Glucosinolate-Containing Plant Tissues as Bioherbicides

Paul D. Brown and Matthew J. Morra*

Division of Soil Science, University of Idaho, Moscow, Idaho 83844-2339

Enzymatic hydrolysis of glucosinolates, a class of compounds found in *Brassica* species, results in a number of products with potential to inhibit seed germination. To investigate the impact of both volatile and water soluble allelochemicals, germination bioassays were conducted with *Lactuca sativa* seeds in the presence of defatted seed meal of *Brassica napus*. Seed meal in which glucosinolates were hydrolyzed to remove volatile glucosinolate degradation products was compared with intact seed meal and water controls. Only tissues containing glucosinolates produced volatiles that inhibited germination. Biologically active volatiles were identified by GC-MS as isothiocyanates and nitriles, products of glucosinolate hydrolysis. Water-soluble components also inhibited germination, with analysis confirming the presence of nonvolatile products of glucosinolate hydrolysis. The results suggest that allelochemical control of germination with glucosinolate-containing plants may contribute to reductions in synthetic pesticide usage if weed seeds are targeted.

Keywords: *Glucosinolates; allelochemicals; Brassica; germination; isothiocyanates*

INTRODUCTION

Herbicides are a highly used class of pesticides, comprising 85% of all pesticides applied to crops in the United States (USDA, 1993). Alternative weed control strategies would minimize pesticide use and the associated potential for environmental contamination. Glucosinolates, compounds that occur in agronomically important crops, may represent a viable source of allelochemical control for a variety of soil-borne plant pests, including weeds.

Glucosinolates are sulfur-containing organic anions with a β-D-thioglucose moiety and various differentiating side-groups. They are found exclusively in dicotyledonous plants, with the highest concentrations in the families Resedaceae, Capparidaceae, and Brassicaceae (Fenwick et al., 1983). Biological effects are not usually attributed to glucosinolates directly, but rather to products such as organic cyanides (CN), isothiocyanates (ITC), oxazolidinethiones (OZT), and ionic thiocyanate (SCN^-) that are released upon enzymatic degradation by myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) in the presence of water. Reaction pathways and products have been reviewed (Chew, 1988; Larsen, 1981; Kjaer, 1976).

Insecticidal (Lichtenstein et al., 1964), nematicidal (Lazzeri et al., 1993; Mojtahedi et al., 1991), fungicidal (Angus it al., 1994; Gamliel and Stapleton, 1993; Chan and Close, 1987; Papavizas and Lewis, 1971), and phytotoxic (Vera et al., 1987; Oleszek, 1987; Mason-Sedun et al., 1986; Bell and Muller, 1973; Campbell, 1959) effects have been reported in association with *Brassica* tissues. In addition, isolated compounds of glucosinolate degradation, such as ITC, or synthetic analogues are known to have varying degrees of biological activity (Williams et al., 1993; Bialy et al., 1990; Wolf et al., 1984; Bell and Muller, 1973; Drobnica et al., 1967). Indeed, a few commercially available fumigants depend on the activity of methyl ITC (not known to be common in nature) either as the parent compound or produced from a dithiocarbamate precursor. Although water extracts of plant tissues have been shown to have some activity (Kasting et al., 1974; Bell and Muller, 1973), it has often been assumed that volatile products such as ITC are responsible for the observed biological impacts, an effect recently referred to as biofumigation (Angus et al., 1994). The role of glucosinolate degradation products in biological impact has proven difficult to determine, however. Assessment is complicated by the potential participation of other compounds and by inconsistent and conflicting results (Choesin and Boerner, 1991; Jessop and Stewart, 1983; Harper and Lynch, 1982; Waddington, 1978; Lewis and Papavizas, 1971). Our goal was to demonstrate allelochemical activity by coupling a clear example of biological impact with the necessary chemical analyses to determine if glucosinolate degradation compounds from plant tissues inhibited seed germination. We investigated both volatile and water-soluble components.

* Author to whom correspondence should be addressed [telephone (208) 885-6315; fax (208) 885-7760; e-mail mmorra@uidaho.edu].

MATERIALS AND METHODS

Plant Tissues. Defatted rapeseed meal (RSM) was obtained by extracting the oil from machine-harvested mature seed of *Brassica napus* L. (variety Dwarf Essex) with a CeCoCo press (Brown et al., 1991; Peterson et al., 1983). RSM, used without further alteration, served as the intact glucosinolate source (iRSM). Prior to experiments, glucosinolates were hydrolyzed in a portion of the defatted rapeseed meal (hRSM). To hydrolyze glucosinolates and produce hRSM, iRSM was moistened (1.4 mL of H_2O/g of meal) and covered, but vented to allow the escape of volatile products, for 48 h, and then dried at 38 °C for 24–30 h with stirring.

Germination Bioassay. To examine effects of volatile degradation products apart from water-soluble compounds that might be present in the tissue, hRSM, iRSM, and H_2O (control) treatments were separated from the lettuce seed in enclosed bioassay chambers so that no contact could occur except through the vapor phase (Figure 1A). Translucent to clear, covered, plastic containers (1450 cm^3) served as germination chambers. Thirty-six lettuce seeds (var. Simpson's Curled) were placed in a 6 × 6 grid on 8.5 × 8.5 cm germination blotter papers. Control and plant tissue treatments (5.5 g) were placed in small trays separated to the side of the blotter paper. Deionized water was added to all treatments (8.8 mL) and blotter paper (10 mL). All treatments were incubated at 18 °C with 14 h of fluorescent light per 24 h. Each treatment was replicated three times.

To examine the effects of water-soluble compounds apart from volatile products of degradation, only solutions from hRSM were allowed to contact the blotter paper and seed in another germination test (Figure 1B). Water-soluble contact

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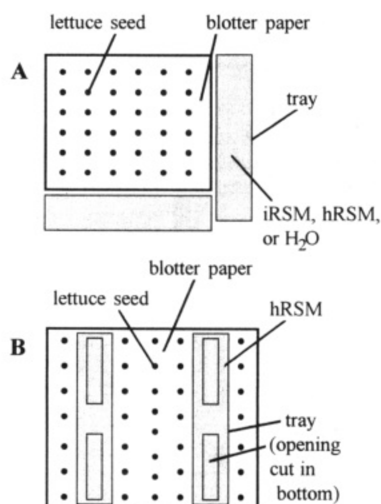

Figure 1. Germination bioassay setups for inhibition through contact with (A) volatile compounds and (B) water soluble compounds.

tests were similar to those conducted in volatile compound tests except that trays with holes cut in the bottom were placed directly on 10 × 10 cm blotter paper with seeds arranged around them. The entire 18.8 mL of H_2O was added to the hRSM, and excess water flowed into the blotter paper. Seeds not germinating within 16 days in all bioassays were considered dead.

Glucosinolate Analysis. Glucosinolates were quantified according to a modified version of the method used by the Canadian Grain Commission (Daun and McGregor, 1983). Glucosinolates from 0.2 g of plant material were desulfated on Sephadex columns (benzyl glucosinolate as the internal standard). A silylation mixture (350 μL) was added to the dried desulfated glucosinolates in 1.5-mL gas chromatography (GC) autosampler vials. The silylation mixture consisted of water-free acetone, bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), and 1-methylimidazole in a 56:20:2:1 ratio (J. P. Raney, Agriculture Canada, Saskatoon, SK, personal communication). A Hewlett-Packard 5890 Series II GC equipped with a flame ionization detector (FID) and a 15-m methylpolysiloxane column (DB-1, J&W, Folsom, CA; 0.32 mm i.d., 1.0-μm film) was used for analysis. The GC-FID operating conditions were as follows: injector, 290 °C; detector, 300 °C; initial oven temperature, 270 °C for 9 min, ramped 5 °C/min to 290 °C and held for 10 min; flow, 1.4 mL/min; and split ratio, 38:1.

Identification of trimethylsilyl (TMS) derivatives was accomplished by GC-mass spectroscopy (Hewlett-Packard 5890 Series II GC; 5972 quadrupole mass selective detector [MSD]; MS Chemstation software; NIST/EPA/NIH Mass Spectral Database). The GC-MSD operating conditions were as follows: injector, 260 °C; interface, 320 °C; initial oven temperature, 130 °C for 1 min, ramped 15 °C/min to 320 °C and held for 10 min; purge (splitless injection), 0.5 min; He flow, 1.14 mL/min; electron energy, 70 eV; emission, 50 μA; repeller, 30 V; scan range, m/z 25−470; and column, 5% phenyl-substituted methylpolysiloxane (HP-5MS), 30 m, 0.25 mm i.d., 0.250-μm film. Glucosinolates observed in trace quantities were resolved only when using the GC-MSD procedure.

Trapping of Volatiles. Volatiles were collected on traps consisting of ~100 mg Super Q porous polymer (Alltech, Deerfield, IL) in 16 × 0.4 cm i.d. glass tubes. Traps were preconditioned with N_2 at 230 °C for 15 min. Moistened rapeseed meal in proportion to that used in seed germination bioassays was placed in a 2-L Teflon chamber. Initial collection started 20 min after wetting of the meal and continued for 2 h. Purified air was swept through the chamber and the in-line trap at a flow of 80 mL/min. A second collection was started at 5 h and continued for 16 h, and a third collection was started at 22 h and stopped at 26 h. Flow was 50 mL/min for these collections. Volatiles were rinsed off the traps with CH_2Cl_2 (2 mL). Analysis was performed in the same manner as described for other extracts of degradation products.

Glucosinolate Degradation Product Analysis. For most glucosinolate degradation products, analysis was done according to procedures already described (Brown et al., 1994) with modification of the GC temperature programming as follows: injector, 220 °C; detector (FID), 280 °C; initial oven temperature, 36 °C for 3 min, first ramp, 20 °C/min to 96 °C, second ramp, 8 °C/min to 216 °C, third ramp, 16 °C/min to 280 °C and held for 7 min. Rapeseed meal was extracted (CH_2Cl_2) 4 h after wetting the meal to determine the glucosinolate degradation products. Identification was accomplished by comparison with known standards, sulfur detection (GC-flame photometric detector), and GC-MSD. In a few cases where identification on GC-FID chromatograms was difficult, quantitation was accomplished by direct comparison with the phenyl ITC peak (internal standard) with GC-MSD chromatograms.

The GC-MSD operating conditions were as follows: injector, 225 °C; interface, 280 °C; initial oven temperature, 36 °C for 2 min, first ramp, 15 °C/min to 96 °C, second ramp, 8 °C/min to 216 °C, third ramp, 18 °C/min to 270 °C and held for 4 min; purge, 0.5 min; He flow, 1.14 mL/min; electron energy, 70eV; emission, 50 μA; repeller, 30 V; scan range, m/z 30−280; column, HP-5MS, 30 m, 0.25 mm i.d., 0.250 μm film.

RESULTS AND DISCUSSION

Compound Identification. Mass spectral identification of several TMS derivatives of desulfoglucosinolates by electron-impact ionization has been accomplished (Christensen et al., 1982; Olsson et al., 1977), but spectra are limited. Glucosinolate identification by this means has often been avoided in favor of other techniques because of the abundance in the spectra of ions from the sugar moiety (Shaw et al., 1989; Hogge et al., 1988; McGregor et al., 1983; Eagles et al., 1981). However, GC analysis of TMS derivatives is often used, and mass spectral information of the derivatives can be useful in verifying glucosinolate identification.

As with previous researchers, we found the following peaks and approximate abundances to be common to TMS-derivatized desulfoglucosinolates: m/z 45(5), 59(7), 73(100), 103(12), 117(6), 129(10), 147(19), 169(10), 191(6), 217(17), 243(7), 271(8), 361(28). The base peak ion at m/z 73 is by far the largest in abundance, the next most abundant ion being only 28% of this peak. With a few exceptions, ions specific to the side chain also occur in low abundance but allow identification in many cases. Comparisons with previously published spectra were similar (Christensen et al., 1982; Olsson et al., 1977). Additional spectral information for several glucosinolates is shown in Table 1.

Spectra for glucosinolate degradation compounds were also compared with the literature where possible (Spencer and Daxenbichler, 1980; Cole, 1980; MacLeod and Islam, 1976; Kjaer et al., 1963). A notable difference appeared in the 4-pentenyl ITC spectrum, which had a prominent peak at m/z 99 ($m^+ - 28$).

The mass spectrum for an eight-carbon alkyl ITC (tentatively identified as 5-methylhexyl ITC) that has not previously been identified in *B. napus* is shown in Figure 2. We also identified 2-hydroxy-3-butenyl ITC with major ions at m/z 57(100), 73(24), 72(20), 101(14), 31(13), 129(10), however, the compound appeared to be mainly an artifact of injection temperature. Lowering the injection temperature greatly reduced the proportion of the ITC in relation to the alternative cyclic OZT product, suggesting the OZT ring was opening in the injection port at higher temperatures. Small amounts of at least four other suspected, but unidentified ITC were also observed in CH_2Cl_2 extracts of iRSM.

Analyses and Bioassays. Rapeseed varieties not bred for canola-quality oil often contain high concentrations of glucosinolates. Defatted rapeseed meal (RSM), a byproduct from seed pressed for industrial quality oil,

Table 1. Diagnostic Ions of Low Resolution Electron Ionization Mass Spectra of Trimethylsilyl Derivatives of Desulfoglucosinolates[a]

glucosinolate	$R-C(=S^+)-N-OSiMe_3$	$R-C{\equiv}\overset{+}{N}-OSiMe_3$	$R-C{\equiv}N^{+\bullet}$ [b]	R^+	other ions
4-methylpentyl		200(5)		85(1)	69(7), 55(8), 43(6), 41(7)
2-hydroxy-4-pentenyl[d]	304(1)	272(4)	182(1)	157(3)	143(6), 41(2)
4-methylthiobutyl		218(19)		103(15)[c]	204(3), 89(3)[c], 87(8), 61(10), 47(1)
phenylethyl			130(16)	105(5)	91(9), 65(1)
5-methylthiopentyl		232(8)	117(8)[c]		69(3), 67(7), 61(13), 55(3), 47(1), 41(5)
4-hydroxyindolyl-3-methyl[d]	437(2)		316(10), 315(20)	290(23)	202(4)
4-methoxyindolyl-3-methyl[d]	379(3)		258(9), 257(20)	232(25)	

[a] Numbers in parentheses are percent relative abundance compared to the base peak at m/z 73. [b] Usually as the fragment ion − 1. [c] Mass also from glucose moiety. [d] R is silylated in the mass spectrum.

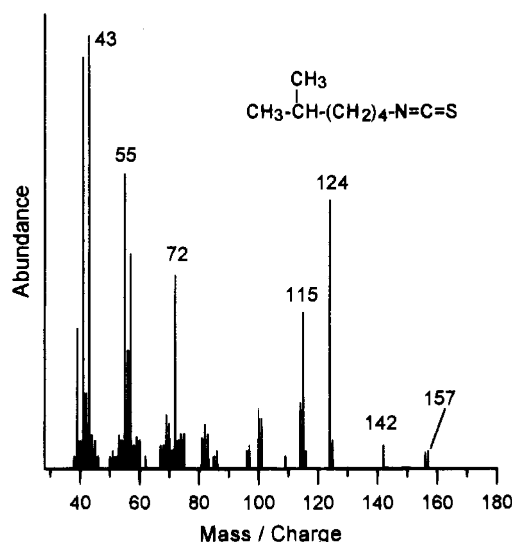

Figure 2. Mass spectrum of an alkyl isothiocyanate produced from intact rapeseed meal.

was used as a model tissue in bioassays and analyses. This represents one form in which *Brassica* tissues could be used in specialized horticultural or agricultural applications. Myrosinase, normally sequestered from glucosinolates in plant tissues, is released upon crushing of the seed. The glucosinolates remain stable in the defatted meal for extended periods of time, however, because of its low moisture content. The addition of water initiates hydrolytic degradation. To obtain a matrix similar to the RSM but without the glucosinolates, water was added to a portion of the RSM prior to the germination study. Analysis and comparison of glucosinolate content with the intact RSM (iRSM) showed most of the glucosinolates had been hydrolyzed in the previously wetted RSM (hRSM) (Table 2).

Volatiles from the hRSM had little or no effect on germination, whereas volatiles from iRSM severely limited germination (Figure 3). Water-soluble compounds from hRSM also severely limited germination (Figure 3). In water-soluble hRSM bioassays, a fair amount of fungal growth occurred on the blotter paper after ~10 days, potentially altering germination towards the end of the bioassay period.

Volatile compounds likely responsible for inhibition of germination would only be those produced in greater quantity from the iRSM than the hRSM. Trapping and analysis of vapors produced from moistened iRSM and hRSM showed that volatile glucosinolate degradation products were produced in much greater quantities from the iRSM than from the hRSM (Table 3). Furthermore, although other volatiles were collected on traps and detected, those evolved in greater quantity from the iRSM than from the hRSM were exclusively isothiocyanates and nitriles produced from glucosinolate hydrolysis. Volatile compounds not associated with glucosinolate hydrolysis and present in both hRSM and iRSM were not inhibitory because volatiles produced from the hRSM had little impact. It is possible that our procedure did not collect all volatiles evolved from RSM. However, the same trapping material has been used to collect other plant volatiles (Turlings and Tumlinson, 1992) and, because hRSM is iRSM wetted roughly 3

Table 2. Glucosinolates in Defatted Seed Meal of *B. napus* Var. Dwarf Essex[a]

glucosinolate R	μmol/g of tissue iRSM[b]	hRSM
allyl	0.3	0.0
isopropyl	tr[c]	0.0
3-butenyl	34.1	0.3
4-pentenyl	11.6	0.1
4-methylpentyl	tr	0.0
2-hydroxy-3-butenyl	75.1	0.4
2-hydroxy-4-pentenyl	6.1	0.0
4-methylthiobutyl	0.9	0.0
phenylethyl	2.2	0.0
5-methylthiopentyl	0.7	0.0
3-indolylmethyl	0.2	0.0
4-hydroxy-3-indolylmethyl	4.6	0.0
1-methoxy-3-indolylmethyl	tr	0.0
4-methoxy-3-indolylmethyl	tr	0.0

[a] Compounds are listed in order of GC elution time. [b] iRSM, intact defatted rapeseed (*B. napus*) meal; hRSM, glucosinolate-hydrolyzed defatted rapeseed meal. [c] tr, <0.05 μmol/g tissue; mass spectra were confirmed by more intense spectra obtained from leaf or root tissues of *B. napus*.

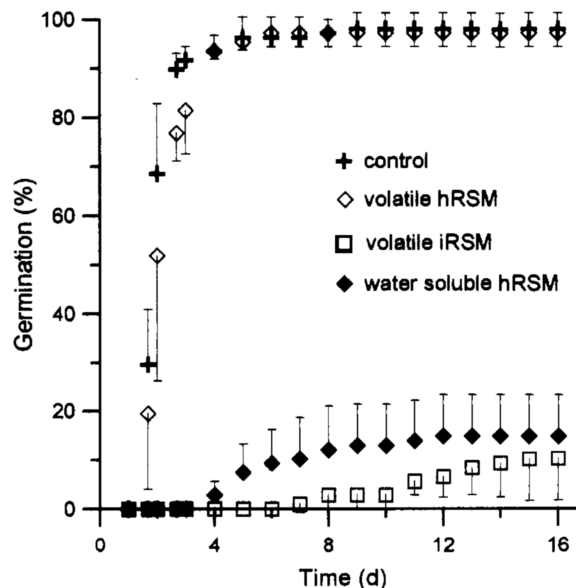

Figure 3. Effect of volatile compounds on seed germination in the presence of water (control), glucosinolate-hydrolyzed rapeseed meal (volatile hRSM), and intact rapeseed meal (volatile iRSM) and effect of water soluble compounds on seed germination (water soluble hRSM). Bars represent ±1 SD.

Table 3. Volatile Glucosinolate Degradation Products Trapped in Headspace

	collection time interval (h)				
	iRSM[a] (nmol/g of meal)			hRSM[a] (nmol/g of meal)	
glucosinolate degradation product[b]	0.3−2.3	5−21	22−26	0.3−2.3	5−21
3-butenyl CN	20.4	58.6	25.1	0.0	0.9
isopropyl ITC	0.8	1.4	0.4	0.0	0.0
4-pentenyl CN	2.5	8.7	3.7	0.0	0.5
allyl ITC	4.0	10.5	1.6	0.0	0.0
1-methylpropyl ITC	1.1	2.3	1.0	0.0	0.0
2-hydroxy-3-butenyl CN	tr[c]	0.6	0.2	0.0	6.0
2-methylpropyl ITC	0.0	0.5	0.0	0.0	0.0
3-butenyl ITC	228.1	260.2	26.8	0.4	2.5
4-pentenyl ITC	23.9	8.7	1.5	tr	1.1
3,4-epithiobutyl CN	1.5	11.6	2.2	0.0	2.4
4-methylpentyl ITC	0.5	0.4	0.0	0.0	0.0
phenylethyl CN	0.1	0.2	0.0	0.0	0.4
4,5-epithiopentyl CN	0.0	0.5	0.0	0.0	0.0
5-methylhexyl ITC	tr	0.2	0.0	0.0	0.0
phenylethyl ITC	1.0	2.4	0.6	0.0	0.4

[a] iRSM, intact defatted rapeseed (*B. napus*) meal; hRSM, glucosinolate hydrolyzed defatted rapeseed meal. [b] Compounds are listed in order of elution time: CN, cyano group; ITC, isothiocyanate group. [c] tr, <0.1 nmol/g of meal.

Table 4. Glucosinolate Degradation Products in Extracts of Defatted *B. napus* Seed Meal

glucosinolate degradation product[b]	μmol/g of meal iRSM	μmol/g of meal hRSM
3-butenyl CN	0.28	0.06
isopropyl ITC	tr[e]	0.00
4-pentenyl CN	0.05	0.05
allyl ITC	0.09	0.00
1-methylpropyl ITC	0.02	0.00
2-hydroxy-3-butenyl CN	0.97	2.63
3-butenyl ITC	5.07	0.05
4-pentenyl ITC	0.54	0.03
3,4-epithiobutyl CN	0.82	0.61
4-methylpentyl ITC	0.02	0.00
4-methylthiobutyl CN	tr	0.00
2-hydroxy-3,4-epithiobutyl CN1[c]	0.58	0.72
phenylethyl CN	0.15	0.13
2-hydroxy-3,4-epithiobutyl CN2[c]	0.57	1.20
4,5-epithiopentyl CN	0.06	0.04
5-methylhexyl ITC	0.02	0.00
5-methylthiopentyl CN	tr	0.00
4-methylthiobutyl ITC	0.11	0.00
phenylethyl ITC	0.73	0.05
5-vinyl OZT	39.17	10.30
3-methylthiopentyl ITC[d]	>0.05	0.00
5-allyl OZT	4.29	1.26
4-methylsulfinylbutyl ITC	0.49	0.05
5-methylsulfinylpentyl ITC	0.60	0.10

[a] iRSM, intact defatted rapeseed (*B. napus*) meal; hRSM, glucosinolate-hydrolyzed defatted rapeseed meal. [b] Compounds are listed in order of elution time: CN, cyano group; ITC, isothiocyanate; OZT, oxazolidinethione; compounds highlighted in bold type were also identified in water extracts of RSM. [c] Diastereomers. [d] This peak was obscured by the large 5-vinyl OZT peak, but its presence was confirmed by earlier extraction and single ion monitoring. [e] tr, <0.01 μmol/g of meal.

days in advance, the evidence clearly suggests that volatiles from glucosinolate degradation are the agents responsible for the inhibition observed.

Very little product from the major glucosinolate in iRSM (R = 2-hydroxy-3-butenyl; Table 2) was measured by headspace trapping. However, several products of this glucosinolate, including OZT, were present in CH_2Cl_2 extracts of both iRSM and hRSM (Table 4). These oxygen-containing hydrolysis products have low volatility and are also more easily solubilized in water. When hRSM was rinsed with water and analyzed, most of these oxygen-containing products were removed. Water extracts of RSM also contained high amounts of these compounds (data not shown). Aqueous extracts of RSM were previously found to contain SCN^- (Brown et al., 1991). These water-soluble products would have been rinsed into the blotter paper to contact the seed and inhibit germination (Figure 3), although contributions from other compounds were not eliminated.

For *Brassica* tissues to be most effectively used in natural pest control systems as a plow down, amendment, or rotation crop, the influence of soil factors on product formation and biological impact must be determined. However, ITC, OZT, and SCN^- are produced in soil amended with iRSM (Brown et al., 1991, 1994). In addition, soils amended with allyl glucosinolate (sinigrin) produce primarily the ITC as opposed to the cyano product (Borek et al., 1994). ITC production and disappearance in both cases were fairly rapid, as were concentrations of the volatile products we observed in headspace (Table 3). It would thus appear that a narrow window is available for bioactive volatile effects in plant tissue-amended soils; this narrow window possibly contributes to the variability of results observed by others.

Little is known of the potential contribution to pest control by root exudates of *Brassica* species. Similarly, the relative importance of the volatile versus water-soluble components in determining biological impact remains to be determined, but both may play important roles. Although lettuce has weedy relatives, further work targeting specific weeds is necessary. We suggest that attempts to increase glucosinolate content in varieties of *Brassica* designed as rotation crops, or to increase the content in certain portions of the plant such as the root, could be beneficial toward decreasing weed populations through the inhibition of germination and thereby contribute to a sustainable agricultural system.

ABBREVIATIONS USED

ITC, isothiocyanate; OZT, oxazolidinethione; SCN^-, ionic thiocyanate; SCN, thiocyanate; CN, (organic) cyanide; RSM, defatted rapeseed meal; iRSM, intact RSM; hRSM, glucosinolate hydrolyzed RSM.

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