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Benzoxazinoids in roots and shoots of cereal rye (*Secale cereale*) and their fates in soil after cover crop termination

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Abstract

Cover crops provide many agroecosystem services, including weed suppression, which is partially exerted through release of allelopathic benzoxazinoid (BX) compounds. This research (1) characterizes changes in concentrations of BX compounds in shoots, roots, and soil at three growth stages (GS) of cereal rye (*Secale cereale* L.), and (2) their degradation in soil over time following termination. Concentrations of shoot dominant BX compounds, DIBOA-glc and DIBOA were lowest at GS 83 (boot). The root dominant BX compound, HMBOA-glc, concentration was least at GS 54 (elongation). Rhizosphere soil BX concentrations were 1000 times smaller than in root tissues. Dominant compounds in soil were HMBOA-glc and HMBOA. Soil BX compound concentrations were similar near root crowns and between-rows. Soil BX concentrations following cereal rye termination declined exponentially over time in three of four treatments: incorporated shoots (S) and roots (R), no-till S + R (cereal rye rolled flat), and no-till R (shoots removed); no-till S had consistently low concentrations. In treatments showing changes, soil concentrations of HMBOA-glc and HMBOA increased above initial concentrations on the day following cereal rye termination. Concentrations of these two compounds decreased more rapidly than the other compounds. Placement of shoots on the surface of an area where cereal rye had not grown (no-till S) did not increase soil concentrations of BX compounds. The short duration and complex dynamics of BX compounds in soil prior to and following termination illustrate the limited window for enhancing weed suppression directly by cereal rye allelochemicals; valuable information for programs breeding for enhanced weed suppression.

Introduction

Cover crops work synergistically with herbicides to suppress weeds (Teasdale et al. 2005; Nord et al. 2011). Cereal rye (*Secale cereale* L.) is well adapted to a range of climates and growing conditions making it the most widely grown cover crop in the United States (Clark 2007). Cereal rye

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provides both physical weed control through limitations of light and temperature (Mirsky et al. 2013), and to a lesser extent through allelopathy (Teasdale et al. 2012).

Two groups of chemicals are responsible for the allelopathic properties of cereal rye, simple phenolic acids (Blum et al. 1999) and benzoxazinoids (BX) (Niemeyer 2009). Extensive literature suggests that BX compounds suppress early growth and development of summer annual weeds (Barnes and Putnam 1983; Kruidhof et al. 2014; Niemeyer 1988; Schulz et al. 2013). These findings have stimulated research to identify which compounds are responsible for the observed weed suppression (Singh et al. 2003); however, the answer has remained elusive. Benzoxazinoid compounds exist as a complex chemical family; at least 12 different compounds were identified in a recent study of cereal rye (Hazrati et al. 2020), all of which may exert allelopathic effects (Macías et al. 2005a; Hazrati et al. 2020).

Plant-derived BXs are noted for their ease of degradation (Krogh et al. 2006; Macías et al. 2005b). In plants, BXs

are typically stored as non-toxic glucose conjugates. However, when the cellular structure of the plant is damaged (e.g., through tillage), enzymatic reactions quickly remove the protective glucose to yield the toxic aglycone groups. These aglycones can also readily degrade to form benzoxazolinones, BOA and MBOA (Krogh et al. 2006). Identifying and quantifying BX compounds in samples, therefore, requires care to avoid post-collection degradation.

In the rhizosphere of actively growing roots, BX glucosides are hydrolyzed to more biologically active aglucones (Macias et al. 2007; Belz et al. 2005). The soil concentrations found beneath growing cereal rye have been reported in the nanogram per gram (ng g^{-1} dry wt.) range (Reiss et al. 2018a) and these compounds are prone to rapid decay (Eljarrat et al. 2004; Cambier et al. 2000), Krogh et al. 2006). Recently, Reiss et al. (2018b) found that BXs in soil are primarily members of the methoxy substituted (BX + M) grouping, rather than the non-methoxy substituted forms (Fig. 1). Similarly, the predominance of the BX + M form is more common in cereal rye roots than shoots (Reiss et al. 2018b). We also found greater concentrations of the BX + M group in soils under a cereal rye cover crop (Rice et al. 2012) where HMBOA and MBOA occurred in the highest concentrations.

Several studies of BXs originating from cereal rye roots have focused on root exudates. Hydroponic culturing methods allowed researchers to monitor the release of exudates (Belz and Hurle 2005); however, the procedure does not foster normal root physiology and can alter the chemical forms released. Recently, a method employing micro glass beads was developed (Hazrati et al. 2020), which allowed for the release of more of the glycosylated BX forms, similar to concentration levels observed under cereal rye grown in situ. Soil is the critical site for toxic action in a cover crop system but evaluating these compounds in situ is much more difficult due to the difficulty in separating roots from soil, which must be done quickly given the rapid changes in BX form and the tediousness of identifying small root fragments in soil.

The goal of this study was to increase our understanding of BX compounds in plant tissues and their release to soils in situ where cereal rye is grown as a cover crop and then terminated. We quantified changes in 10 BX compounds (Fig. 1) in shoots and roots at three growth stages of cereal rye and then following termination, quantified the degradation of nine of these in soil over 56 days under various tillage and cereal rye mulch treatments. A previously published companion study tracked phenolic acid release into soil, using the same samples (Otte et al. 2020).

Methods

Experimental design

The experiment was conducted at the Beltsville Agricultural Research Center in Beltsville, Maryland (39.031759 N, −76.934591 W). The soil at the site is classified as a Downer–Hammonton complex, which is primarily made up of a loamy sand (~20% or less clay content, ~85% or less sand, ~30% or less silt contents). The cereal rye cover crop, 'Aroostook' cultivar, was drill seeded on 24 September 2014 at 125.5 kg ha^{-1} on 19-cm row spacing in an area large enough to establish four replications of the growth stage and termination studies described below.

Fig. 1 Chemical structures of benzoxazinoids measured in this study. Panel **A** shows the group of compounds which lack methoxy substitution on the benzene ring. Panel **B** shows the group of compounds having a methoxy group substituted on the benzene ring. Columns R1 and R2 provide the molecules substituted at the respective locations on the benzene ring with illustrations for BOA and MBOA at the bottom
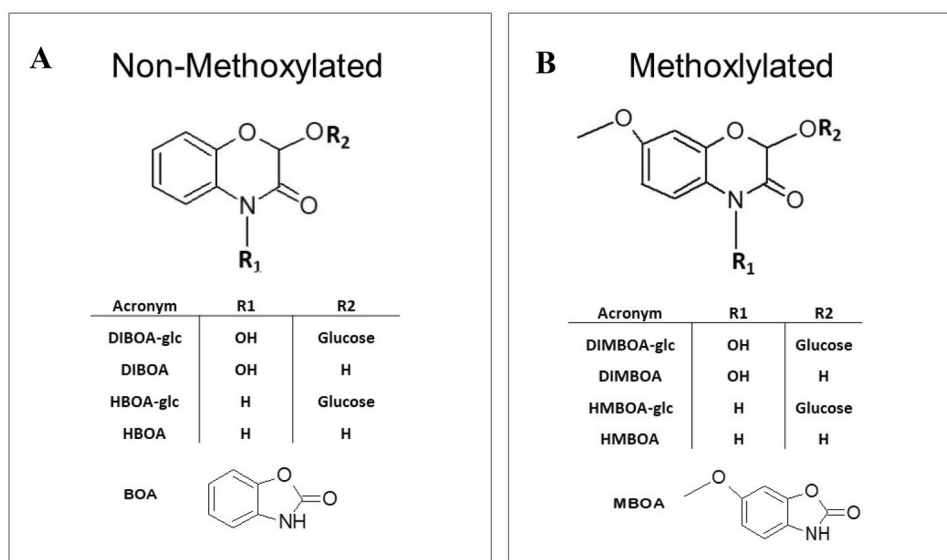
Growth stage study

The growth stage study plots were 3 × 3 m (10 × 10 ft) and were replicated four times. Each plot was split into three 1.2 m² sections, to facilitate sampling cereal rye at three Zadoks growth stages (GS) (Zadoks et al. 1974): (1) early: Zadoks GS 32 (tillering); (2) mid: Zadoks GS 54 (elongation); (3) late: Zadoks GS 83 (boot) (Fig. 2A). At each growth stage, four cereal rye plants were removed from the appropriate section by cutting the shoots at the soil surface and placing them immediately on ice in coolers. The roots of each plant along with attached soil were removed from the ground using a spade and placed in a plastic bag on ice in a cooler. Additionally, intact soil cores were collected from two in-row and two between-row locations near the root collection site by pushing plastic cylinders (4.8 cm diameter × 10 cm depth) into the soil. Four soil cores were also collected from random locations in the bare soil plots. Soil cores were placed on ice in a cooler and transported to the lab. Samples were collected on 15 Apr 2015 (early GS 32), 22 Apr 2015 (mid GS 54), and 11 May 2015 (late GS 83).
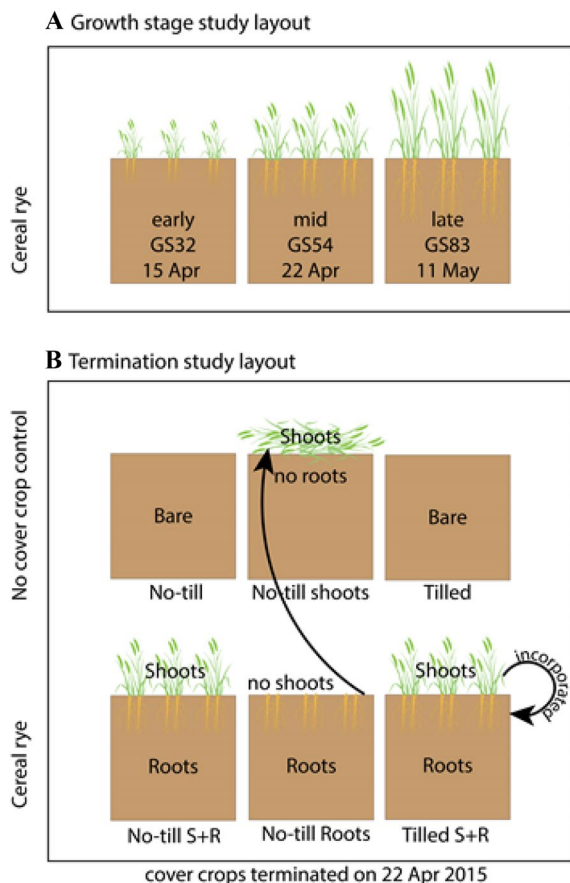

A Growth stage study layout

early GS32 15 Apr · mid GS54 22 Apr · late GS83 11 May

Cereal rye

B Termination study layout

No cover crop control

Bare — No-till · Shoots no roots — No-till shoots · Bare — Tilled

Cereal rye

Shoots / Roots — No-till S+R · no shoots / Roots — No-till Roots · Shoots / Roots — Tilled S+R · incorporate

cover crops terminated on 22 Apr 2015

Fig. 2 Schematic of (**A**) cereal rye growth at the three growth stages and (**B**) tillage/biomass manipulations to create treatments for the termination study initiated at GS 53 in the growth stage plots

Samples were processed in a minimum amount of time by the team to preserve their chemical integrity. Roots were rinsed with chilled water to remove soil and placed in whirl–pak bags (Ft. Atkins, WI) before storing them at −20 °C for later processing. Shoots were also placed in whirl–pak bags and stored at −20 °C. Soil cores remained on ice in coolers until processed. The in-row cores were homogenized and divided into two classes: (a) root debris included ('in-row w roots'), and (b) root debris removed by sieving (0.64 cm) and hand-picking ('in-row w/o roots'). Root debris was not visible in the between-row samples. The division of the in-row soil into two classes resulted in four soil treatments at each growth stage: (1) in-row w roots, (2) in-row w/o roots, (3) between-row, and (4) bare soil.

Termination study

Immediately following collection of the mid GS samples (GS 54; April 22) the remaining cereal rye in that section was terminated with paraquat (2.2 kg/ha) to begin the termination study. Within this area, bare no cover crop plots 3 × 3 m (10 × 10 ft) had been established at the time of planting the GS experiment by not planting cereal rye and occasionally spraying the plots with paraquat (1,1′-dimethyl-4,4′bipyridinium ion) at 2.2 kg ha⁻¹ to suppress weed growth. The day after terminating the cereal rye (DAT 0), each plot was subdivided into six regions using a randomized complete block design with the following treatments: no-till bare, no-till shoots and roots (no-till S + R), no-till roots only, no-till shoots only, tilled bare, and tilled (incorporated) shoots and roots (tilled-S + R) (Fig. 2B).

The no-till roots-only plots were established by clipping shoots at the soil surface and removing them, thus creating roots-only plots. The shoots-only plots were established by spreading the clipped shoots on the soil surface of no-till bare (no cover crop) plots (Fig. 2B). Cereal rye shoots were covered with netting to ensure they were not blown away by wind. In the no-till S + R plots, the above-ground biomass was flattened to simulate a roller-crimper. Finally, the tilled S + R plots were established by tilling shoot and root biomass into the soil with a tractor-mounted rotovator to 10 cm depth. Establishing the treatments took about four hours after which treatment specific soil samples (DAT 0) were collected. Subsequent sampling times at 3, 7, 14, 32, and 56 DAT were based on the expected decomposition of the cereal rye and previous observations showing that allelopathic compound concentrations decline rapidly after cover crop termination (Rice et al. 2012).

As described above, two soil cores (to 10 cm) were collected from in-row (where plant crowns were present) and two from between-rows to obtain representative samples for all treatments. To reduce microbial activity, which could degrade compounds, soil cores were capped and placed

upright in a cooler with ice packs immediately after collection and stored in the laboratory at 4 °C until processed (< 12 h). The four cores from each plot were each split into three depths (0–3 cm, 3–6 cm, and 6–10 cm) and composited by depth. Within each depth, samples were homogenized, passed through a 0.635 cm sieve, and frozen for later extraction and analysis.

Sample preparation and analytical methods

Soils

A solvent shake and sonication method was used for soil extraction, modified from Macías et al. (2005b) as follows: sample size was increased from 0.5 to 2 g of fresh soil; soil was mixed fewer times (two vs. three) with each solvent series, first 2×20 mL methanol and then 2×20 mL ethyl acetate. Sonication in a sonication bath (E100H Elmasonic, Elma Schmidbauer GmbH, Singen, Germany), for ten minutes was carried out after each solvent mix. Extracts were reduced by nitrogen blowdown and cleaned using silicic acid columns (Sep-Pak Vac 6cc part # 186004616) from Waters Corp (Milford, Mass). Separation cartridges were pre-cleaned with 5 mL of methanol followed by 5 mL of water and then allowed to dry by drawing air through with suction. Extracts were loaded in 2 mL of solvent and eluted with 5 mL of methanol. These cleaned extracts (~ 7 mL) were concentrated to 3 mL and injected into the LC/MS column for analyses.

Tissue samples

In preparation for analysis, frozen tissue samples were freeze-dried, weighed, and ground using a model 6750 SPEX Freezer/Mill grinder (Metuchen, NJ) reducing each to powder of < 0.05 mm diameter. For extraction of the benzoxazinoids, an accelerated solvent extraction method (Carlsen et al. 2009) was modified, using sand as the inert support in the extraction cells. Extracts were passed through solid phase cartridges using silicic acid as described above to remove materials that might interfere in the analyses.

We targeted and identified 10 BX compounds in this study (Fig. 1). The sources of analysis standards for nine of these are given in Rice et al. (2012). The standard for an additional compound not measured previously, HMBOA-glc, came from Dr. Gaétan Glauser, Institute for Chemistry, University of Neuchatel, Neuchatel Switzerland. Recent studies have found moderate concentrations of HMBOA-glc in roots and soils where cereal rye was grown (Hazrati et al. 2020; Reiss et al. 2018a). All analyses were by multiple reaction monitoring performed on a LC Quattro Ultima triple quadrupole mass spectrometer (Micromass Ltd., Manchester, UK). Samples were introduced into the mass spectrometer using a

Waters 2695 liquid chromatograph and separated on a Kinetex F5 (2.6 μm 100 Å, 2.1×100 mm) column (Phenomenex, Torrance, CA). The running solvents were mixtures of the following: A-30:70 methanol:0.05% formic acid in deionized water, B-deionized water, and C-methanol. For separating compounds, the running solvent gradient was as follows: initial 0.5:95.5 A:B followed by a linear gradient in 2 min to 7:93 of A:B; then for next 20 m, a linear increase to 16:84 A:B; then a linear gradient for next 5 min to final mix of 90:10, B:C to flush the column. The column was returned to the starting mix for 7 min to equilibrate it for the next injection. Flow rate was 0.5 mL min^{-1} with column temperature maintained at 45 °C. All analytes were introduced to the MS in electrospray negative ionization mode, and the transition used for multiple reaction monitoring followed Tanwir et al. (2013). Table 1 lists the typical retention times and mass transition classes of compounds determined by the LC column.

Detection limits

Washed sand, baked at 450 °C for 4 h, was used as blanks in the analytical process to provide detection limits for each compound. This provides rigorous method to determine detection limits, since the sand samples are normalized to all handling factors as well as weight of sample extracted. The baked sand samples were added randomly within the sequence of samples. Values for the baked sand in ng g^{-1} were as follows [$n = 10$; average ($\pm 95\%$ CI)], HBOA-Glc 0.11 (0.07, 0.14); DIBOA-Glc 0.26 (0.10, 0.42); HMBOA-Glc 0.57 (0.20, 1.0); DIMBOA-Glc 0.19 (0.04, 0.33);

Table 1 Liquid chromatography mass spectrometry instrument BX compound retention time and mass transition parameters using negative electrospray introduction and separation with a Kinetex F5 (Phenomenex) LC column

BX compound	Retention time (min)	Mass transition parent m/z > fragment m/z
HBOA	4.7	164 > 108.4
DIBOA	5.1	180.1 > 134.1
BOA	7.6	134.11 > 42.9
BOA-glc	8.4	326 > 164
DIBOA-glc	8.4	342 > 134.2
HMBOA	9.8	194 > 138
DIMBOA	11.5	210 > 149
MBOA	16.6	164 > 149
HMBOA-glc	19.5	356 > 194
DIMBOA-glc	19.4	371.7 > 164.3

Mass transition is the specific pair of mass (m) to charge (z) ratios used to monitor for a particular fragment ion of a selected precursor ion and is written as parent m/z > fragment m/z

DIBOA 0.39 (0.23, 0.55); HBOA 0.28 (0.10, 0.46); BOA 0.10 (0.02, 0.13); MBOA 0.09 (0.03, 0.15); HMBOA 0.12 (0.03, 0.20).

Overall statistical analyses

All data were analyzed using the R statistical software (R-Core Team 2021). Differences among treatments or compounds were considered significant at $P < 0.05$ (P values adjusted for multiple comparisons). All BX concentration data were transformed to log scale, the optimal λ was close to zero over a series of Box–Cox power transformations (Box and Cox 1964) based on the R *MASS* package (Venables and Ripley 2002). Values below the detection limits (which differed for each compound, see above) were replaced by a random number between zero and the detection limit prior to log transformation. The transformed data were analyzed using ANOVA (in a mixed model framework, allowing for random plot-to-plot variability) conducted using the R *nlme* package (Pinheiro et al. 2020) for linear mixed effects modeling followed by, where appropriate, Tukey's multiple comparison tests using the R *emmeans* package (Lenth 2021) to estimate means and paired contrasts. Back-transformed data for tables and figures are provided in related supplemental tables as indicated in the "Results and Discussion" section. Note that means on the analysis (log) scale are back-transformed to medians on the original scale. Data and R code are available at https://doi.org/10.15482/USDA.ADC/15263 30 (Ag Data Commons).

Growth stage study—tissues and soils

For the statistical analysis of BX compound concentrations in growing cereal rye, tissue type (root or shoot), GS (32, 54, 83), and their interaction were independent variables. For the analysis of BX compound in soils from the same study, the four soil sample classes (in-row w roots, in-row w/o roots, between-row, and bare soil), GS and their interactions were independent variables. The R *emmeans* package (Lenth 2021), with a multiple comparisons adjustment, was used to estimate model means and their confidence intervals. Note that these, when given for one independent variable, are statistically adjusted for the main effects of other independent variables.

Termination study—soils

Changes in the BX compounds in soil following termination were evaluated with regression (in the mixed models framework) by estimating the approximately linear relationship between transformed days after termination (DAT) and log-transformed BX compound concentrations. DAT was transformed as log (DAT + 5) for model estimation.

Had log (DAT) been used instead, it would have yielded a pronounced "hump" at the second time point; adding the constant 5 reduced this effect. On the transformed scale, the linear mixed effect model produced estimates of the intercept that, because of the offset in this transformation, are not at DAT 0. The estimated slope, which is not affected by the offset of the intercept, represents the decline in concentration for each BX compound when negative. More negative slopes indicate faster rates of decay.

While log transformed BX concentration data display a linear decline over (transformed) time, data on the original scale display an exponential decay curve over (transformed) time. To illustrate the relationship between the log transformed data and the original data and how the models fit the data, we preview results for HMBOA-glc in Fig. 3. The left panels show data and fitted models on the log transformed scale, the right panels show the same, but on the original
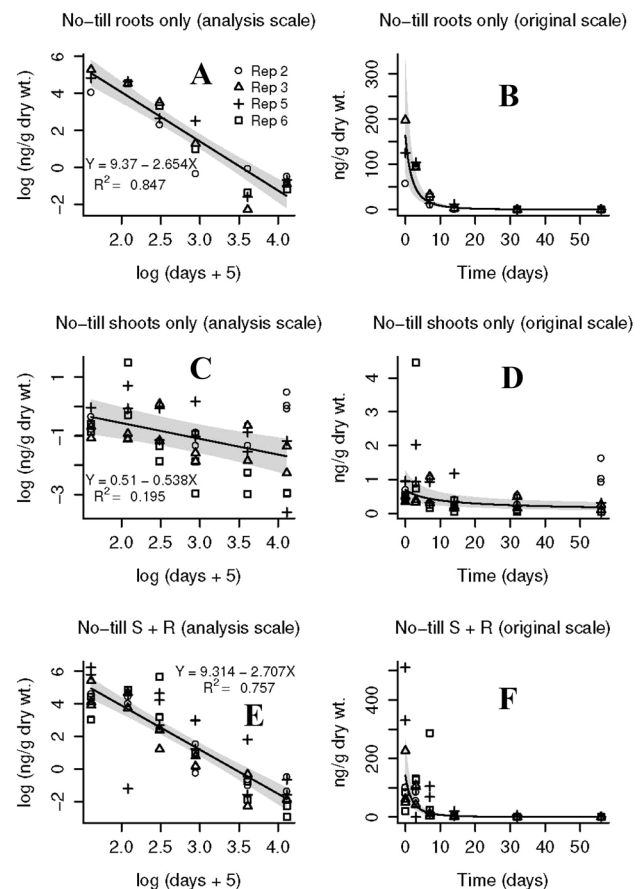

Fig. 3 Example of data transformation effects for soil HMBOA-glc concentrations from three of the cereal rye biomass manipulations in the termination study; no-till roots only, no-till shoots only, and no-till shoots and roots. Panels **A**, **C**, **E** provides log concentration values, best linear fit lines with coefficients, and 95% confidence intervals. Panels **B**, **D**, **F** illustrate the same information, back-transformed to the original scale

scale. Measured values from each plot are shown using symbols to better visualize plot-to-plot variability.

The model fit was used to obtain BX compound concentration estimates and their 95% CI for each compound at DAT 0. Differences in the decline in concentration among BX compounds were statistically compared using linear contrasts of the estimated slope coefficients using the R *emmeans* package (Lenth 2021), with a multiple comparisons adjustment.

Results and discussion

Growth stage effects on benzoxazinoid concentrations in plant tissue and surrounding soil

Changes in BX compound concentrations in cereal rye roots and shoots over time are illustrated in Fig. 4 (Table S1, data in Fig. S1). Concentrations of BX compound were greater in shoots for three BX compounds and greater in roots for five compounds. Similar concentrations in roots and shoots of DIBOA-glc and HBOA-glc were observed at all three GS and across GS. There was a general trend for decreases in BX compounds concentrations in both roots and shoots at the later GS although this was not always significant. Total BX compound concentrations in shoots remained similar for GS 32 and 54 then decreased by 33% at GS 83. Total BX compound concentrations in roots increased from GS 32 to GS 54 and then decreased slightly, with the final increase

being 54% from GS 32 to GS 83. Several researchers have established that cereal rye BX concentrations decline with increasing growth stage (Burgos et al. 1999; La Hovary 2011; Reberg-Horton et al. 2005). These observations were almost entirely based on BX concentrations in plant shoots, and for the dominant DIBOA + DIBOA-glc compounds. In our study, we observed a moderate decline in plant shoot concentrations across growth stages (538, 542, 378 µg g^{-1} d wt., at GS 32, 54 and 83 respectively) for combined DIBOA-glc and DIBOA concentrations, similar to Reberg-Horton et al. (2005). Similar changes were also observed in the roots for these two compounds.

Changes in BX concentrations for soil across the three GS are shown in Fig. 5 (Table S2). Soil concentration data in Fig. 5 are averaged for the in-row without roots (roots removed) and between-row locations; the two locations resulted in similar values and the interactions between GS and soil sample location were negligible. Few differences were observed over the three GS stages for BX concentrations in these soils (Fig. 5, Table S2, data in Fig. S2). The somewhat consistent levels of the compounds over time indicate that they were being replenished either from the roots or shoots because BX compounds have been observed to decrease rapidly in soil following termination of a cereal rye cover crop (Rice et al. 2012, and this study).

Results for the BX compound concentrations in the soil were very different from the patterns observed for root tissues (compare Figs. 4, 5). For example, HMBOA-glc concentrations increased markedly in root tissues from GS 32

Fig. 4 Mean log concentrations of BX compounds (with ± 95% CI) in shoots and roots at three growth stages. Lowercase letters indicate, within a compound, the multiple comparisons groupings
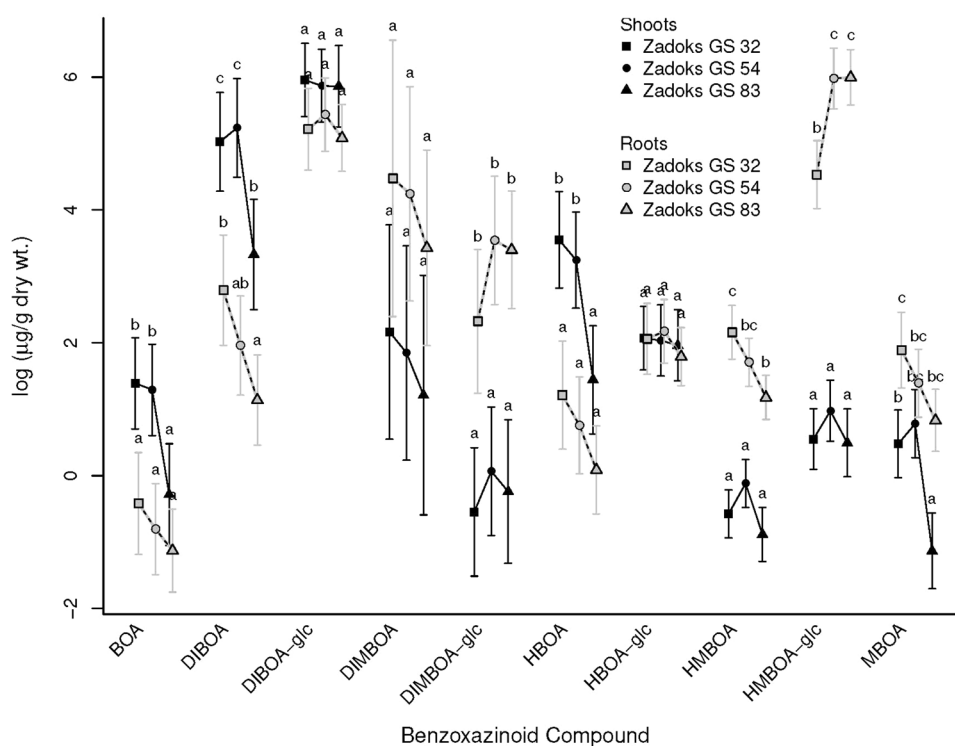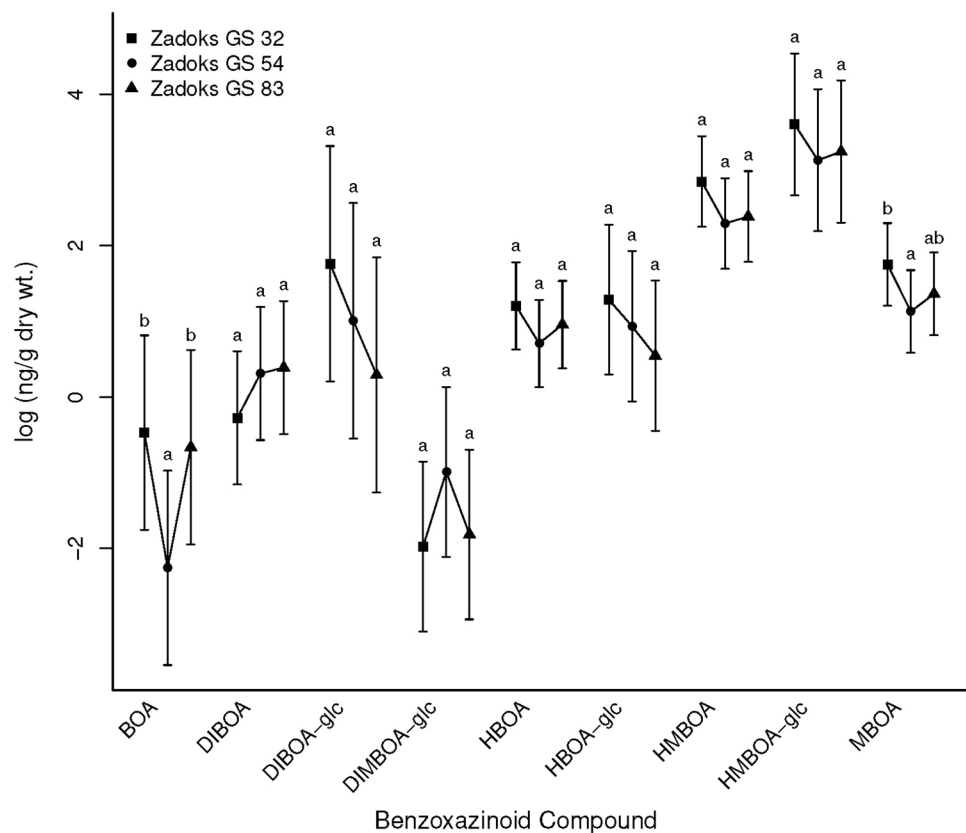
Fig. 5 Mean log concentration of BX compounds in soil without roots at each of the three growth stages. Data are averages of the in-row without roots and mid row locations with ± 95% CI. Lowercase letters indicate, within a compound, the multiple comparisons groupings

to GS 54 but in soil little change was observed. Relative amounts of HMBOA in soil were greater than in root tissues. This could be the result of enzymatic degradation from HMBOA-glc (Sue et al. 2000; Schuetz et al. 2019). Similar to Hazrati et al. (2020), we found HMBOA-glc to be the dominant BX compound in root tissues and exudate in the soil. DIBOA-glc concentrations were relatively large in root tissues, and much smaller in soil without root fragments.

Our sampling for BX compounds in soil assumed that concentrations should vary with density of the root mass, greater in areas of greatest root density (i.e., in-row) and declining to the between-row region of each plot. This dependence on root mass is reinforced by the lack of diffusive transport of these compounds in soil (Rice et al. 2012; Meyer et al. 2009). The concentrations for the in-row with roots samples show how much these fragments added to the exuded BX concentrations (Fig. 6, Table S3; means and confidence intervals have been statistically adjusted for GS, see data in Fig. S3). The presence of roots in the in-row with roots samples resulted in the greatest concentrations of all BX compounds, which differed statistically from the between-row samples for six of nine BX compounds and from the in-row without roots samples in three cases. These findings are not surprising based on the results above, where BX compounds in the roots were present at substantial concentrations. As expected, the bare soil where cereal rye was

not grown had the smallest concentrations of BX compounds and were consistently different from those in the in-row w roots location.

Soil BX concentrations changes over time following termination of cereal rye

Concentrations of BX compounds in soil were determined over time to evaluate their rates of change following cereal rye termination. Sampling depth (0–3, 3–6 and 6–10 cm) was not a significant effect so depth was ignored in further analyses and fitting of statistical models. Lack of a depth response for BX compound concentrations in soil with growing cereal rye agrees with findings of Reiss et al. (2018b) who observed concentrations of BX compounds varied by depth for wheat (*Triticum aestivum*) and triticale (*Tritico-secale*) but not for cereal rye in the first year of their study. In our study, termination of cereal rye resulted in increased soil concentrations of some BX compounds where cereal rye had been grown compared to the previous day, although not always significant (Table 2, Table S4). The previous day's BX concentrations were the averages of the in-row-w/o-roots and between-row samples from GS 54. Increases in concentrations of DIBOA-glc, HBOA, HBOA-glc, HMBOA, HMBOA-glc, and MBOA were somewhat similar for the tilled S + R, no-till S + R and no-till roots only treatments.

Fig. 6 Mean log concentrations (with ± 95% CI) of benzoxazinoid compounds in soil during the growth stage study. Concentrations are from four plot locations; in-row w roots, in-row w/o roots, between-rows, and bare soil (no plants present); the main effects of growth stage have been adjusted for statistically. Lowercase letters indicate the multiple comparisons groupings within a compound. Gray bars above the *x*-axis group the four sample locations for each compound
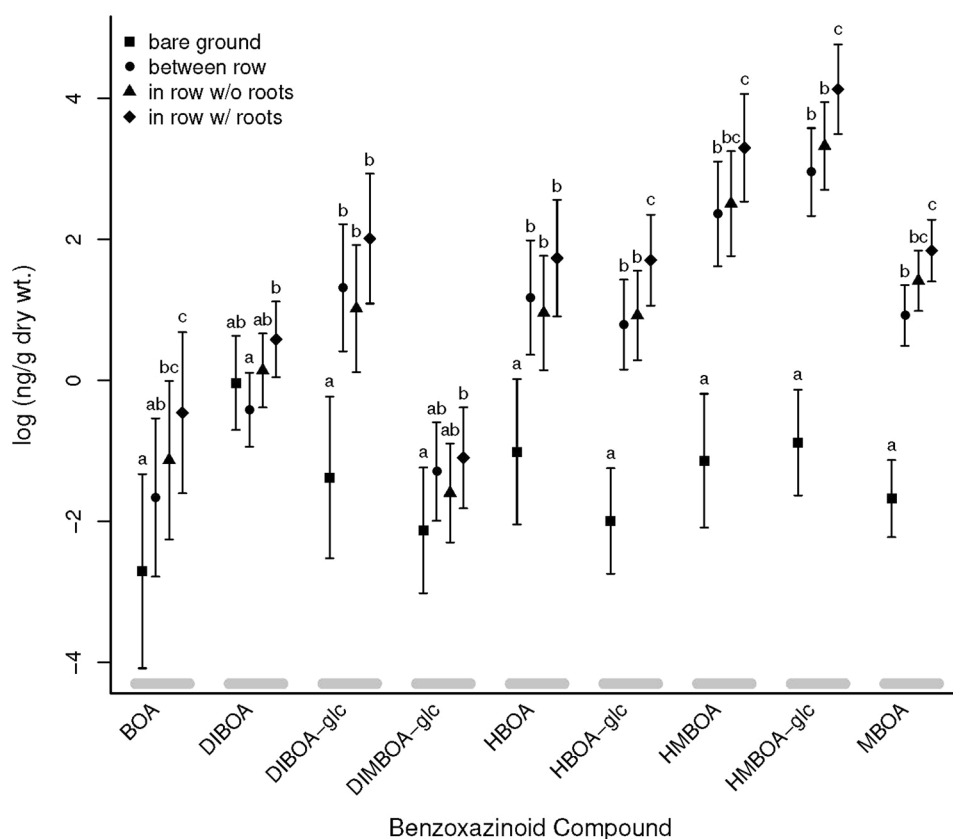

Table 2 Mean and 95% confidence intervals for log (concentration) of soil BX compounds at the start of the termination study

BX Compound	Initial		Tilled S+R			No-till S+R			No-till roots			No-till shoots		
	--- log(ng g⁻¹ dry wt.) ---													
BOA[a,b]	-2.00	(-3.03,-0.96)	0.04	(-2.08,2.15)	a	-0.26	(-1.72,1.19)	a	0.45	(-1.99,2.89)	a	-3.54	(-5.08,-2.01)	b
DIBOA	0.03	(-0.33,0.39)	-0.42	(-0.93,0.10)	a	-0.25	(-0.59,0.09)	a	-1.56	(-2.16,-0.97)	b	-0.43	(-0.80,-0.07)	a
DIBOA-glc	1.27	(0.47,2.07)	2.82	(1.01,4.64)	a	2.75	(1.54,3.96)	a	3.06	(0.96,5.16)	a	-0.98	(-2.27,0.30)	b
DIMBOA-glc	-1.11	(-1.63,-0.58)	-1.63	(-2.64,-0.63)	a	-1.45	(-2.12,-0.77)	a	-0.55	(-1.71,0.62)	a	-1.21	(-1.92,-0.50)	a
HBOA	0.82	(0.46,1.17)	1.93	(1.03,2.83)	a	2.24	(1.60,2.87)	a	2.18	(1.15,3.22)	a	-1.39	(-2.05,-0.72)	b
HBOA-glc	0.97	(0.57,1.38)	1.72	(0.94,2.50)	a	2.02	(1.51,2.54)	a	2.17	(1.27,3.07)	a	-1.68	(-2.23,-1.13)	b
HMBOA	2.29	(1.90,2.67)	2.88	(1.88,3.87)	a	3.65	(2.88,4.41)	a	3.71	(2.60,4.83)	a	-1.92	(-2.71,-1.13)	b
HMBOA-glc	2.96	(2.50,3.43)	3.99	(3.22,4.77)	a	4.69	(4.12,5.27)	a	4.66	(3.78,5.54)	a	-0.56	(-1.15,0.04)	b
MBOA	1.07	(0.55,1.58)	2.2	(1.24,3.16)	a	2.47	(1.72,3.22)	a	2.34	(1.26,3.41)	a	-1.77	(-2.55,-1.00)	b

Initial values are averages of the in-row without roots and mid-row soil samples collected for the growth stage study at GS 54 and represent BX concentrations on the day of cereal rye termination (Fig. 6). Values for the four termination treatments are from soils collected on DAT 0 when the termination treatments were established. Back transformed data are in Table S4

[a]Lower-case letters indicate differences among treatments within a soil BX compound based on Tukey's HSD

[b]Shaded cell with bolded values indicates differences in soil BX compound concentrations between soil samples collected the day before (initial) and immediately following treatment establishment based on pairwise model contrasts

In some cases, the increases were significant indicating a rapid release of BX compounds in less than 24 h following termination of the cereal rye.

In the no-till shoots only treatment (where cereal rye had not been grown) soil concentrations of BX compounds were less on DAT 0 compared to the initial soil (where cereal rye had grown) for six of the compounds and for all of the compounds when compared to the other cover crop treatments (Table 2). Soil concentrations of BX compounds for the no-till shoots only treatment were all similar to the bare soil concentrations in the termination experiment, Table S5.

Concentrations of BX compounds in soil following termination of the cereal rye cover crop rapidly declined over time (Fig. 7). The rates of decay of the BX compounds on the log scale are given in Table 3. For treatments established in plots where cereal rye had grown (tilled S + R, no-till
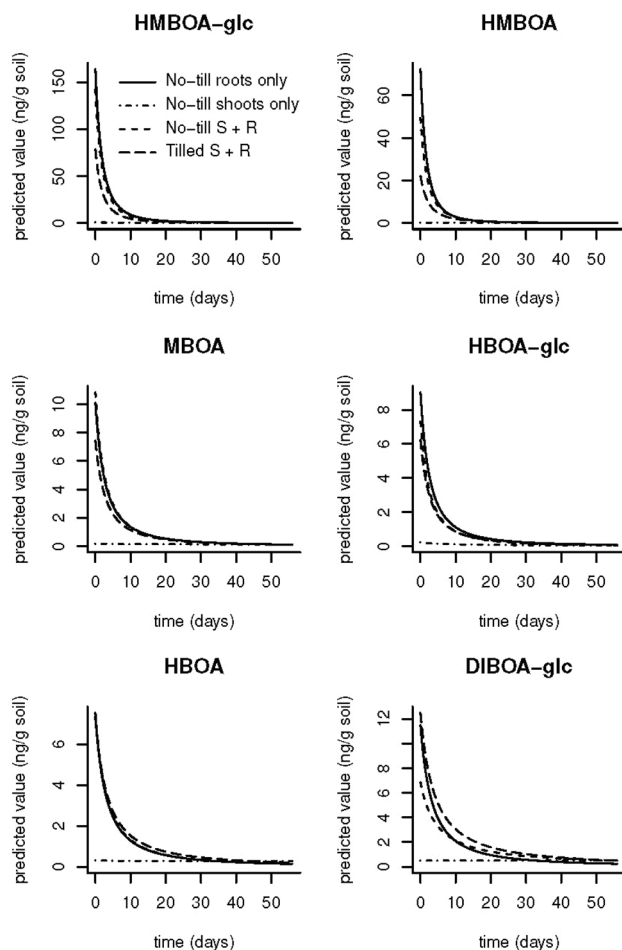
Fig. 7 Fitted exponential relationships showing the decline of BX compound concentrations over time in soil of the four-biomass manipulation treatments following cereal rye termination. Curves are shown on the back-transformed (original) scale. Changes in BX compound concentrations over time for soil from plots where cereal rye had not grown (bare plots) were small and not included in the figure. Those data are provided in Table S6

S + R, no-till roots), rates of decay of individual BX compounds were not significantly different among these three treatments. The concentrations of BX compounds in the no-till shoots only treatment were small and the rates of change for this treatment were near zero (Fig. 7). Differences among compounds in decay rates within a treatment are presented in Table 3. In general, compounds with faster decay rates (more negative slopes) started with greater initial soil concentrations. Considering the data from the treatments where cereal rye had grown (tilled S + R, no-till S + R, and no-till roots), HMBOA-glc and HMBOA concentrations declined the fastest (steepest negative slope estimates), thus, persisting in the soil for the shortest amounts of time. The fast decline in concentrations implies their direct weed suppressive activity would be short-lived. Literature reports that HMBOA-glc and HMBOA will degrade to MBOA (Mwendwa et al. 2021)

and that MBOA is the primary precursor for methoxylated phenoxazinone formation (Etzerodt et al. 2006). The latter compound does have high weed suppression activity which could lead to enhanced weed suppression. While tillage might be hypothesized to encourage more rapid breakdown of HMBOA-glc and HMBOA, possibly caused by greater aeration of the soil, we found no evidence for this (slopes were similar for no-till S + R and tilled S + R), though initial values for both were greater in no-till S + R. Concentrations of HBOA, BOA and DIBOA-glc tended to persist longer than HMBOA-glc and HMBOA, which would allow these compounds to be more effective in weed suppression although this was not determined in this study.

The very small BX concentrations in soil from the no-till shoot only treatment suggests that BXs contained in the shoots failed to move into the soil over time. Rain did not seem to leach them into the soil either, since rain occurred one day after DAT 0 (5.3 mm for 2 h) and 23 days after DAT 0 (17.8 mm). Even the uppermost soil layer in the soil stratified samples showed no evidence of increased BX concentrations (data not shown).

Some of the BX compounds increased from the initial sampling to DAT 0 (Table 2) indicating degradation of primary to secondary metabolites (Schuetz et al. 2019). Rapid decay of the BX compounds was observed, with from 65% to 90% of the initial concentrations lost by DAT 7 (Fig. 7). An explanation for the more rapid decay of the more abundant soil compounds, e.g., HMBOA-glc and HMBOA, is that the soil microbiota was primed for degradation of these compounds as they were being released while the plants were growing, see Table S4. Concentrations of BX compounds had declined to near-background levels by DAT 20. Daily sampling during the first 5 days would have better captured the decay rate. However, decay rates were much slower in a previous experiment (Rice et al. 2012), on which we based our sampling frequency design. The rapid decay observed in this experiment may have been the result of more favorable conditions for decomposition. We did not evaluate BX compound effects on weed suppression, so any lasting effects are unknown. Teasdale et al. (2012) observed weed suppression lasted two weeks in soil from a no-till cereal rye termination study, which is similar to that reported in other field studies (e.g., Korres and Norsworthy 2015). A better understanding of the conditions that affect residence time of different BX compounds would be useful in evaluating toxicity potentials of BXs for weed suppression.

Trace amounts of BX compounds were found in some soil samples which we had expected to be free of BX compounds. The cereal rye plots used in this study were part of a larger cover crop species study. As a result, there were cover crops (either rye or other species) growing adjacent to the bare plot areas. Even though the plots were 10 × 10 ft, and we sampled toward the middle of this area, it is possible

Table 3 Decay trends (slopes, ± 95% CI) for soil BX compound concentrations in treatments following cereal rye termination

BX compound	Tilled S + R			No-till S + R			No-till roots			No-till shoots		
	Slope	LCI UCI		Slope	LCI UCI		Slope	LCI UCI		Slope	LCI UCI	
BOA[b]	−1.29	(−1.71, −0.87)	b	−0.99	(−1.41, −0.56)	cd	−1.56	(−2.11, −1.01)	b	−0.11	(−0.46, 0.25)	a
DIBOA	0.20	(−0.22, 0.62)	c	−0.20	(−0.62, 0.23)	de	0.11	(−0.45, 0.66)	c	0.06	(−0.30, 0.42)	a
DIBOA-glc	−1.29	(−1.71, −0.87)	b	−1.07	(−1.49, −0.64)	bcd	−1.55	(−2.11, −1.00)	b	0.00	(−0.35, 0.36)	a
DIMBOA-glc	0.49	(0.08, 0.91)	c	0.23	(−0.20, 0.65)	e	−0.31	(−0.86, 0.25)	c	0.08	(−0.29, 0.44)	a
HBOA	−1.42	(−1.84, −1.01)	b	−1.59	(−2.02, −1.17)	bc	−1.60	(−2.15, −1.04)	b	−0.04	(−0.40, 0.31)	a
HBOA-glc	−1.82	(−2.23, −1.40)	ab	−1.95	(−2.37, −1.52)	ab	−1.91	(−2.47, −1.36)	ab	−0.71	(−1.06, −0.35)	a
HMBOA	−2.22	(−2.63, −1.80)	ab	−2.62	(−3.05, −2.20)	a	−2.93	(−3.48, −2.37)	a	−0.25	(−0.61, 0.11)	a
HMBOA-glc	−2.58	(−2.99, −2.16)	a	−2.71	(−3.13, −2.28)	a	−2.65	(−3.21, −2.10)	ab	−0.53	(−0.88, −0.17)	a
MBOA	−1.72	(−2.13, −1.30)	ab	−1.88	(−2.31, −1.46)	abc	−1.84	(−2.39, −1.28)	ab	−0.14	(−0.50, 0.21)	a

Slopes were estimated from the regression of BX log (concentration) on log (DAT + 5). More negative slopes indicate a faster rate of decay. All estimated positive slopes (except one[a]) do not differ from zero and indicate no change in concentration over time. Within all compounds, no-till shoots differs from the other three treatments

[a]The estimated positive slope for DIMBOA-glc in the Tilled S + R treatment was significantly greater than zero

[b]Lower case letters indicate differences among soil BX compound concentrations within a treatment based on Tukey's HSD

that roots from cover crops in adjacent plots could have extended into the bare plot area. In addition, some contamination could have occurred inadvertently from walking on the plots during soil sample collection. While levels were far below concentrations that might be biologically meaningful, it shows that in open field situations, biological materials are readily transferred in space, often in unpredictable ways, leading to additional noise in analyses. While this was not an important issue in this experiment, it might be in others where one is measuring low concentrations of biological compounds and reinforces the need for appropriate controls in open field experiments.

While there is much interest in phenoxazinones, especially APO and AMPO, which are formed by bacteria from soil-released BOA and MBOA, our focus here was on the BX compound from *Secale cereale* and their release to soil. Phenoxazinones are not released from the plant but may play an important role in allelopathy after their microbial formation since they are much more toxic than the BX compounds (Venturelli et al. 2016).

Summary and conclusions

The contribution of BX compounds to soil was greater from cereal rye roots than shoots. Concentrations of BX compounds in soils where cereal rye had been grown appeared to persist only for a short time after the cereal rye was terminated. Soil BX concentrations increased from just before the initial pre-burn-down up till the following day's sample (DAT 0) when the cover crop placement was completed. After this time all BX concentration declined regardless of tillage practice. Comparing our results with other published

data (Carlsen et al. 2009; Reiss et al. 2018a), we found larger proportions of glycosylated BX forms, especially DIBOA-glc and HMBOA-glc, in both the tissues and soil samples, possibly due to careful sample processing. HMBOA-glc and HMBOA were the dominant BX compounds in soil growing cereal rye and after termination. HMBOA and HMBOA-glc decayed the fastest; DIBOA-glc decayed the slowest.

Our results provide information on the presence of a diversity of BX compounds (not just DIBOA) in soils where cereal rye is grown. The observed diversity indicates that transitions from primary to secondary metabolites may be an important factor for cover crop allelopathic effects. Root exudates have been highlighted by several authors (Hu et al. 2018; Kong et al. 2018; Korenblum et al. 2020) as being important sources of metabolic compounds in the rhizosphere. Our results suggest that BX concentrations are similar from near the root crown to areas some distance away, suggesting a larger influence for the rhizosphere surrounding the roots for allelopathic activity. Breeding to increase the flux of BX compounds from cereal rye cover crops could be a way to enhance allelopathic weed suppression (Bais et al. 2006).

Declarations

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