 min, 5−5% A; 23−25 min, 5−70% A; 25−30 min, 70−100% A. Total run time was 35 min with the benzoxazinoid derivatives eluted over 8−20 min, and the final 15 min was used for column cleaning and regeneration. Detection was carried out by a UV−visible detector using a HP 1040 M diode array detector over the range of 190−500 nm (*15*).

Mass Spectrometry Conditions. MS analyses were carried out in selected ion monitoring (SIM) mode. The LC-MSD HP 1100 mass selective detector equipped with an atmospheric pressure ionization source was used with electrospray interface. The ESIMS was operated in negative ion mode with the following instrument settings: nebulizer pressure, 5 V; gas temperature, 350 °C; capillary voltage, 3500 V; fragmentor, 0−15 min, 250 V, and 15−35 min, 70 V (*15*).

Quantification. In this study, internal calibration was used for quantification. The use of internal standards has not been described for the quantification of benzoxazinoids. The only quantitative method describing the use of an internal standard for the analysis of these compounds used the naturally occurring degradation product BOA as internal standard (*12*). As this product can potentially occur in samples,

Table 1. Levels (Micrograms per Gram of Dry Weight) of Benzoxazinoid Derivatives in Wheat Foliage, Roots, and Seeds: Total Allelochemical Content in Each Tissue of Wheat Seedlings of the First Sampling Campaign[a]

		DIBOA-Glc	DIMBOA-Glc	HBOA	DIBOA	HMBOA	DIMBOA	BOA	MBOA	Σ tissue
First Stage, Conventional Cultivation										
Astron	foliage	<LOD	<LOD	<LOD	<LOD	4	<LOD	<LOD	28	32
	root	<LOD	<LOD	<LOD	<LOD	3	4	<LOD	90	97
	seed	<LOD	<LOD	<LOD	<LOD	7	<LOD	<LOD	62	69
Ritmo	foliage	<LOD	<LOD	<LOD	<LOD	13	2	<LOD	38	53
	root	<LOD	<LOD	<LOD	<LOD	12	0.5	0.7	117	130
	seed	<LOD	<LOD	<LOD	<LOD	3	<LOD	0.5	45	49
Bill	foliage	<LOD	<LOD	<LOD	<LOD	3	<LOD	<LOD	29	32
	root	<LOD	<LOD	<LOD	<LOD	4	0.6	<LOD	80	85
	seed	<LOD	<LOD	<LOD	<LOD	4	<LOD	0.2	46	50
Solist	foliage	<LOD	<LOD	<LOD	<LOD	19	19	<LOD	86	124
	root	<LOD	<LOD	<LOD	<LOD	17	4	<LOD	128	149
	seed	<LOD	<LOD	6	<LOD	15	38	<LOD	66	125
Second Stage, Conventional Cultivation										
Astron	foliage	<LOD	<LOD	0.5	<LOD	0.8	<LOD	<LOD	26	27
	root	<LOD	<LOD	<LOD	<LOD	0.5	0.7	0.6	57	59
	seed	<LOD	<LOD	<LOD	<LOD	2	<LOD	0.4	69	72

[a] <LOD, below limit of detection; <LQD, below limit of quantification.

it cannot be considered as an appropriate internal standard. In a previous study (*15*), different internal standards were tested: a non-naturally occurring structural analogue of HBOA (2-MeO-HBOA), a natural flavonoid (quercetin-3-*O*-rutinoside), and indoxyl-β-D-glucoside. A non-naturally occurring structural analogue of HBOA (2-MeO-HBOA) showed the best results and was used as internal standard for benzoxazinoid derivatives in the current study. Two ions for each analyte were selected according to specificity and sensitivity, with the primary ions used for quantification and the secondary ion providing confirmation. The ions selected for each analyte were the following: DIBOA-β-D-glucoside (134, 342); DIMBOA-β-D-glucoside (164, 372); HBOA (164, 108); DIBOA (134, 78); HMBOA (194, −); DIMBOA (164, 149); BOA (134, −); and MBOA (164, 149). The instrumental detection limits (LOD_{inst}) ranged between 0.010 and 0.002 ng/μL (*15*).

The applied methodology to plant material gave recoveries of the analytes between 66 and 110% with coefficients of variation ranging from 1 to 14%. The method detection limits (LOD) ranged between 1 and 27 μg/g of dry weight and gave limits of quantification (LQD) between 3 and 89 μg/g of dry weight (*15*).

RESULTS AND DISCUSSION

The benzoxazinoid derivatives were detected in foliage, roots, and also in seeds. None of the previous results presented in the bibliography (*10*−*13*) detected allelochemicals in germinated seeds.

First Sampling Campaign. Levels of benzoxazinoid derivatives are presented in **Table 1**. Five allelochemicals were detected, but only HMBOA and MBOA were present in all wheat extracts, whereas the glucoside derivatives and DIBOA were not detected in extracts. MBOA was the major metabolite detected in foliage, root, and seed extracts and present at the highest levels in root tissue with 128 μg/g of dry weight in So, at 117 μg/g of dry weight in Ri, at 90 μg/g of dry weight in As, and at 80 μg/g dry of weight in Bi. The total amount of allelochemicals in seed extracts was detected at a concentration similar to those found in foliage and root extracts. The variety with highest levels was So followed by Ri, As, and Bi. The total amount of allelochemicals found in the As seedling in the second stage of cultivation was lower than in the first one. **Figure 2a** shows the distribution of the total amount of allelochemicals (expressed as a percentage) in wheat seedlings. As can be observed, the total amount of the allelochemicals was distributed evenly among foliage, roots, and seeds in all cases. However, the percentage was slightly higher in root extracts. The percentages obtained for foliage, roots, and seeds ranged from 16 to 31%, from 49 to 56%, and from 21 to 35%, respectively, in wheat seedlings at the first stage of conventional cultivation.

Second Sampling. Table 2 presents the levels of individual allelochemicals as well the total amount of allelochemical content in each tissue. Whereas five allelochemicals were detected in the first sampling, all of the target compounds (DIBOA-β-D-glucoside, DIMBOA-β-D-glucoside, DIBOA, DIMBOA, HBOA, HMBOA, BOA, and MBOA) were detected in wheat seedlings of the second sampling. **Figure 3** shows the chromatogram of methoxy derivatives (DIMBOA-β-D-glucoside, DIMBOA, HMBOA, and MBOA) present in foliage, root, and seed extracts of the Ri variety.

The differences observed in the content of the allelochemicals between the first and second sampling campaigns were ascribed to the stability of the compounds. In a previous study (*16*), the stability of selected compounds was checked using different acidified standard solutions stored at different temperatures (−20, 4, and 20 °C). The trials showed that in order to stabilize the analytes, the solution must be stored at −20 °C, including the glucoside derivatives. For this reason, freezing the samples immediately after harvesting prevented the degradation of glucoside derivatives, and it was possible to detect the glucoside derivatives in sample extracts. A similar behavior was observed by Baumeler et al. (*17*).

Differences between benzoxazinoid concentrations in conventionally and organically grown wheat in this study are influenced by the soil types and cultivation history as well. Some correlations between allelochemicals content and cultivation characteristics were found as discussed below.

Conventional Cultivation. The major metabolites detected were DIMBOA, MBOA, HMBOA, and DIMBOA-β-D-glucoside. Nevertheless, MBOA was the only metabolite detected in all extracts. The detected compounds in foliage samples showed a different distribution between the first and second stages. In the first stage, DIMBOA presented the highest levels followed by MBOA and HMBOA, whereas DIMBOA-β-D-glucoside was not detected. In the second stage, HMBOA was the major metabolite present in the Ri and Sa varieties, and HBOA presented 54% of the contribution of allelochemicals in the As variety. The results obtained from root samples showed a different distribution of compounds among varieties and between

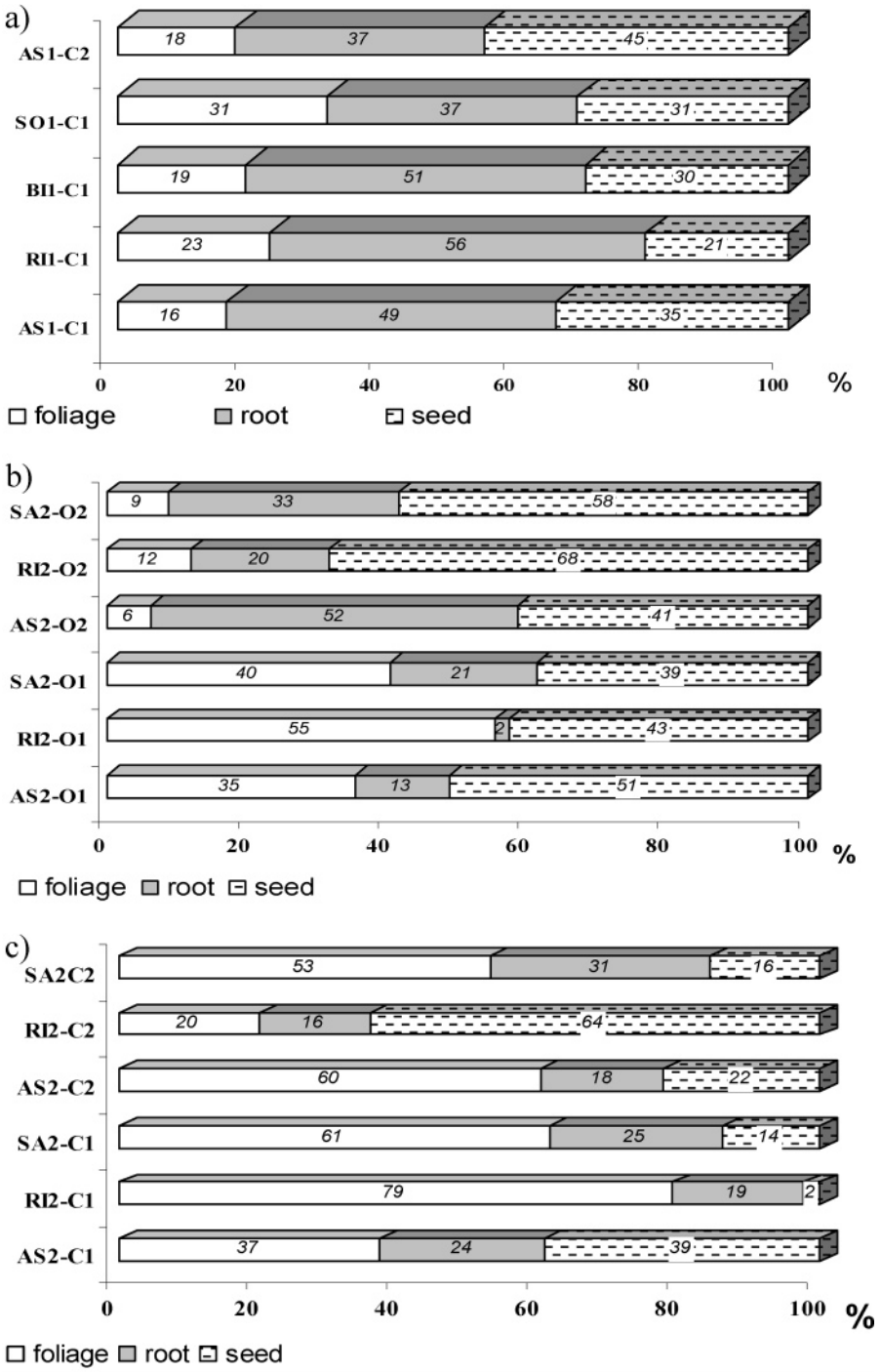

Figure 2. Distribution of total allelochemical content (expressed as percentage) among foliage, roots, and seeds: (**a**) first sampling; (**b**) second sampling, organic cultivation; (**c**) second sampling, conventional cultivation.

the first and second stages. Whereas DIMBOA-β-D-glucoside was not detected in foliage, this metabolite was detected in root extracts. In the first stage, MBOA, DIMBOA-β-D-glucoside, and DIMBOA were the major metabolites detected in the As, Ri, and Sa varieties, respectively. In the second stage, DIMBOA was the major metabolite detected in As, DIMBOA-β-D-glucoside in Ri, and MBOA in Sa of root extracts. Several benzoxazinoid derivatives were also detected in seed extracts. DIMBOA was the major metabolite detected in the first stage of the As and Sa varieties, whereas MBOA was the major one in Ri. Concerning the second stage, the major metabolite detected in the As and Sa varieties was MBOA, whereas DIMBOA presented the highest levels in seed extract of the Ri variety (**Table 2A**).

Organic Cultivation. The most relevant metabolites detected were DIMBOA, MBOA, HMBOA, and DIMBOA-β-D-glucoside. Nevertheless, the results obtained for foliage samples showed also different distributions of compounds between the first and second stages of cultivation. In the first stage of cultivation, DIMBOA was the major metabolite detected, whereas MBOA presented the highest levels in foliage extracts of the second stage for the As and Ri varieties; HMBOA was the major metabolite detected in the foliage extract of the second stage of the Sa variety. The main metabolites in root extracts were MBOA and DIMBOA for the As and Sa varieties, respectively, in each stage of cultivation, whereas for the Ri variety a difference existed between the first and second stages. Whereas MBOA was the main metabolite in the first stage,

Table 2. Levels (Micrograms per Gram) of Benzoxazinoid Derivatives in Wheat Foliage, Roots, and Seeds: Total Allelochemical Content in Each Tissue of Wheat Seedling of the Second Sampling Campaign[a]

A. Conventional Cultivation

		DIBOA-Glc	DIMBOA-Glc	HBOA	DIBOA	HMBOA	DIMBOA	BOA	MBOA	Σ tissue
					First Stage					
Astron	foliage	<LOD	<LOD	<LOD	<LOD	270	833	<LOD	552	1655
	root	46	155	<LOD	<LOD	59	303	11	476	1050
	seed	89	59	<LOD	60	48	1380	<LOD	119	1755
Ritmo	foliage	<LOD	<LOD	<LOD	<LOD	489	1775	<LOD	942	3206
	root	<LOD	318	<LOD	<LOD	34	124	<LOD	290	766
	seed	<LOD	<LOD	<LOD	<LOD	<LQD	<LOD	17	72	89
Stakado	foliage	<LOD	<LOD	<LOD	<LOD	326	3261	<LOD	695	4282
	root	27	329	<LOD	<LOD	83	860	<LOD	420	1719
	seed	<LOD	<LOD	<LOD	<LOD	<LQD	896	<LOD	70	966
					Second Stage					
Astron	foliage	<LOD	<LOD	177	66	56	<LOD	<LOD	30	329
	root	<LOD	<LOD	<LOD	<LOD	<LQD	62	<LOD	34	96
	seed	<LOD	<LOD	<LOD	<LOD	42	<LOD	<LOD	80	122
Ritmo	foliage	<LOD	<LOD	<LOD	<LOD	161	58	<LOD	94	313
	root	<LQD	134	<LOD	<LOD	<LQD	57	<LOD	57	248
	seed	<LOD	97	<LOD	<LQD	59	721	<LOD	130	1007
Stakado	foliage	<LOD	<LOD	<LOD	<LQD	126	51	<LOD	91	268
	root	<LOD	<LQD	<LOD	<LOD	<LQD	55	<LOD	103	158
	seed	<LOD	<LOD	<LOD	<LQD	<LQD	<LOD	10	69	79

B. Organic Cultivation

		DIBOA-Glc	DIMBOA-Glc	HBOA	DIBOA	HMBOA	DIMBOA	BOA	MBOA	Σ tissue
					First Stage					
Astron	foliage	<LQD	122	<LOD	<LQD	179	413	<LOD	197	911
	root	<LOD	<LOD	<LOD	<LOD	<LQD	<LOD	23	324	347
	seed	164	<LOD	<LOD	50	50	925	<LOD	130	1319
Ritmo	foliage	<LOD	<LOD	395	65	355	1570	<LOD	142	2527
	root	<LOD	<LQD	<LOD	<LOD	<LOD	<LOD	<LQD	97	97
	seed	75	77	<LOD	43	67	1544	<LOD	134	1940
Stakado	foliage	<LOD	<LOD	<LOD	<LQD	237	1270	<LOD	433	1940
	root	<LOD	144	<LOD	<LOD	82	463	<LOD	319	1008
	seed	<LOD	<LOD	<LOD	105	91	1536	<LOD	128	1860
					Second Stage					
Astron	foliage	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	24	24
	root	<LQD	<LOD	<LOD	<LOD	48	<LOD	<LOD	152	200
	seed	<LOD	<LOD	<LOD	<LQD	68	<LOD	<LOD	89	157
Ritmo	foliage	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	279	279
	root	<LOD	365	<LOD	<LOD	<LOD	<LOD	10	82	457
	seed	<LOD	165	<LOD	<LQD	69	1166	20	170	1590
Stakado	foliage	<LOD	<LOD	<LOD	<LQD	89	<LOD	<LOD	56	145
	root	<LOD	<LOD	<LOD	<LOD	116	311	<LOD	125	552
	seed	371	<LOD	<LOD	<LQD	<LQD	462	<LQD	147	980

[a] <LOD, below limit of detection; <LQD, below limit of quantification.

DIMBOA-β-D-glucoside presents the highest level in the second stage. In seed extracts DIMBOA was the main compound found for all varieties in the first stage, and a different result was obtained in the second stage between varieties. In this case, MBOA presents the highest levels in the As and Sa varieties and DIMBOA in the Ri variety (**Table 2B**).

As can be observed, our results showed that the type of benzoxazinoid derivatives found depends strongly on plant growth stage and the working procedure of the plant material. Several authors showed different methodologies for the quantification of benzoxazinoid derivatives (*17*−*23*). In our case, the glucoside derivatives were detected in the wheat seedlings of the second sampling campaign; the samples were frozen immediately after harvesting. This fact also explains why the major metabolite detected in samples of the first sampling was MBOA, originated from the degradation of DIMBOA and HMBOA. Nevertheless, DIMBOA and HMBOA were the major metabolites detected in wheat extracts of the second sampling.

Conventional versus Organic Cultivation. **Figure 4** presents the total amount of allelochemical content of wheat plants in conventional and organic cultivation. As can be observed, similar levels were found for conventional and organic cultivation. The As2-C1 (As variety, second sampling, first stage of conventional cultivation), Sa2-C1, and As2-C2 showed slightly higher levels in conventional cultivation than in organic cultivation. However, the Ri2-O1 (Ri variety, second sampling, first stage of organic cultivation), Ri2-O2, and Sa2-O2 presented higher levels in organic cultivation.

Stage of Cultivation. A general decrease of the total amount of allelochemical content in plants was observed from the first to the second stage. The decrease was also observed in each tissue. Like the total amount of allelochemical content in plants and tissues, the general behavior of individual compounds was a decrease of concentration from the first to the second stage except for some metabolites. The first exception was DIMBOA-β-D-glucoside, HMBOA, DIMBOA, and MBOA, which presented an increase of concentration from the first to the second stage in seed extracts of the Ri variety in conventional cultivation. This increase ranged from <LOD to 97 μg/g of dry weight for DIMBOA-β-D-glucoside, from <LQD to 59 μg/g

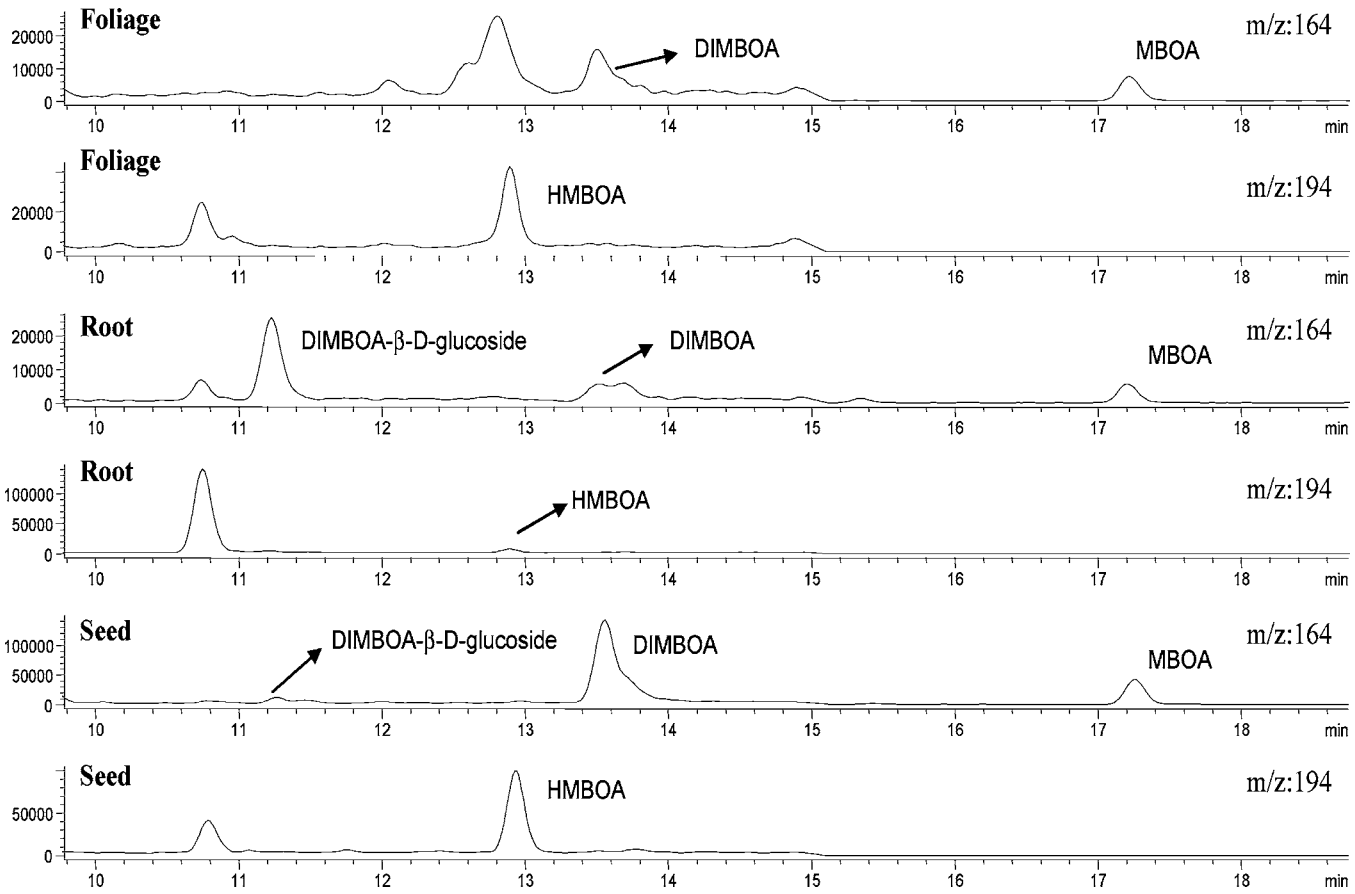

Figure 3. Chromatogram of methoxy derivatives (DIMBOA-β-D-glucoside, DIMBOA, HMBOA, and MBOA) present in foliage, root, and seed extracts of Ritmo variety.

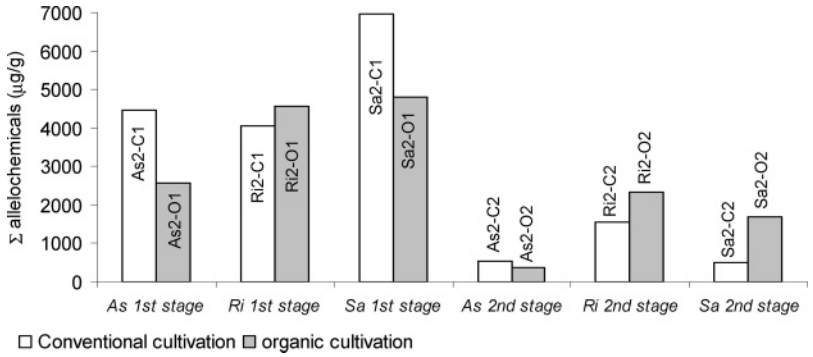

Figure 4. Correlations of total allelochemical content between conventional and organic cultivation in wheat seedlings of the second sampling.

of dry weight for HMBOA, from <LOD to 721 μg/g of dry weight for DIMBOA, and from 72 to 130 μg/g of dry weight for MBOA. Whereas in conventional cultivation the increase was present only in one variety, in organic cultivation the increase was present in all varieties. Then, DIBOA-β-D-glucoside showed an increase from <LOD to 371 μg/g of dry weight in seed extract of Sa variety, DIMBOA-β-D-glucoside increased in concentration from 144 to 365 μg/g of dry weight in root extract of Ri and from <LOD to 165 μg/g of dry weight in seed extract of the Ri variety, HMBOA showed an increase from <LQD to 48 μg/g of dry weight in root extract of the As variety and from 82 to 116 μg/g of dry weight in root extract of the Sa variety and, finally, MBOA showed an increase from 134 to 170 μg/g dry weight in seed extract of Ri and from 128 to 147 μg/g of dry weight in seed extract of the Sa variety.

A general decrease of the total allelochemical content was observed in wheat seedlings. These results were consistent with those presented in the literature. According to Argandona et al. (*9*), concentration in the plant increases abruptly a few days after germination and then decreases progressively with plant age. Nevertheless, an increase of concentration of some metabolites from the first to the second stage indicated that the allelochemicals were synthesized during the growth of plant.

Wheat Varieties. The highest levels of total amount of allelochemicals were found in Sa in the first stage of conventional cultivation and for Ri in the second one (**Table 2A**). For organic cultivation, the highest levels were also found for Sa and Ri in the first and second stages, respectively (**Table 2B**). The content of benzoxazinoid derivatives in wheat plants showed variations between different species (*9*, *17*).

Distribution in Foliage/Roots/Seeds. The distribution of the total amount of allelochemicals (expressed as a percentage) among different tissues in the three varieties is shown in **Figure 2**. In organic conditions (**Figure 2b**), the large amount of allelo-

chemicals was distributed between foliage and seeds during the first stage, and the highest percentage of compounds was distributed in the seeds for the second stage of cultivation. The distribution of the total amount of allelochemicals was not regular in wheat seedlings cultivated in conventional conditions (**Figure 2c**).

Copaja et al. (*13*) suggested that the allelochemical production must be initiated during germination because they observed that the total amount of benzoxazinoids increased during early growth and that no allelochemiclas were detected in germinated seeds. In the current study, allelochemicals found in germinated seeds were detected at levels similar to those found in foliage and root. These findings suggest that this matrix must be taken into account for evaluation of total content of allelochemicals in wheat seedlings. It is important to take into account that the occurrence of benzoxazinoids in seeds 10 days after sowing does not prove that the benzoxazinoids were present in the dry seeds. Our results suggest that the dry seeds could contain precursors from which the benzoxazinoids are formed as soon as they are put into the soil. Future work will be focused in this direction, to determine the occurrence of benzoxazinoids in dry seeds. This information could be important from the health perspective.

ACKNOWLEDGMENT

We thank Prof. D. Sicker (University of Leipzig), Prof. S. Chilton (University of North Carolina), Dr. F. Macias (University of Cadiz), Prof. Dr. Hajime Iwamura (Kyoto University), and Prof. Dr. Lisbeth Jonsson (Södertörn University College) for kindly providing standards and Dr. V. Cambier (Université Catholique de Louvain) and Dr. C. Werner (University of Zurich) for helpful discussions. We greatly appreciate the generous gift of the Phenomenex Synergi MAX-RP HPLC column from Daniel Lopez (JASCO Analytica, Spain) and technical assistance provided by D. Fanjul and R. Chaler (IIQAB-CSIC). Finally, we thank Merck for supplying the SPE cartridges.

LITERATURE CITED

(1) Torres, A.; Oliva, R. M.; Castellano, D.; Cross, P. Introduction. A science for the future. *Proceedings of the First World Congress on Allelopathy*, Cadiz, Spain, Sept 16−20, 1996; p 7.

(2) Narwal, S. S. Potentials and prospects of allelopathy mediated weed control for sustainable agriculture. *Proceedings of the International Conference on Allelopathy*, Jodhpur, India, 1996; pp 23−65.

(3) Ahman, I.; Johansson, M. Effect of Light on DIMBOA-glucoside concentration in wheat (*Triticum aestivum* L). *Ann. Appl. Biol.* **1994**, *124*, 569−574.

(4) Epstein, W. W.; Rowsemitt, C. N.; Berger, P. J.; Negus, N. C. Dynamics of 6-methoxybenzoxazolinone in winter-wheat—effects of photoperiod and temperature. *J. Chem. Ecol.* **1986**, *12*, 2011−2020.

(5) Richardson, M. D.; Bacon, C. W. Cyclic hydroxamic acid accumulation in corn seedlings exposed to reduced water potentials before, during, and after Germination. *J. Chem. Ecol.* **1993**, *19*, 1613−1624.

(6) Niemeyer, H. M. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones) defence chemicals in the Gramineae. *Phytochemistry K* **1988**, *27*, 3349−3358.

(7) Nicol, D.; Copaja, S. V.; Wratten, S. D.; Niemeyer, H. M. A screen of worldwide wheat cultivars for hydroxamic acid levels and aphid antixenosis. *Ann. Appl. Biol.* **1992**, *121*, 11−18.

(8) Copaja, S. V.; Niemeyer, H. M.; Wratten, S. D. Hydroxamic acid levels in Chilean and British wheat seedlings. *Ann. Appl. Biol.* **1991**, *118*, 223−227.

(9) Argandona, V. H.; Niemeyer, H. M.; Corcuera, L. J. Effect of content and distribution of hydroxamic acids in wheat on infestation by the aphid *Schizaphis graminum*. *Phytochemistry* **1981**, *20*, 673−676.

(10) Argandona, V. H.; Luza, J. G.; Niemeyer, H. M.; Corcuera, L. J. Role of hydroxamic acids in the resistance of cereals to aphids. *Phytochemistry* **1980**, *19*, 1665−1668.

(11) Cambier, V.; Hance, T.; de Hoffmann, E. Variation of DIMBOA and related compounds content in relation to the age and plant organ in maize. *Phytochemistry* **2000**, *53*, 223−229.

(12) Cambier, V.; Hance, T.; Hoffmann, E. Non-injured maize contains several 1,4-benzoxazin-3-one related compounds but only as glucoconjugates. *Phytochem. Anal.* **1999**, *10*, 119−126.

(13) Copaja, S. V.; Nicol, D.; Wratten, S. Accumulation of hydroxamic acids during wheat germination. *Phytochemistry* **1999**, *50*, 17−24.

(14) Niemeyer, H. M. Hydroxamic acid content of *Triticum* species. *Euphytica* **1988**, *37*, 289−293.

(15) Villagrasa, M.; Guillamon, M.; Eljarrat, E.; Barcelo, D. Determination of benzoxazinone derivatives in plants by combining pressurized liquid extraction−solid-phase extraction followed by liquid chromatography−electrospray mass spectrometry. *J. Agric. Food Chem.* **2006**, *54*, 1001−1008.

(16) Eljarrat, E.; Guillamon, M.; Seuma, J.; Bugel, B.; Formsgaard, I.; Oleszek, W.; Stochmal, A.; Shakaliene, O.; Barcelo, D. First European inter-laboratory study on benzoxazinone derivatives in plants: phase I. *J. Chromatogr. A* **2004**, *1047*, 69−76.

(17) Baumeler, A.; Hesse, M.; Werner, C. Benzoxazinoids—cyclic hydroxamic acids, lactams and their corresponding glucosides in the genus *Aphelandra* (Acanthaceae). *Phytochemistry* **2000**, *53*, 213−222.

(18) Grambow, H. J.; Luckege, J.; Klausener, A.; Muller, E. Occurrence of 2-(2-hydroxy-4,7-dimethoxy-2*H*-1,4-benzoxazin-3-one)-*β*-D-glucopyranoside in *Triticum aestevium* leaves and its conversion into 6-methoxy-benzoxazolinone. *Z. Naturforsch. C* **1986**, *41*, 684−690.

(19) Cambier, V.; Hance, T.; de Hoffmann, E. Non-injured maize contains several 1,4-benzoxazin-3-one related compounds but only as glucoconjugates. *Phytochem. Anal.* **1999**, *10*, 119−126.

(20) Mayoral, A. M.; Gutierrez, C.; Ruiz, M. L.; Castanera, P. A high-performance liquid-chromatography method for quantification of DIBOA, DIMBOA, and MBOA from aqueous extracts of corn and winter cereal plants. *J. Liq. Chromatogr.* **1994**, *17*, 2651−2665.

(21) Xie, Y. S.; Atkinson, A. J.; Arnason, J. T.; Morand, P.; Philogene, B. J. R. Separation and quantification of 1,4-benzoxazin-3-ones and benzoxazolin-2-ones in maize root extract by high-performance liquid chromatography. *J. Chromatogr. A* **1991**, *543*, 389−395.

(22) Lyons, P. C.; Hipskind, J. D.; Wood, K. V.; Nicholson, R. L. Separation and quantification of cyclic hydroxamic acids and related compounds by high-pressure liquid chromatography. *J. Agric. Food Chem.* **1988**, *36*, 57−60.

(23) Bucker, C.; Grambow, H. J. Alterations in 1,4-benzoxazinone levels following inoculation with stem rust in wheat leaves carrying various alleles for resistance and their possible role as phytoalexins in moderately resistant leaves. *Z. Naturforsch. C* **1990**, *45*, 1151−1155.

Received for review April 19, 2005. Revised manuscript received September 16, 2005. Accepted December 6, 2005. This work was financially supported by the program "Quality of life and management of living resources (1998 to 2002)" of the European Union, FATEALLCHEM Contract QLK5-CT-2001-01967. The funding provided to IIQAB-CSIC (AGL2001-4952-E) by the Ministerio de Ciencia y Tecnologia is gratefully acknowledged.

JF050898H