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Quantification of Benzoxazinone Derivatives in Wheat (*Triticum aestivum*) Varieties Grown under Contrasting Conditions in Denmark

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Three varieties of winter wheat (*Triticum aestivum*) were grown in both conventional and organic farming systems. The contents of the benzoxazinone derivatives 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-Glc), 6-methoxybenzoxazolin-2-one (MBOA), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA), benzoxazolin-2-one (BOA), and 2-hydroxy-1,4-benzoxazin-3-one (HBOA) were analyzed at five growth stages (BBCH 9−10, 12, 21, 31, and 53). Major differences were found between the varieties, with Stakado exhibiting the highest contents. In contrast, only minor and erratic differences were found between the two farming systems, suggesting that the inherent differences in the content of benzoxazinone derivatives of the varieties were not significantly affected by the use of pesticides and synthetic fertilizers. The concentration of benzoxazinone derivatives in the foliage was considerably higher at the early growth stages than later in the growing season, with DIMBOA being the most abundant of the benzoxazinone derivatives. An increase in the concentration was observed in early spring compared to late autumn, suggesting that plants synthesized benzoxazinone derivatives at the commencement of growth in early spring. The concentrations in the roots were considerably lower than in the foliage at the early growth stages but remained relatively constant over time, resulting in a higher concentration than in the foliage at the late growth stages. The results are discussed in relation to previous findings that predominantly originate from experiments done under controlled conditions in either growth cabinets or greenhouses.

KEYWORDS: DIMBOA; wheat varieties; allelochemicals; LC-MS; sonication

INTRODUCTION

Benzoxazinone derivatives, in particular the hydroxamic acids 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), have been shown to be implicated in the resistance of wheat and rye to insects (*1*, *2*) and diseases (*3*, *4*) and the allelopathic activity of wheat and rye against weeds (*5*−*7*). Hence, growing wheat varieties with high levels of benzoxazinone derivatives could reduce the problems with pests, diseases, and weeds and thus reduce the use of pesticides.

Little information is available on the content of benzoxazinone derivatives in modern wheat cultivars. Niemeyer (*8*) analyzed the content of hydroxamic acids in various *Triticum* species and found the highest content in *T. speltoides* (16 mmol/kg of fresh weight) and the lowest in *T. taushii* (0.21 mmol/kg of fresh weight), whereas the modern wheat varieties contained intermediate levels of benzoxazinone derivatives (1.44−2.77 mmol/kg of fresh weight). Copaja et al. (*9*) analyzed a large number of Chilean and British wheat cultivars, mostly *T. aestivum*, and found nearly a 10-fold difference in the levels of DIMBOA. In this study the content of DIBOA was on average a magnitude lower than the content of DIMBOA; however, in a subsequent study by the same research group involving only three wheat (*T. aestivum*) cultivars, much smaller differences in the contents of the two hydroxamic acids were reported (*10*).

DIMBOA and DIBOA are not present in the seeds (*10*, *11*) but appear upon germination. The highest concentrations of DIMBOA and DIBOA are found shortly after germination and then decrease (*1*, *10*, *12*). Copaja et al. (*10*) found that the absolute quantity of DIMBOA and DIBOA remained stable over time, indicating a growth dilution effect. Hydroxamic acids have been found both in leaves and in roots of wheat. Copaja et al. (*10*) found higher concentrations of DIMBOA in the leaves than in the roots in two of three *T. aestivum* cultivars and no

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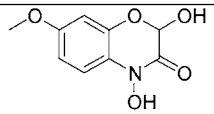
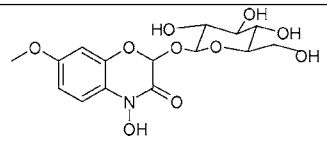
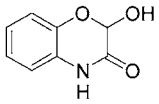
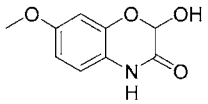
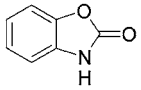
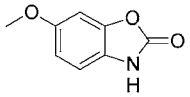
Table 1. Systematic Names, Acronyms, and Structures of Benzoxazinone Derivatives Analyzed in Wheat Plants

Systematic name	Acronym	Structure formula
2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one	DIMBOA	
2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one	DIMBOA-Glc	
2-hydroxy-1,4-benzoxazin-3-one	HBOA	
2-hydroxy-7-methoxy-1,4-benzoxazin-3-one	HMBOA	
benzoxazolin-2-one	BOA	
6-methoxy- benzoxazolin-2-one	MBOA	

differences in the third cultivar, whereas the opposite was true for DIBOA. Wu et al. (*13*) analyzed the concentration of DIMBOA in 58 wheat accessions (*T. aestivum*) and found a higher concentration in the roots compared to the shoots in 50 of the accessions.

DIMBOA content in wheat was shown to be genetically inheritable (*14*), and genetic studies in maize have shown that the accumulation of DIMBOA could be monogenic or polygenic depending on the population (*15*, *16*). However, the concentration was also influenced by environmental parameters such as temperature (*17*) and light intensity (*18*), and hence it seems likely that cultivation practices may also influence the concentration of DIMBOA and other benzoxazinone derivatives in wheat; however, no information is available on this aspect.

In most of the previous studies only the contents of the aglucones DIMBOA and DIBOA in wheat were quantified, whereas the contents of glucosides of DIMBOA and DIBOA and metabolites such as 6-methoxybenzoxazolin-2-one (MBOA), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA), benzoxazolin-2-one (BOA), and 2-hydroxy-1,4-benzoxazin-3-one (HBOA) were not determined. The objective of the present study was therefore to determine the concentration of a broad range of benzoxazinone derivatives in three modern winter wheat varieties grown under two contrasting farming systems, a conventional system in which synthetic fertilizers and pesticides were used and an organic system with no use of pesticides and synthetic fertilizers, only manure. The experiment was conducted over two years. In the first year six wheat varieties replicated twice were included, whereas in the second year only three varieties were cultivated, but each variety was replicated four times. The results of the first year of experimentation were published elsewhere (*19*). In this paper we report the results of the second year of experimentation.

MATERIALS AND METHODS

Reagents and Chemicals. Methanol and acetonitrile were of LiChroslv gradient grade for liquid chromatography from Merck; acetic acid was 100% glacial acetic acid pro analyse from Merck. Water was Milli-Q water from Millipore. Water/methanol was acidified with 1% acetic acid. DIMBOA, MBOA, HMBOA, and HBOA were received from Professor F. Macias from University of Cadiz, Spain, DIMBOA-Glc was received from Prof. Dr. Hajime Iwamura at Kyoto University and Prof. Dr. Lisbeth Jonsson at Södertörn University College. BOA was purchased from Acros Organics. Systematic names and structures of the benzoxazinone derivatives are displayed in **Table 1**.

Table 2. Retention Times and Ions Used for Quantification and Identification of Benzoxazinone Derivatives

compound	retention time (min)	quantification ion (*m*/*z*)	confirmation ion (*m*/*z*)
HBOA	12.65	148	166
DIMBOA-Glc	12.81	166	212
HMBOA	13.31	196	178
DIMBOA	13.52	212	166
BOA	14.31	136	
MBOA	14.69	166	

Cultivation and Sampling of Wheat Varieties. Three varieties of wheat (*T. aestivum*), Astron, Ritmo, and Stakado, were cultivated under conventional as well as organic farming conditions, each variety being sown in four random plots in each of the two fields. The experimental fields were located at Research Centre Flakkebjerg, Zealand, Denmark. Two different fields were used for the experiments as the organic field was located in an area cultivated organically for more than 10 years. Consequently, the cropping histories of the two fields were different, whereas the soil type in both fields was a sandy loam soil. Wheat was sown on September 12, 2002. Samples of 10 wheat plants from each of the 24 plots were collected at five growth stages: BBCH 9−10 (October 1, 2002), BBCH 12 (October 8, 2002), BBCH 21 (December 5, 2002), BBCH 31 (May 5, 2003), and BBCH 53 (June 11, 2003). Immediately after sampling, roots were separated from foliage, and roots and foliage were frozen in separate bags in the field using dry ice. The samples were kept frozen at −20 °C.

Sample Preparation. The samples were lyophilized until dryness. The dry samples were ground in a ball mill of agate, the root samples being cut into smaller pieces before grinding. The benzoxazinone derivatives were extracted from the plant material by ultrasonic extraction using a modification of the method described by Stochmal et al. (*20*). One hundred milligrams of dry sample was weighed into a glass vial, and 5 mL of acidified methanol was added. The mixture was sonicated in a 5510 Branson ultrasonic bath for 5 min and left in a refrigerator for 16 h followed by another 5 min of sonication. The extract was filtered through a 17 mm 0.45 μm nylon membrane filter, and the sample was sonicated for another 5 min with an additional 5 mL of acidified methanol followed by filtration. The combined extracts were evaporated to dryness under a flow of nitrogen at 37 °C and redissolved in 2 mL of acidified water. As described by Villagrasa et al. (*21*), cleanup of the extract is necessary before the instrumental analysis, and a cleanup procedure similar to that described by Villagrasa et al. (*22*) was applied. The extract was cleaned up on a Sep-pak tC18 cartridge from Waters conditioned with 5 mL of acidified methanol followed 5 mL of acidified water. The extract was filtered through a glass fiber prefilter to prevent clogging of the cartridge. The cartridge was eluted with 6 mL of acidified water followed by 2 times 6 mL of 1% acetic acid in methanol/acetonitrile (1:1). The combined eluates were evaporated into dryness and redissolved in 5 mL of 1% acetic acid in methanol/water (60:40). With each batch of samples were one reagent blank and one recovery sample prepared by spiking a sample of foliage from the latest growth stage in the experimental year 2001−2002 as well as an unspiked sample of the same foliage. The recovery sample was spiked with a mixture of standards to give a theoretical concentration of 1 ng/μL in the final extract.

Chromatographic Conditions. LC-MS analysis was carried out on a HP 1100 (Hewlett-Packard) instrument. The analytical column was a Hypersil BDS C18 from ThermoHypersil (250 × 2.1 mm, 5 μm particle size) with an ODS 4 mm × 2 mm precolumn. Column temperature was 25 °C. Flow rate was 0.2 mL/min. Injection volume was 20 μL. Solvent A was 810 mL of water, 90 mL of methanol, and 1 mL of acetic acid. Solvent B was 900 mL of methanol and 1 mL of acetic acid. Total run time was 25 min with the following solvent gradient: 0−1 min, 90% A; 1−8 min, 90−30% A; 8−15 min, 30% A; 15−16 min, 30−90% A; 16−25 min, 90% A. The benzoxazinone derivatives eluted from 12 to 16 min (**Table 2**).

Mass Spectrometry Conditions. The HP 1100 mass selective detector equipped with an atmospheric pressure ionization (API) source was used with an electrospray interface (ES). The ES was operated in positive ion mode. MS analyses were performed in SIM mode. Ions used for quantification and identification are displayed in **Table 2**. The MS conditions are displayed in **Table 3**.

Table 3. Mass Spectrometer Conditions (Specific for HP 1100 MSD)

time (min)	SIM ion	fragmentor	gain (EMV)	dwell time (ms)
10.0	148	28	3.0	108
	164	28		108
	166	80		108
	178	28		108
	182	28		108
	196	28		108
	212	15		108
	396	70		108
13.90	136	60	3.0	145
	148	16		145
	164	40		145
	166	50		145
	180	16		145
	241	15		145

Table 4. Limit of Quantification and Recovery of Benzoxazinone Derivatives in Roots and Foliage

compound	LOQ in foliage (μg/g of dry wt)	LOQ in roots (μg/g of dry wt)	recovery (%) ± SD
HBOA	1.5	0.15	76 ± 12
DIMBOA-Glc	10	1	134 ± 24
HMBOA	1.5	1.5	81 ± 26
DIMBOA	0.5	0.5	69 ± 16
BOA	0.15	0.15	83 ± 11
MBOA	0.15	0.15	87 ± 4

Quantification. Quantification was performed using external calibration with standard solution concentrations of 0.001, 0.005. 0.01, 0.1, 1.0, 2.5, and 10 ng/μL. HP ChemStation rev A.07.01 682 program was used for quantification of the compounds. A pronounced ion suppression from the extract turned out to be dependent on the concentration of the analytes in the extract, to have different influence on the signals from the different analytes, and further to depend on the kind of extract, that is, roots or foliage, the effect in foliage extracts being stronger than that in root extract. The effect of the ion suppression was corrected for in the following way: A series of calibration standards were prepared in pure solvent as well as in extracts of roots and foliage. Signals from pure solvent standards were divided by signals from roots or foliage extracts of the same concentration, and the resulting factors were used to adjust the suppressed signals. The applied factors varied in roots from 1 to 2.9 and in foliage from 1.4 to 9.3 depending on analyte and concentration. The high factors were applied in only a few samples with concentrations close to the limit of quantification. In the same way recovery data, based on spiked foliage, were adjusted for ion suppression. A paper on the ion suppression is in preparation.

Detection Limit and Recovery. The instrumental detection limit was as low as the lowest standard concentration, 0.001 ng/μL, corresponding to 0.05 μg/g of dry weight in 5 mL of extract of 100 mg of plant material. However, the detection limits in plant extracts were higher because of coextracted compounds causing noise and ion suppression. The limits of detection and quantification were determined from the series of standard solutions in extracts of roots and foliage mentioned above. The limit of detection was determined as the lowest concentration providing a signal distinguishable from the signal from the unspiked sample, taking into account both signal height and peak shape. The limit of quantification was determined as 3 times the signal-to-noise. Recovery was determined from analysis of spiked foliage. The average recovery from all analytical batches and the limits of quantification are displayed in **Table 4**. The average recoveries and limits of quantification are acceptable except those for DIMBOA-Glc. This is caused by the very strong ion supression for especially this compound.

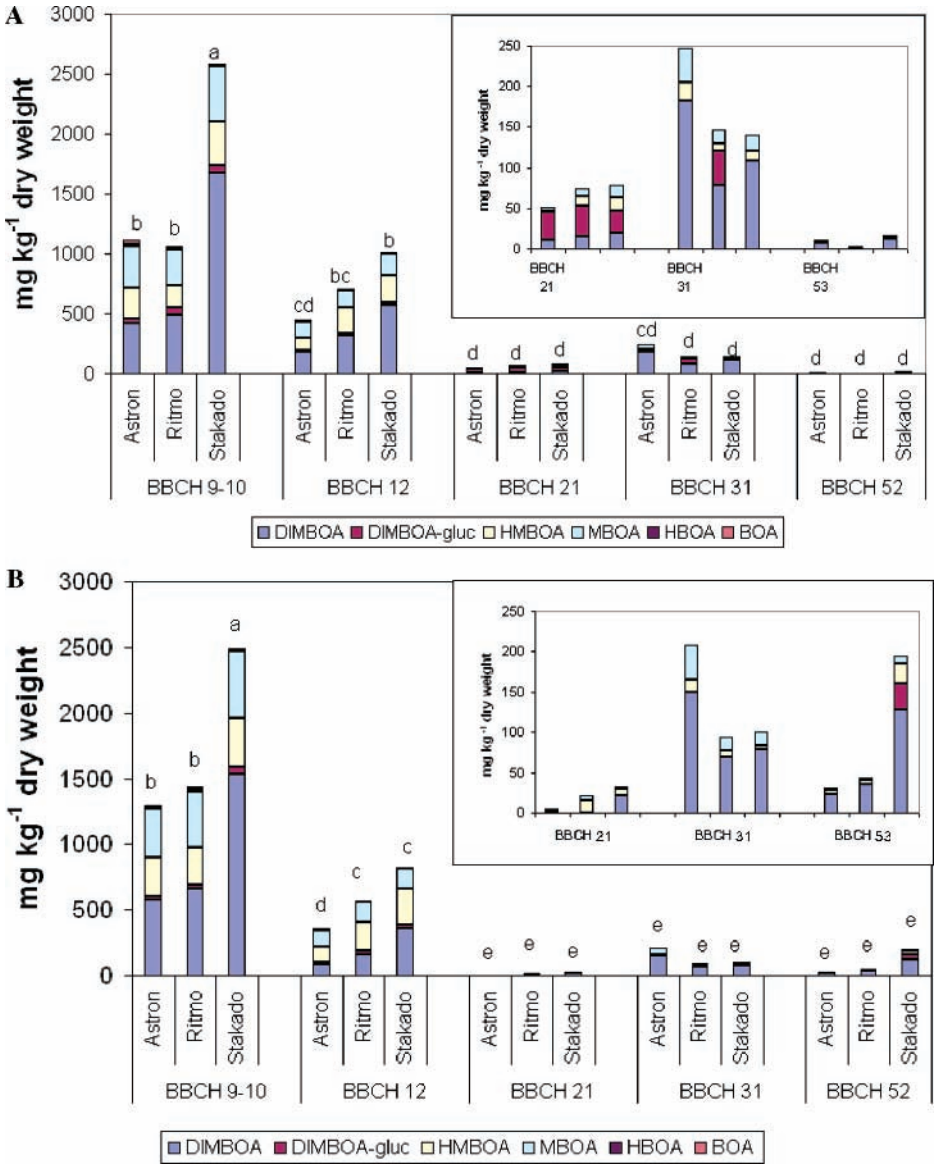


Figure 1. Concentration of benzoxazinones in foliage (milligrams per kilogram of dry weight) of three winter wheat varieties at different growth stages grown organically (**A**) and conventionally (**B**). Inset graphs show enlargements of results at growth stages 21, 31, and 53. The results for bars denoted with the same letter are not significantly different according to Duncan's mulitiple-comparison test.

Table 5. Concentration Range (Milligrams per Kilogram of Dry Weight) of Benzoxazinone Derivatives in Foliage and Roots of Three Wheat Varieties Grown under Contrasting Conditions

plant tissue	growth stage	DIMBOA	MBOA	HMBOA	DIMBOA-Glc	BOA	HBOA
foliage	BBCH 9–10	419–1675	289–518	187–370	26–59	6–37	10–23
	BBCH 12	86–567	120–180	102–271	15–35	3–16	4–6
roots	BBCH 9–10	46–163	50–89	19–33	19–50	2–13	<LOQ–3
	BBCH 12	29–87	54–80	22–39	41–91	2–8	<LOQ–2

RESULTS

Benzoxazinone derivatives were detected in roots and foliage at all growth stages. However, the concentration of the various derivatives fluctuated during the sampling period and varied between plant parts (roots/foliage). In **Figures 1** and **2** are shown the concentration in foliage and roots, respectively, of the six benzoxazinone derivatives included in the chemical analysis. The bars represent mean concentration of samples collected in four replicate field plots in each farming system. The sum of benzoxazinones in foliage and roots, respectively, was compared pairwise for the three varieties and five growth stages using Duncan's multiple-comparison test. The result of this test is shown by a letter above the bars. Bars denoted with the same letter are not significantly different.

Foliage. Generally the concentration of benzoxazinone derivatives was highest at the earliest growth stage, declining at growth stage 12 and being almost negligible at later growth stages except at growth stage 31. The concentration ranges of the first two growth stages in which the highest concentrations of benzoxazinones were found are shown in **Table 5**. DIMBOA was found at the highest concentration of any derivative, and the concentration was significantly higher in Stakado compared to Ritmo and Astron at growth stages 9−10 and 12. The total concentration levels of benzoxazinones in organically and

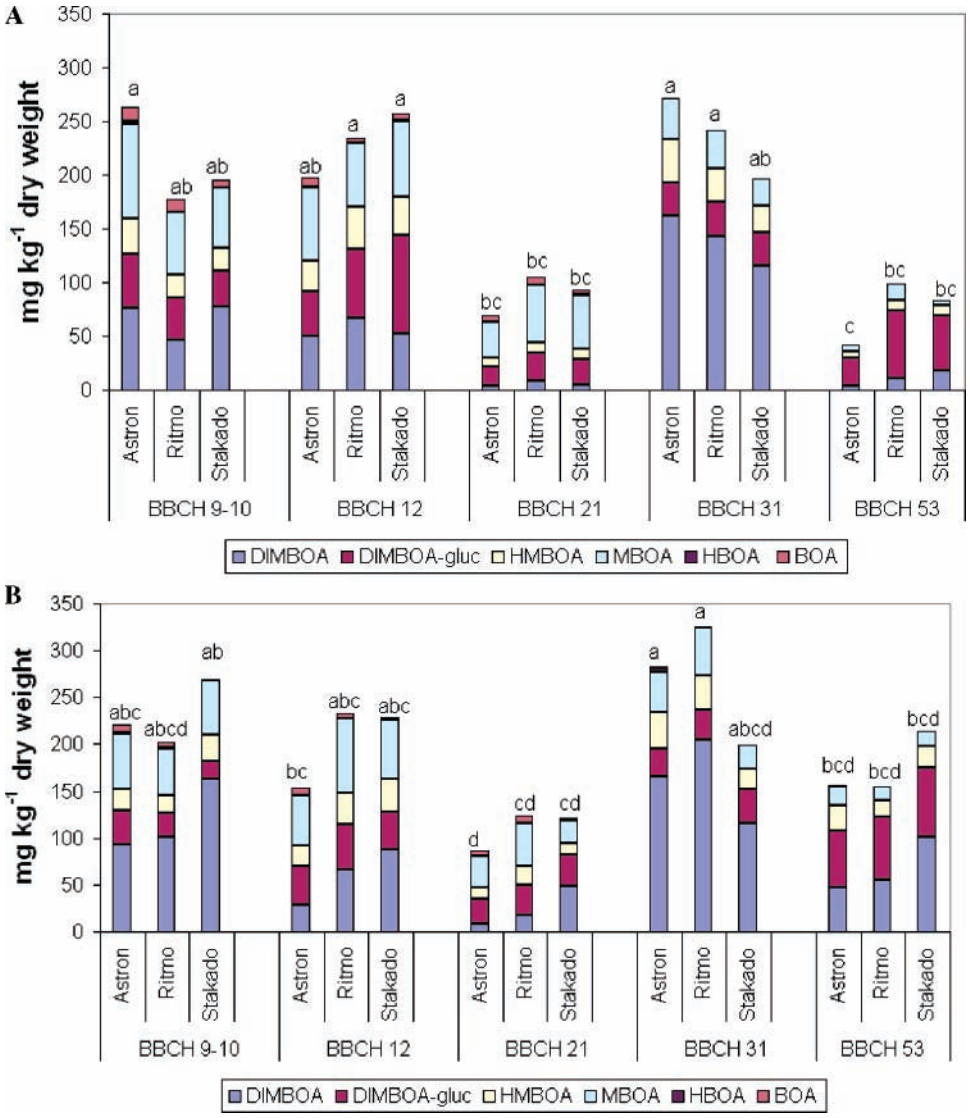


Figure 2. Concentration of benzoxazinones in roots (milligrams per kilogram of dry weight) of three winter wheat varieties at different growth stages grown organically (**A**) and conventionally (**B**). The results for bars denoted with the same letter are not significantly different according to Duncan's mulitiple-comparison test.

conventionally grown wheat were comparable according to a pairwise comparison of the two farming systems using Duncan's test. The concentrations of MBOA and HMBOA were of the same order of magnitude, with slightly higher concentration levels in Stakado compared to Ritmo and Astron and no differences between conventionally and organically grown wheat. In organically grown wheat, DIMBOA-glucoside was detected in all varieties at growth stage 21 and in Ritmo at growth stage 31, whereas it was detected at only the two earliest growth stages in conventionally grown wheat. BOA and HBOA were detected in low concentrations at growth stages 9−10 and 12 and were not found at later growth stages.

Roots. Generally the presence of benzoxazinone derivatives in roots was more evenly distributed over the whole growing period. To illustrate the variation between varieties and growth stages, the concentration ranges of the first two growth stages are shown in **Table 5**. As for the foliage DIMBOA was the benzoxazinone derivative found at the highest concentrations; however, in growth stages 9−10 and 12 the concentration levels in roots were markedly lower than that of the foliage. The concentration of DIMBOA diminished over time from growth stage 9−10 to 21 but then increased at the two latest growth stages. In contrast, the concentrations of MBOA, DIMBOA-Glc, and HMBOA did not fluctuate very much, and the concentrations of the first two derivatives were higher than that of DIMBOA at growth stage 21 when the DIMBOA concentration was at a minimum. Like for the foliage, BOA and HBOA were the benzoxazinone derivatives detected at the lowest concentrations. Overall, only minor differences were found between conventionally and organically grown wheat, and likewise differences in the sum of benzoxazinones between the wheat varieties were less pronounced than for the foliage.

DISCUSSION

In the present study only minor differences were observed between the cultivation systems. The wheat varieties were sown on the same date, and plant samples were collected simultaneously; that is, nearly all variable growing parameters except the previous cropping history and cropping practices were comparable. Hence, our results indicate that the use of synthetic fertilizers rather than manure and the application of pesticides, when required, did not have any pronounced effect on the concentration of benzoxazinone derivatives. This result is somewhat in contrast to the previous season, when the results suggested a higher concentration of benzoxazinone derivatives

in organically grown wheat (*19*). However, in the 2001/2002 growing season factors other than farming practice may have influenced the results, for example, the fact that the soil type of the organic field was a sandy loam soil, whereas the conventional field was a clay loam soil. In 2002/2003 the soil types in the fields were similar.

The total content of benzoxazinone derivatives in milligrams per kilorgram of dry weight was markedly lower in the roots compared to the foliage; however, whereas the benzoxazinone derivatives tended to disappear from the foliage at the three latest growth stages, many of the compounds were found in the roots beyond the third sampling. Wu et al. (*13*) generally found higher concentrations in the roots than in the foliage, and we can provide no explanation why the 3 varieties in our experiment deviated from the overall trend in the previous study including 58 wheat accessions.

Differences between varieties were observed for the foliage concentration of benzoxazinone derivatives, in particular for DIMBOA. The concentration of DIMBOA in Stakado was 2−3 times higher than in Astron and Ritmo in the conventional farming system and 3−4 times higher in the organic farming system. A similar trend was observed for MBOA and HMBOA, but the differences were less than observed for DIMBOA. Similar differences between varieties were observed in 2001−2002 (*19*), suggesting an inherently higher concentration of DIMBOA in Stakado compared to the other two varieties. In contrast, the concentration of BOA was significantly lower in Stakado than in Astron and Ritmo, and a similar trend was observed for HBOA. However, as the content of BOA and HBOA was very low in all varieties, constituting only 1−2% of the total content of benzoxazinone derivatives, these differences did not have influence on the overall conclusion that Stakado was the variety with the highest foliage content of benzoxazinone derivatives. The differences between varieties observed in the content of benzoxazinone derivatives in the foliage were not reflected in the root tissue. Generally only small differences were found between the three varieties in the content of benzoxazinone derivatives in the roots, and no clear trend could be observed.

The maximum foliage concentration of DIMBOA in Stakado was 1675 mg/kg of dry weight at growth stage 9−10 and 567 mg/kg of dry weight at growth stage 12 (**Table 5**). Wu et al. (*13*) found DIMBOA contents in the foliage ranging from 0 to 730 mg/kg of dry weight in 17-day-old wheat seedlings; however, the growth stage of the plants at harvest was not disclosed. In our experiment growth stage 9−10 was ∼17 days after sowing; however, the plants used by Wu et al. (*13*) were grown in controlled growth cabinets at a temperature cycle of 25/13 °C. On the basis of the authors' own experiences it can be envisaged that the wheat plants were closer to growth stage 12 than 9−10; hence, the maximum concentration of DIMBOA in Stakado was comparable to the content of the group of accessions that Wu et al. (*13*) classified as the group with a medium content of DIMBOA. The maximum DIMBOA concentrations of Astron and Ritmo were considerably lower than that of Stakado at growth stage 9−10, but the differences particularly between Stakado and Ritmo were less pronounced at growth stage 12. The maximum DIMBOA concentrations of Astron and Ritmo of 183 and 317 mg/kg of dry weight would place Astron in the group with a low content of DIMBOA, whereas Ritmo would just qualify for the group of varieties with a medium content of DIMBOA.

Wu et al. (*13*) determined only the content of DIMBOA, whereas in the present study we also determined the concentration of DIMBOA-glucoside, the precursor of DIMBOA, and MBOA and HMBOA, two DIMBOA metabolites. If the contents of these compounds are expressed as DIMBOA equivalents and added to the content of DIMBOA in Stakado, the average contents over the conventional and organic farming systems were 2657 and 960 mg/kg of dry weight at growth stages 9−10 and 12, that is, 59 and 69% higher than if only DIMBOA was included. This clearly suggests that these compounds should also be analyzed when the allelopathic potential of wheat varieties is assessed.

Cambier et al. (*23*) studied the effects of sample preparation on the relationship between the gluconoconjugated compounds and the aglucones in maize and concluded that detection of agluconic compounds or their first derivatives, DIMBOA and MBOA, in the analysis was due to the activity of β-glucosidase during sampling preparation. In the present study DIMBOA-Glc was sometimes present at higher concentrations than DIMBOA and MBOA (e.g., organically grown wheat at growth stage 21), whereas in other cases the contents of DIMBOA and MBOA were much higher than the content of DIMBOA-Glc, suggesting that DIMBOA-Glc was well preserved using our sampling procedure of immediately freezing plant samples in the field. Our findings support the hypothesis of Zuñiga and Massardo (*24*) that the gluconoconjugates as well as the aglucones can be found in noninjured wheat plants in the differentiated and undifferentiated tissues, respectively.

In earlier studies the concentration of benzoxazinone derivatives was often expressed as millimoles per kilogram of dry weight or fresh weight. In the present experiment individual plants were not weighed, but on average the fresh weights of the whole wheat plants were 0.1, 0.2, 0.5, 5, and 12 g at growth stages 9−10, 12, 21, 31, and 53, respectively and the average dry matter content of the plants was determined to be ∼10%. Consequently, the maximum contents in Astron, Ritmo, and Stakado of 577, 658, and 1675 mg/kg of dry weight DIMBOA at growth stage 9−10 corresponded to 0.27, 0.31, and 0.79 mmol/kg of fresh weight or 0.27×10^{-4}, 0.31×10^{-4}, and 0.79×10^{-4} mmol/plant. The corresponding values at growth stage 12 were 0.09, 0.15, and 0.27 mmol/kg of fresh weight or 0.09×10^{-4}, 0.15×10^{-4}, and 0.27×10^{-4} mmol/plant. Foliage constituted ca. 25 and 70% of the total fresh weight at growth stages 9−10 and 12 (*25*). As the DIMBOA contents of the roots were generally lower than those of the foliage at the early growth stages, the content of DIMBOA was actually somewhat lower than the values estimated above.

Niemeyer (*11*) found DIMBOA concentrations of up to 2.0 mmol/kg of fresh weight in seedlings of four accessions of *T. aestivum* grown for 10 days in a greenhouse with an average concentration of 1.4 mmol/kg of fresh weight. The growth stage of plants at the time of analysis was not disclosed; however, considering the growth conditions, it seems reasonable to assume that the growth stage of the plants was between BBCH 9−10 and 12. Hence, the contents of these accessions were considerably higher than those of the varieties included in our experiments. Copaja et al. (*9*) found DIMBOA concentrations varying from 1.4 to 10.9 mmol/kg of fresh weight in 52 Chilean *T. aestivum* and *T. durum* varieties, whereas Copaja et al. (*10*) reported DIMBOA concentrations ranging from 1.33×10^{-4} to 2.31×10^{-4} mmol/plant in 7-day-old seedlings of 3 *T. aestivum* varieties. In both studies the concentration of DIMBOA was considerably higher than in the present field study. It should be noted in the two last mentioned studies plants were grown under controlled conditions in growth cabinets. In the first study the light intensity was 75 μEinstein/m^2/s^{-1}, whereas

in the second study no information was given on light intensity. Aahman and Johansson (*18*) found an inverse relationship between DIMBOA concentration and light intensity, and although light intensity was not recorded in our field experiment, there is no doubt that it, on average, was considerably higher than 75 μEinstein/m^2/s^{-1} in the beginning of the growing season. In contrast, high temperatures were found to increase the content of DIMBOA, and a characteristic of most of the studies conducted under controlled conditions is that they were done at relatively high temperatures compared to the average autumn temperatures under Danish conditions. Differences in light intensity and temperature may at least partly explain the pronounced differences in the DIMBOA concentrations between our study and some of the studies conducted under controlled conditions. Hence, experiments done under controlled conditions may provide valuable information concerning the ranking of a large number of accessions or varieties; however, the pronounced differences in the concentration of DIMBOA observed between our field studies and studies done under controlled conditions suggest that studies done under controlled conditions may overestimate the concentration of benzoxazinone derivatives and thus the allelopathic potential of wheat. The possible discrepancy between field-grown plants and plants grown under controlled conditions was also highlighted by the fact that in one instance the content of benzoxazinone derivatives in plants of the variety Stakado grown in a glasshouse was considerably lower than the corresponding content of the varieties Astron and Ritmo (unpublished data), that is, in total contrast to the results from two years of field studies.

It has been hypothesized that plants synthesize benzoxazinone derivatives only at the earliest growth stages around germination and that the reduction in concentration over time observed in all studies so far is merely a dilution process (*10*). This conclusion was based on analyses of up to 7-day-old plants. Our study does not support this as being true for wheat plants growing until maturity. First, the reduction in the concentration of benzoxazinone derivatives from growth stage 9−10 to 21 is significantly larger than can be explained in terms of plant growth. For example, the foliage concentration of the most abundant benzoxazinone derivative, DIMBOA, at growth stage 21 was reduced to 1−3% of the concentration at growth stage 9−10, whereas the fresh weight was increased by a factor 5. Second, at the first sampling in the spring (growth stage 31) the foliage concentration was markedly higher than at growth stage 21, although the wheat plants had increased their fresh weight by a factor 10, indicating that benzoxazinone derivatives were synthesized when growth resumed in early spring.

The results of this study and the study done the year before (*19*) have shown that the concentration of benzoxazinone derivatives varies significantly among commonly cultivated wheat varieties. Copaja et al. (*9*) found differences in the DIMBOA concentration among 52 wheat varieties of a factor ~8. In our study the differences were smaller, up to a factor 4 at the early growth stages, but we included only 6 varieties in the two years of experimentation. Our results therefore indicate that among the cultivated wheat varieties it will be possible to find some with high contents of benzoxazinone derivatives, and these varieties could provide a valuable input to the adaptation of integrated pest management approaches.

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