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Differential Exudation of Two Benzoxazinoids—One of the Determining Factors for Seedling Allelopathy of Triticeae Species

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Benzoxazinoids (Bx) are natural phytotoxins that function as chemical defense compounds in several species. The release of Bx by intact plant roots associated these compounds with root allelopathy in Triticeae species; however, the significance of exudate concentrations of Bx for plant−plant interactions is still a controversial question. A biological screening of 146 cultivars of four Triticeae species (*Triticum aestivum* L., *Triticum durum* Desf., *Triticum spelta* L., and *Secale cereale* L.) demonstrated a high cultivar dependence to suppress the root growth of *Sinapis alba* L. by root allelopathy in a dose−response bioassay. Only a few cultivars possessed a marked high or low allelopathic activity, whereby high-performance liquid chromatography−diode array detection analysis of root exudates revealed that these cultivars differed considerably in their ability to exude the two Bx aglucones, DIBOA [2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one] and DIMBOA [2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one]. The total amount of DIBOA and DIMBOA exuded showed a significant correlation to the growth inhibition in bioassay with a statistically estimated contribution to the overall allelopathic effect of 48−72%. In a bioassay with pure phytotoxins, Bx concentrations consistent with the amounts quantified in the screening bioassay caused detrimental effects on *S. alba* and almost reproduced the statistically estimated contribution. The observed causal association between the allelopathic activity under laboratory conditions and the exudate concentrations of Bx suggests that this association might have implications for the interference of Triticeae species in natural plant communities.

KEYWORDS: Allelopathy; DIBOA; DIMBOA; root exudation; rye; wheat

INTRODUCTION

Benzoxazinoids (Bx) are major secondary metabolites found among Acanthaceae, Lamiaceae, Poaceae, Ranunculaceae, and Scropulariaceae (*1*). Because of their multifunctional toxicity, these metabolites act as natural pesticides against pathogens, insects, and weeds. Bx are stored as inactive (2*R*)-2-β-D-glucosides in the vacuole, and the actual bioactive aglucones are formed by enzymatic deglycosylation. The formation of toxic aglucones occurs if the spatial segregation between glucosides and β-glucosidases is overcome, which generally occurs as the integrity of cell organelles is destroyed following wounding or tissue death (*2*−*4*). Furthermore, recent studies indicated an intrinsic regulation of aglucone formation in intact plant tissue triggered by plant stress or plant age (*5*−*10*). Induced release of free aglucones in intact plant parts prior to systemic translocation is thought to involve spatial reallocation to affected tissues and/or release via root exudation (*5*−*7*). This capacity implicates Bx in constitutive and induced host−plant defenses and suggests an involvement in allelopathy of decomposing plant residues and root exudates (*5*, *11*−*16*).

The discovery that Bx function as natural phytotoxins initiated attempts to utilize these compounds for weed suppression. Because the use of these unstable compounds as natural herbicides is rather limited (*17*, *18*), efforts focused on allelopathy approaches, where the degradation is compensated by a continuous release. In annual crops, root exudation of the phytotoxin by the crop has been suggested to be the most effective method of allelochemical delivery for weed control (*19*). The fact that Bx are released from intact roots expels them as potential first-line allelochemicals responsible for observed allelopathic effects in Triticeae species (*14*−*16*, *20*). However, despite the fact that Triticeae species produce and exude Bx, it is still unclear whether their release into the environment has significant implications for allelopathic interactions.

In Triticeae species, two Bx aglucones are believed to play an important role: DIBOA [2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one] and its 7-methoxy derivative DIMBOA [2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one] (**Figure 1**). Research has so far concentrated on the evaluation of tissue

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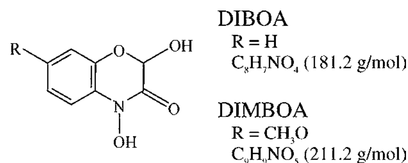

Figure 1. Structures of the two Bx aglucones mentioned in the text.

levels, and only few data exist for exudation of these aglucones by Triticeae species. In *Secale cereale* L., DIBOA is the predominant Bx found in plant tissues as well as in root exudates, while DIMBOA is believed to play a negligible role (*8*, *13*, *18*, *21*). In *Triticum aestivum* L., DIMBOA is the main aglucone and although DIBOA was detected in plant tissues, root exudates contain primarily DIMBOA (*10*, *21*−*25*). In contrast, both aglucones along with other weak bioactive Bx were exuded by a *Triticum durum* Desf. cultivar (*20*). The level of Bx exudation proved to be highly cultivar-dependent (*14*, *23*−*25*) and changed with plant age (*20*). Although investigations with Triticeae species suggested a contribution of Bx to observed overall phytotoxicity of exudates, a causal association between quantified levels of Bx and cultivar or age specific phytotoxicity of exudates could not be established under laboratory conditions (*20*, *25*). Thus, divergent perceptions exist as to whether root exudation of Bx provides concentrations of a phytotoxic level sufficient to cause allelopathic effects, especially since Bx are chemically unstable when in solution and rapidly degraded by microorganisms (*26*−*28*).

The overall aim of the present study was to investigate if the release of Bx by intact plant roots is a determining causal factor for the overall phytotoxicity of root exudates under laboratory conditions. Therefore, a biological screening was conducted to assess the variation in phytotoxicity of root exudates on *Sinapis alba* L. in 146 cultivars of four Triticeae species, namely, *T. aestivum, T. durum, Triticum spelta* L., and *S. cereale*. The bioassay was conducted as a dose−response experiment with varying densities, and the cultivar specific level of phytotoxicity was quantified by the density causing 50% test−plant response (ED_{50} value). Simultaneously, the quantities of total Bx (DIBOA, DIMBOA) in the exudates were analyzed by high-performance liquid chromatography (HPLC) during the course of the bioassay. The capacity of Bx to account for observed test−plant bioassay response was statistically estimated by correlation coefficients for the linkage between the observed variability in ED_{50} values and the corresponding exudate concentrations of Bx. Finally, the statistically estimated contribution was substantiated in pure compound bioassays applying Bx at quantified exudate concentrations. Significant causal relationships would considerably progress the question of whether Bx are part of an allelochemical system used in the interference with surrounding plants in nature.

MATERIALS AND METHODS

Biological Screening. The allelopathic activities of 146 cultivars of *T. aestivum* ($N = 112$), *T. durum* ($N = 19$), *T. spelta* L. ($N = 7$), and *S. cereale* ($N = 8$) on *S. alba* cv. Albatros were evaluated with a plant-by-plant bioassay. The tested germplasm originated from 17 different countries and was comprised of currently used cultivars, as well as breeding lines and landraces. Seeds were obtained from breeders and institutional germplasm collections. The bioassay was conducted according to Belz and Hurle (*29*) in nonaxenic hydroponic culture under greenhouse conditions (24/16 °C, photoperiod 16 L:8 D, 300 $\mu E/m^2/s$). In brief, surface-sterilized donor seeds (1−2 h hot water sterilization at 45 °C, 5 h redrying at 30 °C) were pregerminated in a greenhouse in vermiculite (2/3 mm, BayWa, Germany) for 4−5 days until the first leaf was through the coleoptile. Donor plants were subsequently transferred to aluminum-covered glass beakers (Sturz-Form 290 mL, Weck, Germany) filled with aerated distilled water and raised for 1 day. *S. alba* was pregerminated wrapped in filter paper after surface sterilization [5 min, 70% ethanol; 10 min, 6.5% sodium hypochlorite (13% active chlorine); 30 min rinsing with tap water] starting 4 days prior to bioassay. The bioassay was conducted as a dose−response experiment whereby at each cultivar four *S. alba* plants at a time were cocultured for 6 days with seven different donor plant densities (0−30 plants/pot) in triplicate in a complete randomized design. The 146 cultivars were screened in 11 successive sets, each of 13−14 cultivars. Evaporation of distilled water was adjusted daily. The allelopathic potential was evaluated by recording the root length of *S. alba* after 6 days and calculating dose−response curves for the relative increase in root length according to Michel et al. (*30*) using SPSS regression models [method of Levenberg-Marquardt (*31*), $1/e^8$ convergence of error sum of squares]. Comparison of cultivar specific dose−response curves was done by horizontal assessment using the lack-of-fit F test ($P = 0.05$) (*30*, *32*, *33*). Finally, the 146 cultivars were differentiated based on ED_{50} values (plant density causing 50% inhibition in root growth of *S. alba*) by cluster analysis using SPSS. Initial partitioning was done by hierarchical cluster analysis [Ward method (*34*), squared Euclidean distance] and optimized by the k means method (*35*, *36*).

Chemical Screening. Root exudates were collected across the hydroponics bioassay either directly from the aqueous test medium or by trap solution technique (*37*, *38*).

Density-Dependent Variations. Differences in Bx concentrations in the test medium depending on the applied donor plant density were investigated for *T. aestivum* cv. Granada at the end of the bioassay. An aliquot of 12 mL/replicate was taken from the test medium at each density (x plants/pot/6 days; three replicates). Samples were subsequently concentrated at 40 °C under vacuum until dryness (Rotational Vacuum Concentrator RVC 2-25, Christ, Germany, with cooling trap), redissolved in 200 μL of water (20% acetonitrile), centrifuged (20 min, 20800 rcf), and analyzed by HPLC with a diode array detector (DAD). In addition, for *S. cereale* cv. Amilo, release rates at each density were determined by trap solution technique. Therefore, roots were at first rinsed in distilled water and plants subsequently were transferred for a period of 2 h (8−10 am) to aerated distilled water [x plants/100 mL (Geleeglas 230 mL, Weck, Germany); three replicates]. A pooled aliquot of 150 mL was concentrated 300-fold by solid phase extraction (ISOLUTE ENV^+, 200 mg, IST) and analyzed as above-mentioned by HPLC-DAD.

Diurnal Variations. To investigate diurnal variations in release rates of Bx, the root system of *S. cereale* cv. Amilo was six times bihourly immersed into aerated distilled water (30 plants/100 mL; three replicates; 13 day old plants) over a 12 h period (8 am−8 pm) using the same plants for each consecutive collection. At a time, an aliquot of 12 mL/replicate was concentrated 60-fold at 40 °C under vacuum and analyzed by HPLC-DAD. Means were subjected to analysis of variance with a multiple comparison test (Tukey's test, $P = 0.05$).

Dynamics of Bx within Root Exudates. The accumulation of Bx in the aqueous test medium during the bioassay was evaluated daily at the highest plant density (30 plants/pot; three replicates) by analyzing an aliquot of 12 mL/replicate of one randomly selected cultivar of each species. Samples were subsequently concentrated 60-fold at 40 °C under vacuum and analyzed as a pooled sample by HPLC-DAD. At the same time, changes in release rates for Bx were evaluated by the trap solution technique (30 plants/100 mL/2 h; three replicates). An aliquot of 12 mL/replicate was concentrated 60-fold at 40 °C under vacuum and analyzed as a pooled sample by HPLC-DAD.

Cultivar Specific Variations. At the end of the bioassay, a similar collection in trap solution was done at the highest plant density (30 plants/100 mL/2 h; three replicates; 12 day old plants) for 95 cultivars (seven sets of 13−14 cultivars). Aliquots were concentrated 60-fold at 40 °C under vacuum and analyzed by HPLC-DAD. Roots were harvested and dried at 120 °C to estimate the root dry weight.

HPLC Analysis. HPLC analysis was done on a Waters model chromatograph equipped with a DAD detector (Waters 991). A Synergi polar C-18 reversed phase column [250 mm × 4.6 mm (4 μm), Phenomenex, Germany] was used and was eluted with a gradient of 5% acetonitrile and 95% Na_2HPO_4 buffer (1 mM, pH 2.4, 10%

acetonitrile) for 0−8 min (0.65 mL/min flow rate), 30% acetonitrile and 70% Na_2HPO_4 buffer for 8−26 min (0.7 mL/min flow rate), 100% acetonitrile for 26−29 min (0.7 mL/min flow rate), 100% acetonitrile for 29−31 min (0.7 mL/min flow rate), and then reequilibrated to starting conditions. The injection volume was 50 μL. DIBOA and DIMBOA were identified and quantified at 220 nm. Retention times were 15.1 ± 0.5 min for DIBOA and 18.1 ± 0.5 min for DIMBOA. Quantitative analysis was done by internal calibration curves. The isolation of standard compounds was done from ethyl acetate extracts of *S. cereale* cv. Amilo for DIBOA and of *Zea mays* L. cv. Lorenzo for DIMBOA followed by a purification step using thin-layer chromatography (Alugram SIL G/UV254, 0.2 mm, Macherey-Nagel). Plates were developed in a mixture of *tert*-butyl methyl ether and 2,2,4-trimethylpentane (7:3 volume) ($R_{f\mathrm{DIMBOA}} = 0.2$, and $R_{f\mathrm{DIBOA}} = 0.3$). The purity of standards was verified by HPLC-DAD, and results were confirmed by HPLC-ESI$^-$-MS. Instrumental limits of quantification for Bx and their degradation products were determined at 220 nm according to Frehse and Thier (*39*) (0.05−0.5 μg/mL based on standard solutions) at 0.119 μg/mL for DIBOA, 0.196 μg/mL for DIMBOA, 0.085 μg/mL for benzoxazolin-2(3*H*)-one (BOA; obtained from Sigma-Aldrich), and 0.089 μg/mL for 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA; obtained from Sigma-Aldrich). Recovery rates with RVC concentration (0.01−0.1 μg/mL based on 12 mL aliquot) were 100.9 ± 4.8% for BOA and MBOA, 79.6 ± 10.3% for DIBOA, and 64.8 ± 12.3% for DIMBOA.

Correlation Analysis. The correlation between the cultivar specific allelopathic potential (ED_{50}) and the total release of Bx (DIBOA, DIMBOA) per root dry weight at the end of the bioassay (30 plants/100 mL/2 h) was assessed by linear regression analysis based on log-transformed data. The degree of covariance was quantified by the Pearson correlation coefficient (*r*) using SPSS.

Phytotoxicity of Pure Bx. A hydroponics bioassay investigated the sensitivity of *S. alba* to Bx at concentrations consistent with the amounts quantified in the test medium during the screening bioassay. Pregerminated seedlings of *S. alba* were individually transferred to aluminum-covered scintillation vials (8 mL) filled with nonaerated nutrient solution [nutrients according to Dannel et al. (*40*)] plus 1% (volume) acetone as a solvent for Bx. Controls were performed with acetone only. Concentrations of Bx were equivalent to those quantified for *T. durum* cv. Bani-Sowif, the most suppressive durum cultivar in the biological screening. DIBOA and DIMBOA were tested solitary or as a mixture (1:1 weight) at the highest quantified concentration (11 μg/mL). Furthermore, the aglucones were applied in mixtures equivalent to the observed dynamics of accumulation in the test medium. The sequence of concentrations (**Figure 7a**) was accomplished by a daily exchange of test solution. Isolation of Bx was done from ethyl acetate extracts of *S. cereale* and *Z. mays* as described above. The bioassay was conducted under greenhouse conditions with 10 replications in complete randomized blocks. The root length of *S. alba* was evaluated after 0 and 6 days, and the increase in root length was analyzed by means of an analysis of variance with a multiple comparison test (Tukey's test, $P = 0.05$) using SPSS.

RESULTS AND DISCUSSION

Biological Screening. Cultivars differed significantly in their efficacy to suppress the root growth of *S. alba.* The ED_{50} values for the 146 cultivars tested varied between <3 to >13 plants/pot and could be differentiated by cluster analysis into six clusters (**Figure 2**). The most effective cluster 1 (ED_{50} = 2.7−3.7 plants/pot) consisted of 10.3% of the tested cultivars and contained cultivars of *T. aestivum*, *T. durum*, and *S. cereale.* Clusters 2 and 3, each consisting of 37.4 ± 0.4% of the tested cultivars, made up the biggest groups composed of all species. The cluster size decreased again with increasing ED_{50}, with the weakest cluster 6 (ED_{50} = 12.0−13.7 plants/pot) being the smallest one (2.7%) and containing only cultivars of *T. aestivum* and *T. durum*. A strong or weak allelopathic potential was thus found only in a small percentage of the tested cultivars. The most suppressive cultivar (*S. cereale* cv. Forrajero Baer) was

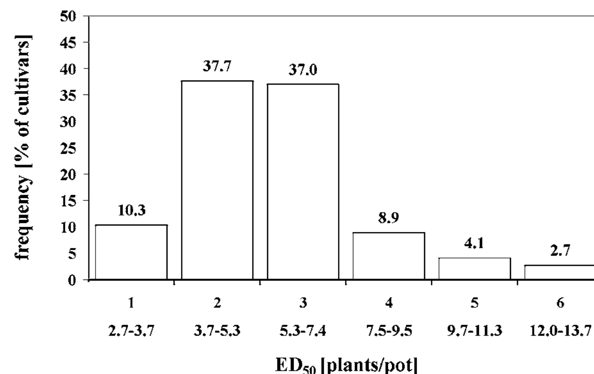

Figure 2. Frequency distribution of the allelopathic activity over six clusters in 146 cultivars of four Triticeae species. ED_{50} = plant density causing 50% inhibition in root growth of *S. alba*.

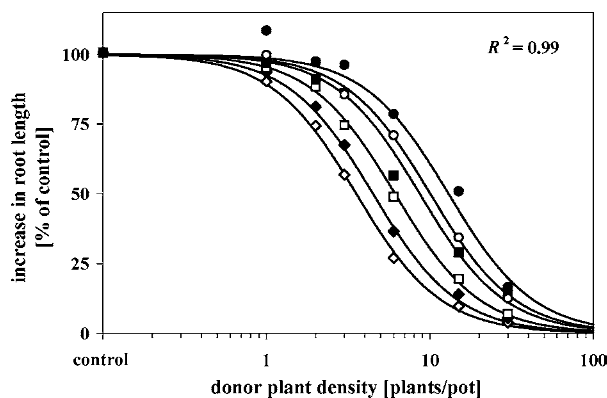

Figure 3. Clustered dose–response curves for 146 cultivars of *Triticum* spp. and *S. cereale* affecting *S. alba*. ◇, cluster 1 (ED_{50} = 3.3 plants/pot a); ◆, cluster 2 (ED_{50} = 4.4 b); □, cluster 3 (ED_{50} = 6.1 c); ■, cluster 4 (ED_{50} = 8.4 d); ○, cluster 5 (ED_{50} = 10.4 e); ●, cluster 6 (ED_{50} = 12.9 f). ED_{50} = mean plant density of clusters causing 50% reduction in root growth of *S. alba*; small letters indicate significant differences (*F* test, $P = 0.05$); R^2 = 1 − residual SS/corrected SS.

five times more effective than the weakest cultivar (*T. durum* cv. Kausnak tohumiu). Frequency distribution of the allelopathic activity was log-normal (skewness = 0.40; standard error = 0.20; Kolmogorow−Smirnow test) and, thus, confirmed a continuous genetic variation for the allelopathic trait among the tested cultivars. This is in accordance with previous observations in *Oryza sativa* L. and *T. aestivum*, leading to the general assumption that allelopathy is a quantitative trait (*41*−*43*).

Investigations with *O. sativa* suggest that several active compounds that are selective against specific weed species might be responsible for the observed selective allelopathic activity of *O. sativa* cultivars (*41*, *44*, *45*). Theoretically, the dose−response design should discriminate such differences in the compositions of active root exudates by nonparallel dose−response relationships. If a similar selectivity exists among the tested Triticeae cultivars, it should be parametrized depending on the slope of test−plant responses. However, none of the 146 cultivars tested in this study showed a significant nonparallel dose−response relationship. Intra- and interspecific comparisons of response curves revealed a parallel displacement along the *x*-axis for all cultivars. **Figure 3** shows the clustered dose−response curves calculated for the means of all cultivars belonging to each of the six clusters. The response patterns did not provide an indication of selectivity in allelopathic effects among and between the tested cultivars of *Triticum* spp. and *S. cereale*. By assuming the parallel line concept (*32*, *46*, *47*), observed parallel dose−response patterns may indicate dose-

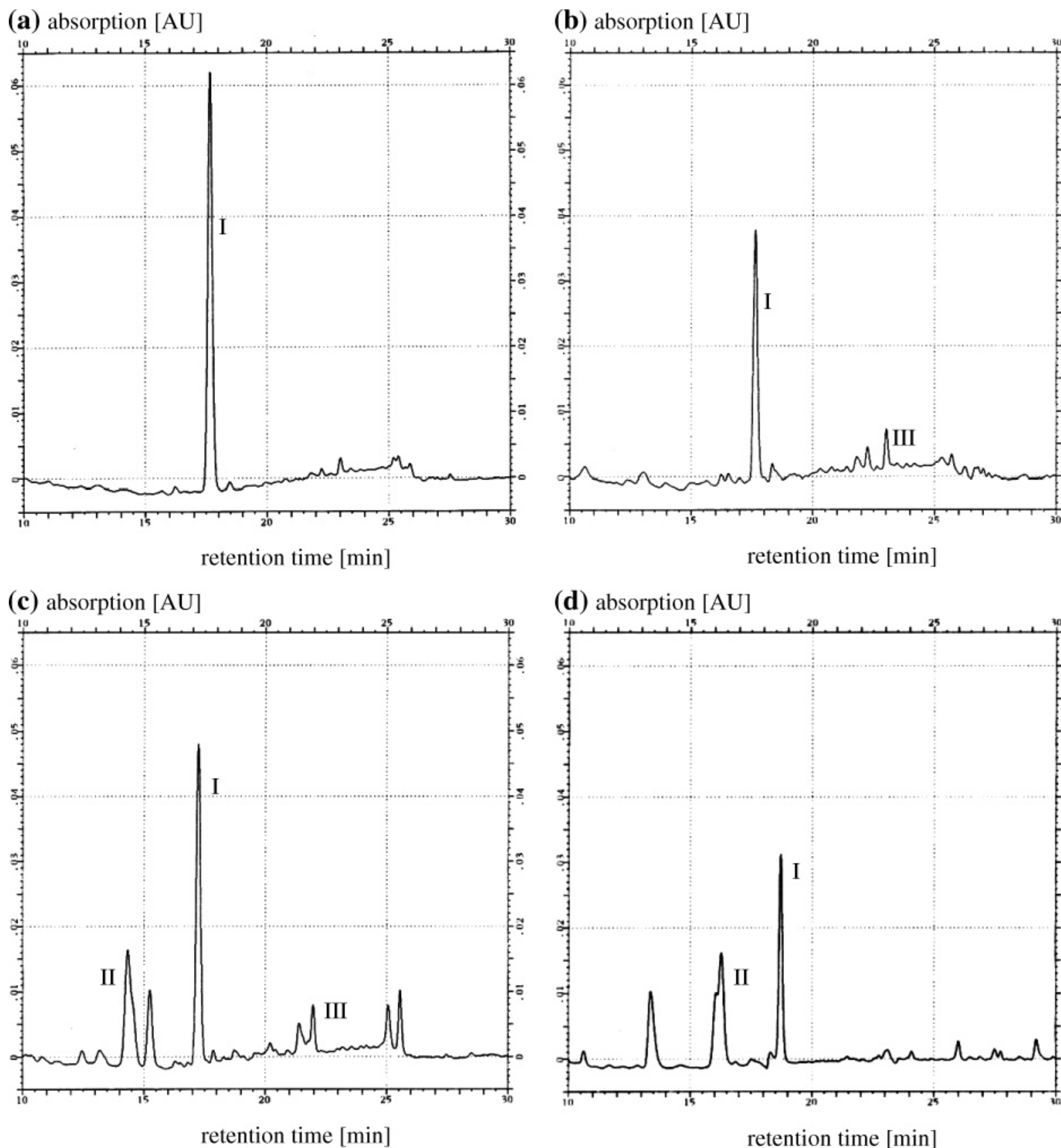

Figure 4. HPLC-DAD chromatograms (220 nm) of the root exudates of 12 day old *Triticum* spp. and *S. cereale* (30 plants/100 mL/2 h; 12 mL aliquot; factor of concentration 1:60). (**a**) *T. aestivum* cv. Visperterminen 644IC; (**b**) *T. spelta* cv. Hercule; (**c**) *T. durum* cv. Bani-Sowif; and (**d**) *S. cereale* cv. Forrajero Baer. I = DIMBOA, II = DIBOA, and III = MBOA.

dependent differences in the growth-inhibiting effect and, thus, quantitatively different but qualitatively similar compositions of active root exudates. This indicates that the growth inhibition by *Triticum* spp. and *S. cereale* may rely upon identical allelochemicals, analogues, or at least allelochemicals similar in their mode(s) of action.

The most suppressive cultivar in this study, the Chilean *S. cereale* cv. Forrajero Baer (Campex Semillas Baer), has been previously characterized by a high exudation of DIBOA, which is supposed to be responsible for the substantial weed suppressive ability of this cultivar under field conditions (*14*, *15*). This suggested that Bx exudation by this cultivar might also have played a significant role for its high allelopathic activity in the current bioassay.

Chemical Screening. For all tested cultivars, DIBOA and DIMBOA were the leading compounds in root exudates of 12 day old plants collected with the trap solution technique. The prevalence of the two Bx analogues would confirm the hypothesis of similarity of active allelochemicals indicated by the parallel dose−response patterns within the biological screening since the bioactivity of the aglucones is based upon identical reactive groups and, thus, similar mode(s) of action (*18*, *48*, *49*).

Specifity of Root Exudates. Qualitative differences in the exudation of both aglucones occurred only between species, while no intraspecific variations were observed between cultivars. In exudates of the two hexaploid wheat species *T. aestivum* ($N = 67$) and *T. spelta* ($N = 7$), DIMBOA was the only Bx detectable (**Figure 4a**,**b**). The distinct appearance of DIMBOA in *T. aestivum* is in accordance with previous findings of Wu et al. (*23*−*25*), whereas to our knowledge no reports exist about Bx exudation by *T. spelta.* Exudates of the tetraploid wheat

species *T. durum* ($N = 19$) contained both aglucones with a predominance of DIMBOA (**Figure 4c**). This confirms recent findings of Huang et al. (*20*). As in *T. durum*, exudates of *S. cereale* ($N = 4$) contained detectable amounts of both aglucones (**Figure 4d**). All results were confirmed by HPLC-ESI^{-}-MS analysis.

It remains speculative what caused the observed qualitative differences in Bx exudation among the Triticeae species. The biosynthetic pathway for Bx has been suggested to be similar in all Triticeae species, with seven Bx genes encoding the appropriate enzymes catalyzing DIMBOA formation via its demethoxylated precursor DIBOA (*50*−*52*). Supposedly because of a species specific regulation of Bx gene expression or even a partial loss of Bx genes, Triticeae species differ in their final Bx metabolite, leading to a characteristic accumulation of Bx glucosides in the tissue (*8*, *14*, *21*, *52*−*54*). Because a high accumulation of glycosidic DIMBOA in the root tissue seemed to be an essential prerequisite for its exudation by wheat (*24*), one could speculate that the appearance of Bx in exudates in general reflects the corresponding accumulation of their glycosidic precursors in the plant. In fact, reports on root tissue levels of Bx glucosides at plant ages concurrent with present exudate collection would support this hypothesis (*9*, *10*, *22*, *55*, *56*).

In *Triticum* spp., the level of polyploidy seems to determine Bx accumulation (*8*, *21*, *54*). Hexaploid species accumulate preferentially glycosidic DIMBOA and only low levels of DIBOA, while the tetraploid *T. durum* accumulates substantial levels of both glucosides (*22*, *52*, *55*−*58*). This would be in accordance with the observed specifity of Bx exudation. However, Pethö (*59*) detected both aglucones in root exudates of a *T. aestivum* cultivar; therefore, as previously assumed for DIMBOA (*24*), the observed absence of detectable quantities of DIBOA in root exudates of both hexaploid species could merely reflect low tissue levels of glycosidic DIBOA by the time of current exudate collection.

Although in *S. cereale* DIBOA is the designated predominant final metabolite, in root exudates of all tested 12 day old rye cultivars, both aglucones were detected. The same was previously reported by Pethö (*59*); while contradictory, a distinct DIBOA exudation by rye was found by other authors (*14*, *60*). In the present work, the exudation of DIMBOA by living rye roots clearly shows that *S. cereale* has a capacity to biosynthesize and exude both aglucones. This assumption is further substantiated by the fact that shoot and root tissue of *S. cereale* seedlings contained low levels of glycosidic DIMBOA (*4*, *9*). Hence, unless there is no alternative pathway for DIMBOA, under the conditions of the present bioassay, conversion of DIBOA to DIMBOA predominated over β-glycosylation for vacuolar storage and, thus, DIMBOA exudation might be even more relevant for observed allelopathic effects in rye. This suggests that besides genetic factors, environmental or methodological factors may impact the specifity of Bx exudation as well.

The conditions and methods employed for collection and analysis of root exudates can significantly impact qualitative and quantitative exudation patterns (*38*). Hence, methodological aspects need a critical evaluation before drawing definite conclusions about exudate constituents. Our approach employed an aqueous trap solution for the collection of water soluble compounds exuded by plant roots after a preceding long-term exposure to distilled water prior to subsequent concentration by vacuum evaporation and final HPLC-DAD detection of Bx. This approach seemed most appropriate to assess the relevancy

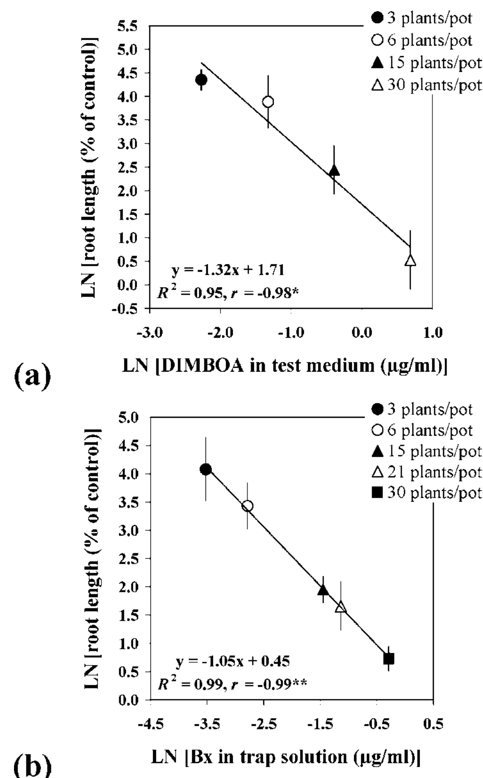

Figure 5. Correlation between density-dependent inhibition of root length of *S. alba* by root exudates of *T. aestivum* (TRZAX) cv. Granada (**a**) and *S. cereale* (SECCE) cv. Amilo (**b**) and density-dependent increase in amount of DIMBOA in the test medium (*x* plants/pot/6 days; 12 day old plants) or DIMBOA and DIBOA (Bx) in trap solution (*x* plants/100 mL/2 h; 13 day old plants). R^2 = coefficient of determination; r = Pearson correlation coefficient (* significant at $P = 0.05$; ** significant at $P = 0.01$).

of Bx exudation under the conditions of the present bioassay and indicated that in this case the exudation of DIMBOA could be relevant for observed allelopathic effects in all tested Triticeae species, while a contribution of DIBOA seemed to be restricted to *T. durum* and *S. cereale*.

Density-Dependent Variations. The analysis of root exudates of *T. aestivum* cv. Granada and *S. cereale* cv. Amilo at different plant densities showed that the amount of Bx in the test medium or in trap solution increased with donor plant density. Accordingly, there appeared to be a strong association between donor density, concentration of Bx in the test medium or in the trap solution, and inhibition of root growth of *S. alba* (**Figure 5**). Although this is not sufficient for assuming a causal contribution of Bx to the observed effects, it is a necessary condition for any plant-generated allelochemical involved in density-dependent phytotoxicity. On the basis of these results, further exudate collections were done at the highest plant density. This approach should also reduce the variability of results with genetically nonhomogeneous cultivars, especially in landraces and population cultivars.

Diurnal Variations. Pethö et al. (*61*) assumed a distinct rhythm of Bx exudation based on the diurnal pattern of secretion of DIMBOA observed for a *Z. mays* cultivar. Hence, the time of exudate collection could be crucial for recovery of Bx especially in trap solution. Investigating the pattern of Bx exudation for *S. cereale* cv. Amilo over a period of 12 h revealed that the release of DIBOA and DIMBOA was quantitative, positively related, and significantly enhanced at the first 2 h collection between 8 and 10 am. In the following five measure-

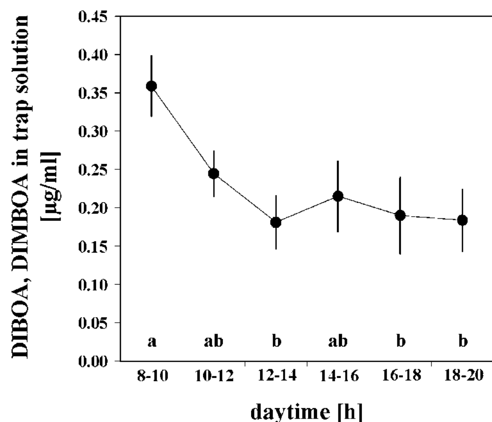

Figure 6. Bihourly exudation of DIBOA and DIMBOA by *S. cereale* cv. Amilo during a 12 h period using the same plants for each collection (30 plants/100 mL/2 h; 13 day old plants). Small letters indicate significant differences (Tukey's test, $P = 0.05$).

ments, plants released on average 43.5 ± 7.5% lower amounts of Bx (**Figure 6**). Diurnal rhythms are well-known for the phytosiderophores of poaceous species (*37*, *62*, *63*), and there might be a regulative link, since Bx can also act as a chelator for iron and copper (*64*, *65*). For the phytosiderophores, it is suggested that the diurnal rhythm indicates a controlled release mechanism (*37*). Thus, an active release as previously suggested by Pérez and Ormeño-Nuñez (*14*) may also be indicated in the case of Bx rather than a simple passive diffusion. Another possible explanation for the observed temporal differences could be an accumulation of Bx at the root surface during the preceding culture period, which would primarily enhance the amount of Bx collected within the first period. A corresponding precipitation on the root surface was previously reported for 2-amino-3*H*-phenoxazin-3-one (APO), a microbial degradation product of DIBOA (*66*), and could be relevant for Bx as well. Although the root system was rinsed in distilled water prior to exudate collection, an adsorption at the root surface cannot be completely excluded. To provide conclusive proof of a distinct diurnal rhythm, it will therefore be necessary to collect exudates over a 24 h period using different plants for each collection. Nevertheless, further exudate collections in trap solution were done between 8 and 10 am following thorough washing of the root system.

Dynamics of Bx within Root Exudates. In grasses, Bx are de novo biosynthesized upon germination and maximum concentrations of glycosidic Bx appear in plant tissues soon after germination. As the plant starts autotrophic growth, concentrations gradually decline and substantial levels are then restricted to young tissues (*8*, *22*, *67*, *68*). A concurrent dynamics occurs for Bx gene expression and the activity of the enzymes involved in vacuolar storage and deglycosylation (*9*, *10*, *51*, *69*). Hence, an intrinsic temporal regulation of Bx biosynthesis and storage by plant age has been suggested (*8*, *9*). Cambier et al. (*7*) assumed that an enhanced activation of glycosidic Bx commences with release of the elevated amounts of free aglucones by the plant, which should inevitably result in a corresponding periodic appearance of Bx in exudates. Huang et al. (*20*) observed a transient dynamics of Bx in the test medium for a *T. durum* cultivar under ECAM conditions (equal compartment agar method), and thus, the exudation of Bx should change across the current bioassay as well.

The consecutive chemical analysis of Bx across the bioassay confirmed the assumption of a transient appearance of Bx in the test environment for all species tested. **Figure 7** shows the

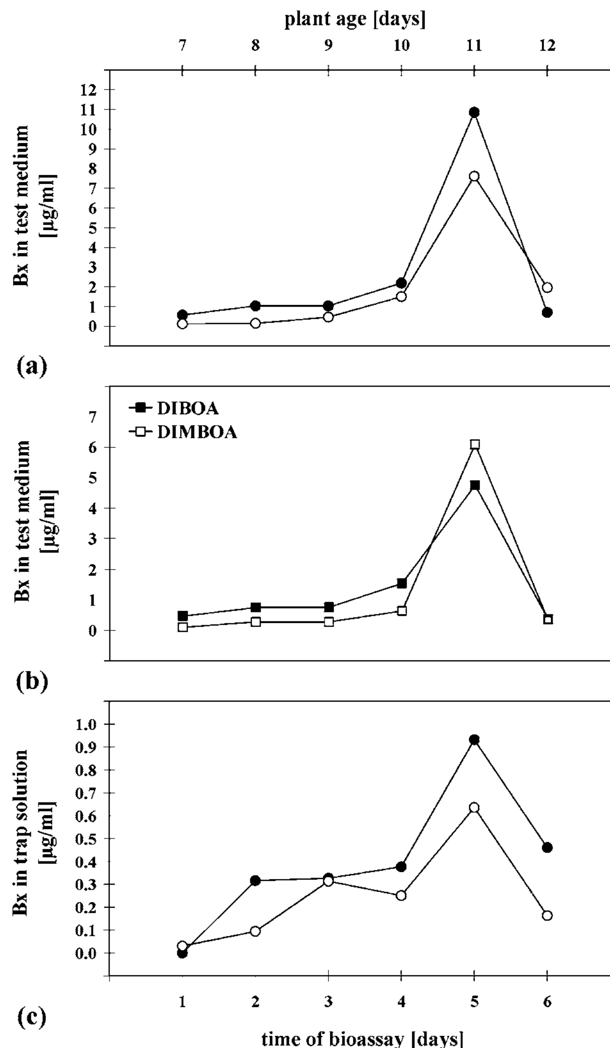

Figure 7. Exudation of Bx (DIBOA, DIMBOA) by *Triticum* spp. depending on time of bioassay. (**a**) Content of Bx in hydroponic culture of *T. durum* cv. Bani-Sowif (●) and *T. spelta* cv. Ostar (○) (30 plants/pot); (**b**) proportions of DIBOA and DIMBOA in hydroponic culture of *T. durum* cv. Bani-Sowif (30 plants/pot); and (**c**) release rates for Bx by *T. durum* cv. Bani-Sowif (●) and *T. spelta* cv. Ostar (○) (30 plants/100 mL/2 h).

dynamics of Bx exemplarily for two Triticeae species, whereby an equivalent trend was observed for the remaining species as well. The concentration of Bx in the test medium gradually increased, reaching a pronounced peak on day five of the bioassay (11 day old plants) and subsequently decreased (**Figure 7a**). Maximum concentrations of total Bx were 10.9 µg/mL for *T. durum* cv. Bani-Sowif ($ED_{50} = 3.1$ plants/pot) and 7.6 µg/mL for *T. spelta* cv. Ostar ($ED_{50} = 4.2$ plants/pot). Huang et al. (*20*) observed a maximum concentration on day six of their bioassay (8 day old *T. durum* plants). For the period of the bioassay, DIMBOA was the only Bx detected for the hexaploid wheat cultivar, while in the case of *T. durum* cv. Bani-Sowif both aglucones appeared continuously in the test medium (**Figure 7b**). The occurrence of DIBOA was positively related to DIMBOA (Pearson correlation coefficient, $r = 0.98$; significant at $P = 0.01$). Huang et al. (*20*) observed changes in specifity of Bx exudation for *T. durum* cv. Khapli. Across a growth period of 15 days, DIMBOA was only detectable between 4 and 6 days, while DIBOA appeared continuously. These findings clearly demonstrate that knowing the kinetics of exudation is essential for the recovery of Bx in root exudates. An et al. (*70*)

stated that such periodic dynamics may be a general reason for disparate results of allelopathic research. It remains to be investigated if this is one of the reasons for the above-mentioned inconsistent reports on DIMBOA exudation by *S. cereale* and DIBOA exudation by wheat (*14*, *59*, *60*).

Analyzing the release rates for Bx by the trap solution technique revealed highly consistent dynamics, with a maximum on day 5 amounting to 0.47 μg/mL/h for cv. Bani-Sowif and 0.32 μg/mL/h for cv. Ostar (30 plants/100 mL) (**Figure 7c**). Extrapolating these release rates for an entire day suggests that theoretically cv. Bani-Sowif should build up a concentration of 11.2 μg/mL in the test environment and cv. Ostar 7.6 μg/mL. The actual concentrations quantified by HPLC-DAD deviated from theses estimates just 3.2% for cv. Bani-Sowif and 0.3% for cv. Ostar. This clearly shows that the active concentration of Bx in the test medium was a direct function of the continuous supply via root exudation without any sustainable accumulation in the aqueous test solution. The concentration of the unstable aglucones rapidly declines in the absence of a subsequent supply, which should be mainly attributable to a chemical or microbial decomposition to the corresponding benzoxazolinones, which are further degraded by microorganisms to form phenoxazinones (*26*, *28*, *71*). In addition, it could be demonstrated that exogenously applied Bx can be taken up by plants and detoxified by the same glucosyltransferases accompanying the biosynthesis of Bx (*72*). Thus, reabsorption and detoxification by the donor plants could have decreased the Bx peak as well.

In summary, it is well-documented that Bx biosynthesis and remobilization in plants follow a distinct transient pattern, which is triggered by plant age. Current findings confirmed an equivalent dynamics in root exudation of Bx and a corresponding fate in the environment. It remains to be evaluated if the transient accumulation pattern of glycosidic Bx in plant tissue is responsible for the observed dynamics of Bx exudation. Nevertheless, results indicate that if Bx exudation contributes to observed allelopathic effects, inhibition of the receiver species at the end of the bioassay results from temporarily changing concentrations of DIBOA and/or DIMBOA and maybe as well as their phytotoxic breakdown products.

Cultivar Specific Variations. The highest variation in Bx exudation between cultivars of all Triticeae species tested resulted for the measurement of relative release rates per root dry weight in trap solution, while the assessment of Bx accumulation in test medium at the end of the bioassay proved to be a relatively coarse parameter to distinguish cultivar specific differences. Therefore, the quantitative chemical screening was done by trap solution technique. The release of Bx in trap solution by 12 day old plants differed significantly for the 95 cultivars, and the frequency distribution of total Bx exudation was log-normal (skewness = 0.04, standard error = 0.25; Kolmogorov−Smirnov test). This indicates that Bx exudation is a quantitative trait as well. Among the five cultivars with the highest exudations were cultivars of *T. aestivum*, *T. durum*, and *S. cereale* with an average maximum release rate of total Bx of approximately 2.0 μg/mg/day referring to root dry weight (**Table 1**). The five cultivars with the highest exudations released on average 15 times more Bx than the five cultivars with the lowest exudations. A high cultivar dependence of DIMBOA exudation was previously shown by Wu et al. (*23*−*25*) analyzing the root exudates of 58 cultivars of *T. aestivum*. However, they found no clear association with phenotypic measurements of allelopathic potential on *L. rigidum*. At present, the five cultivars with the lowest Bx exudations were on average of their ED_{50} values (8.7 plants/pot) 2.4 times less inhibitory in the bioassay than the five cultivars with the highest exudations (3.6 plants/pot). The observed differences in allelopathic activity of cultivars with high/low exudations were the first indication for an association between the Bx exudation and the observed phytotoxic effect on *S. alba*. The following correlation analysis investigated this assumption for the entire spectrum of tested cultivars.

Table 1. Release Rates Per Root Dry Weight for Total Bx (DIBOA, DIMBOA) of the Five Cultivars with the Highest/Lowest Exudations in Comparison with the Five Most/Least Allelopathic Cultivars (30 Plants/100 mL/2 h; 12 Day Old Plants; $N = 95$)

feature	release rate for total Bx per root dry weight (μg/mg/day): mean ($N = 5$)		cultivars
highest exudation	1.98 ± 0.38[a]	2.54 ± 1.21[a]	TRZAX[b] cv. Visperterminen 644IC
		2.14 ± 0.54	TRZDU cv. Imbar
		1.86 ± 0.61	SECCE cv. Forrajero Baer
		1.82 ± 0.32	TRZDU cv. Topdur
		1.55 ± 0.30	TRZDU cv. Grandur
lowest exudation	0.12 ± 0.02	0.14 ± 0.01	TRZAX cv. Runal
		0.14 ± 0.01	TRZAX cv. Reaper
		0.11 ± 0.01	TRZAX cv. Alcedo
		0.10 ± 0.01	TRZAX cv. Ramiro
		0.09 ± 0.03	TRZAX cv. Trakos
most allelopathic	1.58 ± 0.62	2.54 ± 1.21	TRZAX cv. Visperterminen 644IC
		1.86 ± 0.61	SECCE cv. Forrajero Baer
		1.29 ± 0.74	SECCE cv. Protector
		1.14 ± 0.02	TRZDU cv. Bani-Sowif
		1.05 ± 0.06	TRZAX cv. Rütti 40
least allelopathic	0.23 ± 0.17	0.51 ± 0.04	TRZDU cv. Izmir
		0.24 ± 0.04	TRZDU cv. Kausnak tohumiu
		0.18 ± 0.06	TRZAX cv. Zenith
		0.11 ± 0.01	TRZAX cv. Alcedo
		0.10 ± 0.01	TRZAX cv. Ramiro

[a] Standard deviation. [b] TRZAX, *T. aestivum*; TRZDU, *T. durum*; and SECCE, *S. cereale*.

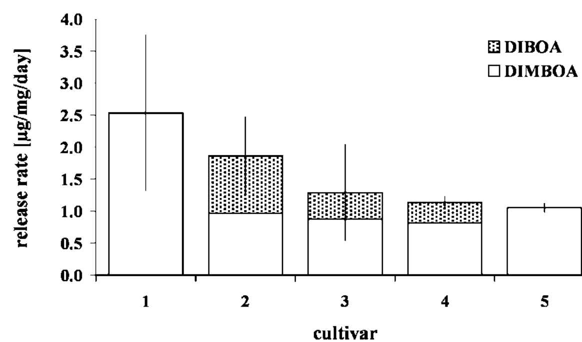

Figure 8. Relative release rates per root dry weight for DIBOA and DIMBOA by the five most allelopathic Triticeae cutivars tested ($N = 95$; 12 day old plants). (1) *T. aestivum* cv. Visperterminen 644IC (ED_{50} = 2.7 plants/pot); (2) *S. cereale* cv. Forrajero Baer (ED_{50} = 2.7); (3) *S. cereale* cv. Protector (ED_{50} = 3.3); (4) *T. durum* cv. Bani-Sowif (ED_{50} = 3.1); and (5) *T. aestivum* cv. Rütti 40 (ED_{50} = 3.5). ED_{50} = plant density causing 50% reduction in root growth of *S. alba*.

Correlation Analysis. The five most suppressive cultivars examined within chemical screening released on average 1.6 μg/mg/day of total Bx at the end of the bioassay, which was just 20% less than for the five cultivars with the highest exudations (**Table 1**). Two of the five most suppressive cultivars were among the five cultivars with the highest exudations. Regarding the specifity of Bx exuded by these five cultivars would suggest that a high allelopathic effect in bioassay could either depend on a high accumulation of DIMBOA alone or a mixture of smaller amounts of both aglucones (**Figure 8**). Hence, the observed specifity of Bx exudation was rather less

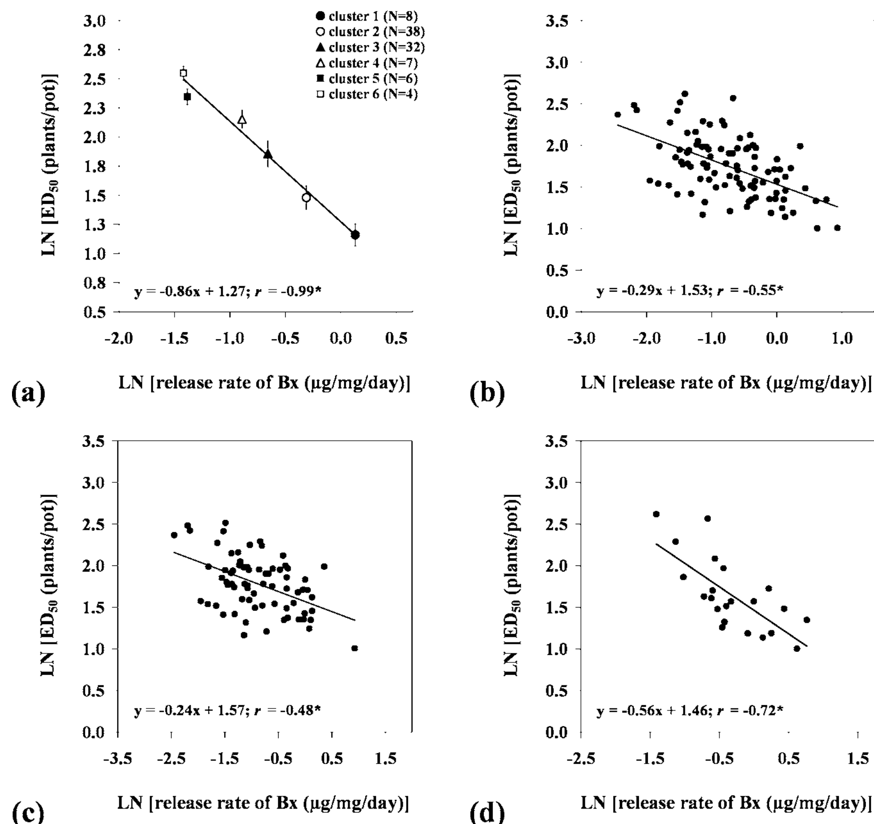

Figure 9. Correlation between relative release rates for total Bx (DIBOA, DIMBOA) in 95 cultivars of *T. aestivum* ($N = 67$), *T. durum* ($N = 19$), *T. spelta* ($N = 5$), and *S. cereale* ($N = 4$) (30 plants/100 mL/2 h; 12 day old plants) and the growth inhibiting ability (ED_{50}) against *S. alba* in bioassay. (**a**) Clustered data ($N = 95$); (**b**) discrete data ($N = 95$); (**c**) discrete data of *T. aestivum* and *T. spelta* ($N = 72$); and (**d**) discrete data of *T. durum* and *S. cereale* ($N = 23$). ED_{50} = plant density causing 50% reduction in root growth of *S. alba*; r = Pearson correlation coefficient for log-transformed data; * significant at $P = 0.01$.

important for the allelopathic effect on *S. alba* than the total quantity of both aglucones. However, the strong electron-donating 7-methoxy group present in DIMBOA enhances its bioactivity as compared to DIBOA (*18*, *49*) and in comparison a predominantly DIBOA-exuding cultivar could be less allelopathic. The most allelopathic cultivars exuded seven times higher amounts of Bx than the five least suppressive cultivars, whereby two of these were among the five cultivars with the lowest exudations.

Correlation Coefficients. Correlating the clustered means of cultivar specific ED_{50} in bioassay with the relative rates of Bx exudation for all tested species showed a strong negative association. Cultivars of the most allelopathic cluster 1 exuded approximately five times higher amounts of Bx than cultivars of the least allelopathic cluster 6 (**Figure 9a**). The variability in exudation between clusters could explain 99% of the observed cluster specific allelopathic activity, which certainly overestimates the real association but evidently shows that there is a significant relationship. Evaluating the linkage for cultivar specific data, which provides a more realistic estimation of covariation, revealed a medium association with a correlation coefficient of −0.55 (**Figure 9b**). This suggested that under the conditions of the present bioassay 55% of the observed phenotypic variation was statistically linked with the differential exudation of total Bx. However, the medium degree of covariation indicates that Bx does not exclusively account for observed phytotoxicity, raising the question of what else might be involved in allelopathic traits expression of the tested cultivars. Wu et al. (*25*) demonstrated that the cultivar specific allelopathic activity of *T. aestivum* on *L. rigidum* could be explained to 51% by the variation of total phenolic acids in root exudates. Taking both studies into account, it is indicated that crop allelopathy of *T. aestivum* could mainly result from a joint action of phenolic acids and Bx. This assumption would be substantiated by the findings of Huang et al. (*20*), demonstrating that the transient allelopathic activity of *T. durum* cv. Khapli on *L. rigidum* could be explained by 91% by the variation of seven phenolic acids, DIBOA, and DIMBOA in its root exudate. Comparative investigations on root exudation of phenolic acids and root allelopathy of *S. cereale* and *T. spelta* are absent.

The correlation analysis for the DIMBOA-exuding species *T. aestivum* and *T. spelta* revealed a slightly lower coefficient of correlation of about −0.48 (**Figure 9c**). In contrast, a stronger association with a coefficient of correlation of −0.72 resulted for *T. durum* and *S. cereale* (**Figure 9d**). Hence, the contribution of Bx to the overall phytotoxicity of root exudates on *S. alba* proved to be species-dependent and increased in the order *T. spelta* < *T. aestivum* < *T. durum* < *S. cereale*. The association of Bx to the overall allelopathic effect seemed to be favored if both, DIBOA and DIMBOA, were exuded. The correlation coefficient calculated for the 19 *T. durum* cultivars ($r = -0.69$) closely coincides with the level of association quantified for DIMBOA and DIBOA by Huang et al. (*20*) for *T. durum* cv. Khapli ($R^2 = 0.68$) under ECAM conditions. Thus, whether the current single-point assessment of exudate data was correlated with the test−plant bioassay response or the dynamics of Bx within root exudates (*20*), the exudation of DIBOA and DIMBOA would correspondingly account for approximately 69% of the variation in phytotoxicity of *T. durum* root exudates.

In summary, the correlation analysis confirmed the hypothesis that Bx are significantly associated with root allelopathy in

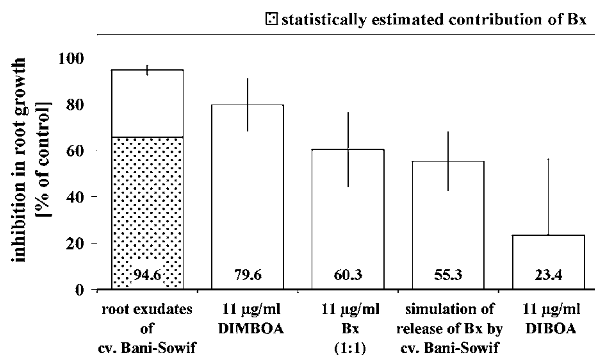

Figure 10. Phytotoxicity on root growth of *S. alba* by pure Bx (DIBOA, DIMBOA) treatments or root exudates of *T. durum* cv. Bani-Sowif (30 plants/pot/6 days).

Triticeae species. Nevertheless, a statistical significant association leaves uncertainty whether there is a direct causal correlation.

Phytotoxicity of Pure Bx. The maximum concentration of total Bx in the test medium quantified during the bioassay for the most inhibitory *T. durum* cultivar Bani-Sowif was approximately 11 μg/mL (30 plants/pot/5 days) (**Figure 7a**). Although Bx have been shown to have broad spectrum phytotoxic activity, a concentration consistent with the observed maximum is most likely insufficient to cause significant growth inhibition in a range of plant species such as *Avena fatua* L., *Echinochloa crus-galli* (L.) P. Beauv., or *Lepidium sativum* L. (*13*, *73*). However, for susceptible species such as *Amaranthus retroflexus* L. or *Lactuca sativa* L., a concentration of 11 μg/mL is adequate to provide a certain extent of growth inhibition (*17*). In the case of *Lemna paucicostata* Hegelm., a one-time application of 11 μg/mL of DIBOA even caused a reduction in leaf area of approximately 46% of control (*74*), suggesting that the observed level might be sufficient to have caused a negative effect on the sensitive species *S. alba* as well. In a hydroponics bioassay, *S. alba* was exposed to an artificial mixture of DIBOA and DIMBOA in concentrations quantified within root exudates of cv. Bani-Sowif. A single application of the maximum concentration of 11 μg/mL of either DIBOA or DIMBOA or a mixture of both inhibited the root growth of *S. alba* (**Figure 10**). Most inhibitory was DIMBOA with a reduction in root growth of 79.6% of control, while DIBOA was least inhibitory with a reduction of 23.4%. Thus, DIMBOA was more bioactive on *S. alba* than its precursor DIBOA. The 60.3% inhibition caused by the mixture of both compounds was not significantly different from the inhibition caused by DIMBOA alone. This would be in accordance with the biological screening, with corresponding ED_{50} values for cultivars exuding equivalent amounts of DIMBOA alone or in combination with DIBOA. Observed inhibition of *S. alba* by pure phytotoxin treatments at the maximum concentration quantified clearly revealed that root exudation of Bx provided concentrations on a phytotoxic level. However, the applied phytotoxic mixture represented the day 5 exudate concentration and did not consider the observed dynamics of exudate composition with much lower concentrations at the beginning of the bioassay.

Simulating the observed dynamics of Bx within root exudates of cv. Bani-Sowif as shown in **Figure 7a**,**b** (30 plants/pot) by a daily adjustment of the test solution caused a significant inhibition in root growth of *S. alba* (55.3%). From the biological screening, we know that at a plant density of 30 plants/pot cv. Bani-Sowif caused an inhibition in root growth of about 94.6% (**Figure 10**). The correlation analysis estimated the putative contribution of Bx to the observed growth inhibition for *T. durum* cultivars at 69.4%; thus, theoretically, Bx should have accounted for an inhibition of 65.6%. Hence, with a discrepancy of 10.3%, the statistically estimated contribution of Bx to the overall allelopathic effect of *T. durum* cultivars could be reproduced by the pure phytotoxin treatment in equivalent concentrations. Consequently, the observed statistical correlation apparently illustrates a direct, causal relationship and constitutes that the accumulation of Bx aglucones in the test medium by Triticeae species was most likely sufficient to have caused detrimental effects on *S. alba*. This provides strong evidence that under the conditions of the present screening bioassay, Bx exudation was one of the crucial factors for observed phytotoxicity. The observed discrepancy may support the assumption that besides Bx aglucones also their phytotoxic, spontaneous, and/or microbial breakdown products (e.g., benzoxazolinones, phenoxazinones) contributed to the overall phytotoxicity of Bx in the screening bioassay, a fact that the present design with daily exchange of test solution did not capture. Furthermore, the moderate recovery rates for Bx within the applied analytical procedure may have slightly underestimated the real active concentration in the screening bioassay.

In conclusion, the current findings provide strong evidence that the release of Bx by intact plant roots of Triticeae species is a determining causal factor for the overall phytotoxicity of root exudates under laboratory conditions. The observed transient dynamics of Bx exudation along with the corresponding fate in the test environment suggest that a significant contribution might be restricted to the early seedling stage. There appeared to be no accumulation of Bx aglucones in the test environment exceeding the amount daily supplied via root exudation; thus, not only DIBOA and DIMBOA but also the decomposition products can act as allelochemicals. The quantified degree of covariation and phytotoxicity of pure aglucones would support this assumption and clearly demonstrated that Bx were not the only allelochemicals involved in observed phytotoxicity of root exudates of Triticeae species. The present state of knowledge on the biochemical basis of crop allelopathy in Triticeae species would suggest a joint action of Bx, phenolic acids (*20*, *23*, *25*, *75*), bioactive coumarins (*75*), and their spontaneous and/or microbial metabolites (benzoxazolinones, phenoxazinones) (*60*). Up to now, attempts to trace the biochemical basis of seedling allelopathy in Triticeae species could not completely explain the overall allelopathic effect. However, the allelochemicals examined in each case would jointly account for a major part of the allelopathic activity in bioassay. Huang et al. (*20*) proposed a fluctuating phytotoxic mixture of several allelochemicals, whereby the contribution by each of the individual constituents to the overall allelopathic effect may change depending on the respective donor species, its developmental stage, and environmental factors. Under the conditions of the present bioassay, the fluctuating exudation of DIBOA and/or DIMBOA proved to account for phenotypic trait expression by 48−72%, whereby a stronger association appeared in case of a prolonged transient release of both aglucones. The pure phytotoxin treatments in equivalent concentrations established a causal association and clearly demonstrated the allelopathic potency of Bx exudation under laboratory conditions. However, the differential exudation of Bx by Triticeae species was not the only, but one of the determining factors for observed allelopathic potency.

The findings demonstrate that Bx exudation is relevant under laboratory conditions in a hydroponic system and this capacity might have implications for allelopathic interactions in a natural setting. However, the present exudate data cannot predict

realistic rhizosphere concentration levels, nor can they predict weed suppressive effects in a field situation, where Bx aglucones are more rapidly degraded by soil microorganisms or adsorbed by soil particles (*18*, *28*). Nevertheless, the fact that the most suppressive cultivar within the biological screening, *S. cereale* cv. Forajero Baer, was among the most potent Bx exuding cultivars and has been found to be highly efficient in reducing the biomass of surrounding weeds under field conditions (*14*, *15*) may allow the speculation that a highly effective Bx exuding cultivar has a potential to leverage crop seedling allelopathy as a new option for integrated weed management.

ABBREVIATIONS USED

Bx, benzoxazinoid(s); DIBOA, 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one; DIMBOA, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one; BOA, benzoxazolin-2(3*H*)-one; MBOA, 6-methoxy-benzoxazolin-2(3*H*)-one.

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