Allelochemicals Released in Soil Following Incorporation of Rapeseed (*Brassica napus*) Green Manures

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Plant-derived allelochemicals such as those produced by glucosinolate hydrolysis in *Brassica napus*, or rapeseed, are viable alternatives to synthetic compounds for the control of soil-borne plant pests. However, allelochemical production and residence times in field soils have not been determined. Soil samples were taken at 0−7.5 and 7.5−15 cm during a period of 3 weeks following plow-down of two winter rapeseed cultivars (Humus and Dwarf Essex). Soil samples were extracted with dichloromethane and analyzed using gas chromatography. Nine glucosinolate degradation products were identified—five isothiocyanates, three nitriles, and one oxazolidinethione. Maximum concentrations were observed 30 h after plow-down. Compounds derived from 2-phenylethyl glucosinolate, the principal glucosinolate in rapeseed roots, dominated the profile of degradation products. Shoot glucosinolates left few traces. This indicates that rapeseed roots may be a more important source of toxic fumigants than above-ground parts of the plant.

Keywords: *Glucosinolates; isothiocyanates; allelochemicals; Brassica spp.; soil fumigation*

INTRODUCTION

Brassica napus, like the mustards, cabbages, and other members of the Brassicaceae, produces a class of chemicals known as glucosinolates (Fenwick et al., 1983; Larsen, 1981) (Figure 1). There are >100 glucosinolates (Brown and Morra, 1997), distinguished from one another by their different organic side chains. They occur in all parts of the plant and can degrade via enzymatic (Bjorkman, 1976; Challenger, 1959; Larsen, 1981; Underhill, 1980) or nonenzymatic (Challenger, 1959; Fenwick et al., 1983; MacLeod et al., 1981) hydrolysis, generally in response to damage to the plant such as that caused by insect feeding. The specific hydrolysis products formed depend on the R group of the parent glucosinolate (Figure 1). The most important degradation pathway at neutral pH involves hydrolysis to produce glucose, bisulfate, and isothiocyanates. The latter are highly toxic compounds that tend to be quite volatile. Other products are also produced. Oxazolidinethione, for instance, results from cyclization of a hydroxylated isothiocyanate (Van Etten et al., 1969). At low pH and/or in the presence of reduced iron, glucosinolates can degrade to give nitriles and free sulfur (Chew, 1988; Van Etten and Tookey, 1979; Van Etten et al., 1969). The upper limit of pH for nitrile formation from rapeseed tissue is 5.8 (Josefsson, 1967), whereas nitriles are favored over isothiocyanates at pH values <3.5 (Larsen, 1981). Organic thiocyanates can also be products of glucosinolate hydrolysis, but the mechanism of their formation has not been adequately explored (Tookey et al., 1980). According to Chew (1988), only three glucosinolates—2-propenyl, benzyl, and 4-methylthiobutyl—can produce organic thiocyanates because only these three have R groups that are capable of forming stable intermediary cations. If the R group of the thiocyanate contains a terminal alkene structure, and a specifier protein is present, the thiocyanate may be converted to an epithionitrile (Chew, 1988).

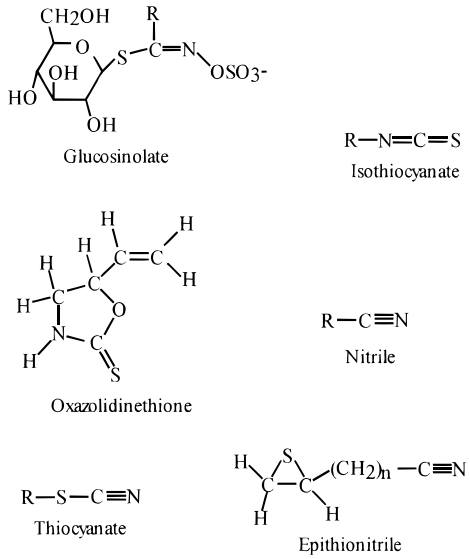

Figure 1. Glucosinolates and their degradation products.

Most of these degradation products are toxic to some degree (Duncan, 1991; Feeny, 1977; Thompson, 1983; Van Etten et al., 1969) because of their reactive functional groups. Insecticidal (Fenwick et al., 1983), fungicidal (Mayton et al., 1996; Papavizas and Lewis, 1971), nematicidal (Mojtahedi et al., 1991), and herbicidal (Brown and Morra, 1996; Stiehl and Bible, 1989; Vera

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et al., 1987) effects have been documented. Toxicity against higher animals has also been demonstrated (Tookey et al., 1980). The mode of action has not in all cases been identified (Duncan, 1991). For isothiocyanates this can involve bonding to protein sulfhydryl groups (Kawakishi and Kaneko, 1985), thus making them broad-ranging toxins. Their toxicity and quick dispersal and degradation to more innocuous compounds make glucosinolate hydrolysis products and the plants that produce them potential alternatives to the synthetic soil fumigants, many of which have been banned because of adverse environmental effects. Significant effort has been devoted to using members of the Brassicaceae as green manures and cover crops in an attempt to control soil-borne plant pests (Kirkegaard and Sarwar, 1988; Sarwar and Kirkegaard, 1998).

Laboratory studies to measure glucosinolate products have been performed with both tissues (Brown and Morra, 1996) and tissue-amended soils (Brown et al., 1991), but field studies are lacking. Such studies are necessary because extrapolation of laboratory studies to predict field results is tenuous. Our objectives were to identify and quantify glucosinolate breakdown products in the field following incorporation of a winter rapeseed green manure. Two cultivars of winter rapeseed with relatively high concentrations of glucosinolates were selected because of their potential use in pest control strategies.

MATERIALS AND METHODS

Field Investigations. Two cultivars of winter hardy rapeseed (*B. napus*), Dwarf Essex and Humus, were planted at the Aberdeen Research and Extension Center of the University of Idaho on August 17, 1994, using a seeding rate of 7.8 kg ha^{-1}. The soil at the experiment station is a Declo sandy loam, consisting of 480 g of sand, 70 g of clay, and 450 g of silt per kilogram of soil. The pH is 8.1 down to at least 45 cm; the soil has 13.7 g kg^{-1} organic carbon in the surface 30 cm and 7.6 g kg^{-1} organic carbon at 30−45 cm. Rapeseed was planted in plots measuring 6 by 18 m in a randomized complete block design with four replications. Fallow plots treated in the same manner as the treatments served as controls. Root and shoot biomass were determined by completely removing rapeseed tissues from two 0.25-m^2 sections in each plot immediately prior to incorporation on April 27, 1995. We excavated to a depth of 30 cm when sampling the roots. Shoots and roots were dried at 50 °C and weighed to express all biomass and respective glucosinolate concentrations on a dry weight basis. Also on April 27 prior to plow-down, 10 additional plants outside the biomass sampling areas were excavated from each plot, roots excised, and subsamples of the roots and shoots immediately frozen in liquid nitrogen. The samples were later freeze-dried in the laboratory and analyzed for glucosinolate concentrations. Rapeseed green manures were incorporated into soil to a depth of ~13 cm using a rototiller. Plants were in the bud to very early flowering stage (<1% flowering).

Soil and Plant Analyses. Glucosinolate measurement was performed using a gas chromatographic (GC) method similar to that recommended by the Canadian Grain Commission (Daun and McGregor, 1983). Glucosinolates were extracted with methanol from 0.2 g of plant tissue. The extracts were placed on Sephadex columns that were first treated with 6 M imidazole in 5 M formic acid and rinsed with water. Other compounds were separated from the sample in rinse steps using 67% methanol and water. Glucosinolates were desulfated overnight, eluted from the columns using 60% methanol, and silylated in acetone. Silylated derivatives were separated and identified using a Hewlett-Packard 5890 Series II GC equipped with a 5972 quadrupole mass selective detector (MSD). GC-MSD operating conditions included the following: injector, 260 °C; interface, 320 °C; initial oven temperature, 130 °C for 1 min, ramped at 15 °C min^{-1} to 320 °C, and held for 10 min; purge (splitless injection), 0.5 min; He flow of 1.14 mL min^{-1}; emission at 50 μA; repeller at 30 V; scan range, m/z 25−470. The column coating was 5% phenyl-substituted methylpolysiloxane (HP-5MS; 30 m, 0.25 mm i.d., 0.250 μm film). Glucosinolates were identified with the assistance of previously reported spectra and quantified using published response coefficients and benzyl glucosinolate as an internal standard (Fenwick et al., 1980; Raney and McGregor, 1990).

Soil samples were taken from Humus, Dwarf Essex, and fallow control plots immediately after tissue incorporation on April 27 and then at intervals thereafter, with the last sampling done on May 17. Only a light infestation of seedling weeds was growing in the control plots. Samples were taken at two depths, 0−7.5 and 7.5−15 cm. The samples were taken with a stainless steel corer having an interior diameter of 1.4 cm and were deposited immediately into Teflon centrifuge tubes containing 5 mL of 0.2 M aqueous $CaCl_2$ and 12 mL of dichloromethane with 0.1% cyclohexane as an internal standard. We used this particular mixture because Brown et al. (1994) determined it to be the most effective for extracting isothiocyanates and other products of glucosinolate degradation from soil without misrepresenting relative abundances and because glucosinolate hydrolysis during the extraction procedure is minimized. Preliminary investigations demonstrated that inclusion of $CaCl_2$ in the mixture inhibited glucosinolate hydrolysis in tissues by 60% in 30-min extractions. In the present case, soil samples were weighed, shaken for 15 min, and centrifuged for 10 min at 1950*g*. This procedure therefore minimizes glucosinolate hydrolysis during the extraction, thereby reducing the formation of hydrolysis products from any rapeseed tissues included in the soil sample.

The dichloromethane fraction of each sample was withdrawn with a syringe and placed in a vial with 0.4 g of sodium sulfate for desiccation. After contact with the sodium sulfate for a minimum of 1 h, 1.5−2.0 mL of the dichloromethane fraction was withdrawn from the vial and passed through a 0.45-μm filter (FP-450 Gelman Sciences, Ann Arbor, MI) into a GC vial. The vial was capped and stored in a freezer pending analysis for glucosinolate breakdown products. Other soil samples were taken concurrently and oven-dried, so that constituent concentrations might be expressed on a dry weight basis. A total of 216 samples was analyzed, representing 2 cultivars × 2 depths × 12 samplings × 4 replications + 1 control × 2 depths × 12 samplings.

Analyses were performed on a Hewlett-Packard 5890 Series II+ GC equipped with a 30-m-long DB-5 column, having an interior diameter of 0.25 mm and a coating thickness of 0.25 μm. The carrier gas was helium. The temperature program began at 40 °C, was held for 2 min, then increased at 15 °C min^{-1} to 285 °C, and was held for 1.67 min. We ran standards of 2-propenyl, propyl, phenyl, benzyl, and 2-phenylethyl isothiocyanate and of goitrin (oxazolidinethione). We used a Hewlett-Packard 5972 quadrupole MSD and adapted the relationship of Brown et al. (1994) between FID peak area and isothiocyanate molecular weight to the GC-MSD analysis of isothiocyanates for which standards were not available. Absolute quantities of nitriles were estimated by comparison of chromatograms with those of corresponding isothiocyanates of similar structure and number of carbon atoms.

Many samples contained only traces of glucosinolate degradation products, so faint that it was not possible to distinguish a peak against a background in the total ion chromatogram. We found, however, that for each compound it was possible to identify one ion, or a suite of ions, rare in the background, that enabled us to confirm the presence of that compound (Table 1). From among the suite of ions representing a given compound, we chose one for quantification. We measured this peak's area and then used a few of the richer samples for which total ion peaks could be clearly discerned to determine ratios between peak areas of diagnostic ions and total ion peak areas. We used these ratios to determine concentrations based on the integrals of the diagnostic ions in the standard curves. In this way we were able to quantify the analyte even in samples in which the concentration was

Table 1. Ions Used for Mass Spectrometric Identification of Glucosinolate Hydrolysis Products

compound	*m/z*
2-phenylethyl ITC[a]	163[b]
3-butenyl ITC	113, 72[b]
4-pentenyl ITC	127, 126, 99, 72[b]
4-methylthiobutyl ITC	161, 115, 72,[b] 61
5-methylthiopentyl ITC	175, 129, 72,[b] 61
goitrin	129,[b] 68
benzene propanenitrile	131, 91[b]
5-methylthiopentanenitrile	129, 82, 61[b]
6-methylthiohexanenitrile	143, 96, 61[b]

[a] ITC, isothiocyanate. [b] Ion used to quantify concentration of respective analyte.

too low to give a discernible total ion peak. Statistical comparisons of the data using SAS (version 6; SAS Institute, Cary, NC) and the General Linear Model procedure were performed.

RESULTS AND DISCUSSION

Glucosinolates in Plant Tissue. The data in Table 2 show that (1) the dominant glucosinolate in the roots of both cultivars was 2-phenylethyl, (2) the alkenyl glucosinolates (3-butenyl, 4-pentenyl, 2-hydroxy-3-butenyl, and 2-hydroxy-4-pentenyl) were prominent in both the roots and tops but were dominant in the tops, (3) methylthioalkyl glucosinolates can be found in modest concentrations in the roots but were nearly absent from the tops, (4) indole glucosinolates were present only at low concentrations in the roots and tops of both cultivars, (5) total glucosinolate concentrations were greater in the roots than in the shoots, and (6) glucosinolate concentrations in Dwarf Essex roots were greater than in Humus roots, while concentrations in the tops of the two cultivars were about the same.

Glucosinolate Degradation Products in Soil. The most abundant degradation product produced in soils collected at both depths, regardless of rapeseed cultivar, was 2-phenylethyl isothiocyanate (Figures 2−4). The second most abundant was benzenepropanitrile (Figures 2−4). Both products were definitively identified using mass spectrometry (Figure 2). Benzenepropanitrile and 2-phenylethyl isothiocyanate are products of the degradation of 2-phenylethyl glucosinolate. This is the dominant glucosinolate in the roots of both Humus and Dwarf Essex cultivars, but it is only a minor constituent of their shoots (Table 2). The production of 2-phenylethyl isothiocyanate is especially significant because its toxicity against insects and fungi has been documented (Borek et al., 1998; Sarwar et al., 1998).

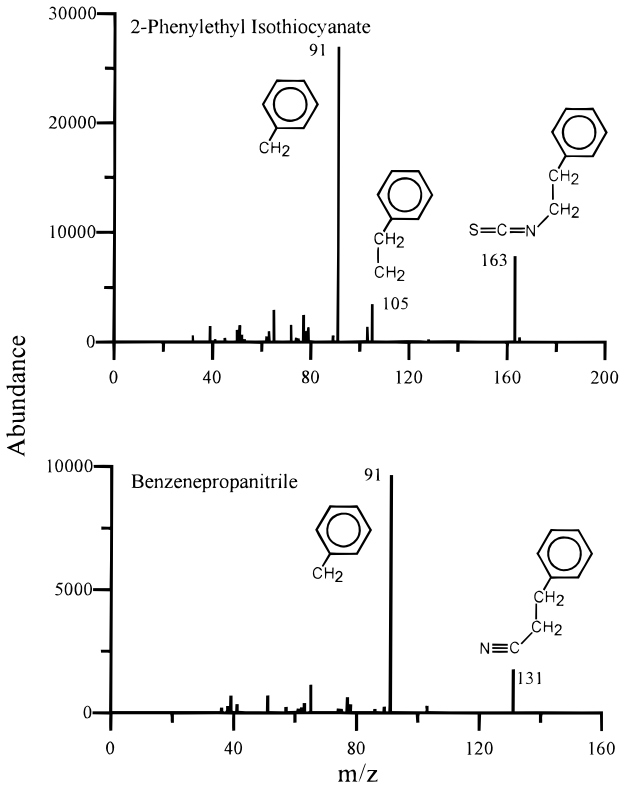

Figure 2. Mass spectra of the principal glucosinolate degradation products observed in soils collected after incorporation of rapeseed green manures.

3-Butenyl, 4-pentenyl, 4-methylthiobutyl, and 5-methylthiopentyl isothiocyanates were found in small quantities in a few samples (Figures 3 and 4). 5-Methylthiopentanenitrile and 6-methylthiohexanenitrile also appeared in a few samples. Four samples held traces of goitrin. Epithionitriles and organic thiocyanates were not detected.

Each nitrile that was identified shares a parent glucosinolate with one of the isothiocyanates that was observed. Benzenepropanenitrile has already been mentioned. 5-Methylthiopentanenitrile derives from the same parent glucosinolate as 4-methylthiobutyl isothiocyanate, and 6-methylthiohexanenitrile derives from the same parent compound as 5-methylthiopentyl isothio-

Table 2. Glucosinolate Concentrations (Micromoles per Gram of Tissue)[a] in Two Cultivars of *B. napus* Tissues at the Time of Incorporation

	Humus		Dwarf Essex	
glucosinolate	tops	roots	tops	roots
1-methylpropyl	ND[b]	ND	tr[c]	ND
3-butenyl	1.01 (25%)	0.42 (61%)	1.08 (30%)	0.79 (23%)
4-pentenyl	5.09 (24%)	2.30 (46%)	5.60 (18%)	2.74 (20%)
2-hydroxy-3-butenyl	3.92 (20%)	2.64 (50%)	4.09 (20%)	4.29 (26%)
2-hydroxy-4-pentenyl	1.48 (26%)	1.36 (24%)	1.55 (21%)	1.98 (21%)
4-methylthiobutyl	tr	0.90 (73%)	tr	1.88 (23%)
2-phenylethyl	0.77 (27%)	8.77 (33%)	0.91 (16%)	12.86 (20%)
5-methylthiopentyl	0.05	1.63 (63%)	0.11 (84%)	2.80 (58%)
indole-3-methyl	0.29 (61%)	0.12 (33%)	0.48 (26%)	0.20 (39%)
1-methoxyindole-3-methyl	0.02	0.02	0.07	0.06
4-hydroxyindole-3-methyl	0.05	0.03	0.04	0.05
4-methoxyindole-3-methyl	0.01	0.06	0.01	0.06
total	12.69	18.25	13.94	27.71

[a] Each figure represents the mean of eight replications. Coefficients of variance are shown in parentheses, except where concentrations were <0.1 μmol g^{-1} of freeze-dried tissue. [b] Not detected. [c] Trace.

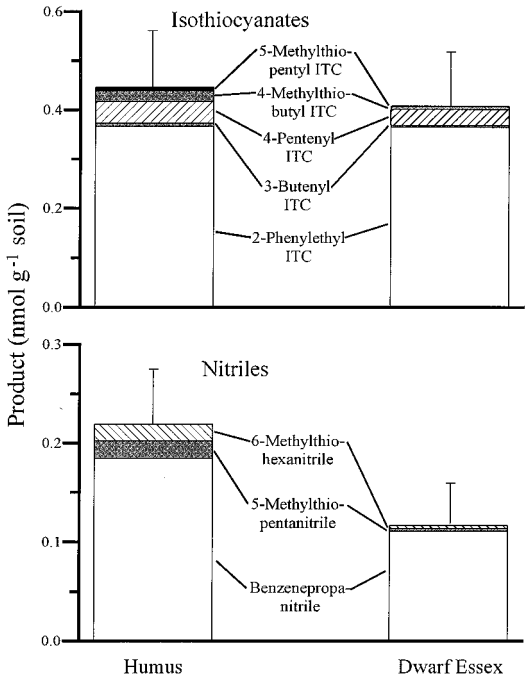

Figure 3. Isothiocyanates (ITCs) and nitriles detected in soils following the incorporation of two different rapeseed cultivars. A lack of covariance was indicated by statistical analyses, and samples collected at both soil depths were therefore pooled to obtain the data shown. Standard error bars of pooled data are shown with each respective mean.

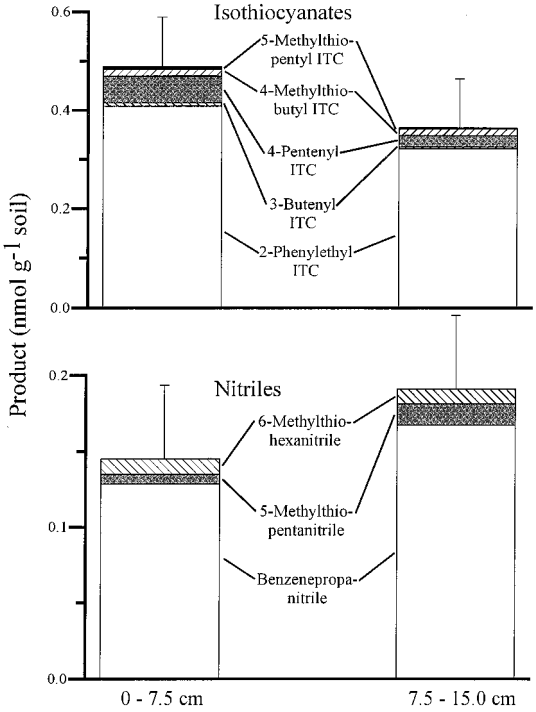

Figure 4. Glucosinolate degradation products detected at different soil depths following the incorporation of rapeseed. A lack of covariance was indicated by statistical analyses, and samples collected from soils cropped with both cultivars were therefore pooled to obtain the data shown. Standard error bars of pooled data are shown with each respective mean.

cyanate. Like the isothiocyanates, these nitriles are potential toxins and are also generally quite volatile.

Comparison of the concentrations of degradation products between cultivars, between depths, and with time yielded very high coefficients of variation. This is to be expected given the heterogeneous distribution of rapeseed tissue in the soil following incorporation of the crop. In Figure 3 it appears that the Humus cultivar produced more of each of the nine glucosinolate degradation products found than did the Dwarf Essex cultivar. For nitriles in particular the differences seem to be appreciable, but according to the LSD test, they are not significant even at the 0.1 level of probability. Figure 4 shows the distribution of products as a function of depth; there is a trend for isothiocyanate production near the surface and nitrile production at greater depths, although again there is no significance at the 0.1 level of probability. Goitrin was more abundant in the Humus samples and nearer the surface, but the quantities were small, in the picomole per gram of soil range, and are not shown.

Nitriles were not expected, because they are released in abundance from their respective glucosinolate precursors only in a much more acid environment than prevails in this soil (Josefsson, 1967; Larsen, 1981). Borek et al. (1994) amended sinigrin (2-propenyl glucosinolate) in pure form to soils and observed predominantly isothiocyanate formation in soils with pH values ranging from 5.65 to 8.30. In contrast, sinigrin hydrolysis in unbuffered aqueous solutions resulted in nitrile evolution. They attributed nitrile formation to the rapid decrease in pH that resulted from deprotonation of bisulfate released by the degrading glucosinolate. However, Van Etten and co-workers also associated nitrile evolution with tissue freshness (Van Etten and Tookey, 1979; Van Etten et al., 1969). Thus, if degradation takes place in pockets of plant material largely insulated from surrounding soil, products of such reactions may be expected to reflect the vegetal rather than the edaphic environment. Nitrile detection in our soils indicates that glucosinolate hydrolysis in green manures may produce nitriles at the expense of the respective isothiocyanates.

It is remarkable that the bulk of the isothiocyanates detected, and all of the nitriles, should derive from glucosinolates that are nearly absent from the tops of the plants. This is especially surprising given the fact that shoot biomass was ~4 times root biomass when expressed on a dry weight basis. Shoot biomass for Dwarf Essex was 5230 kg ha^{-1} and root biomass 1221 kg ha^{-1}. Shoot biomass for Humus was 5440 kg ha^{-1} and root biomass 1447 kg ha^{-1}. The total amount of glucosinolates contributed from Dwarf Essex shoots as calculated using concentration data in Table 2 was 73 mol ha^{-1}, whereas roots contributed only 34 mol ha^{-1}. Similarly, glucosinolates contributed to the soil from Humus shoots totaled 69 mol ha^{-1} and roots only 26 mol ha^{-1}. Thus, soil contributions of glucosinolates from shoot biomass of both varieties were more than double those of the root biomass.

The dominance of root-derived hydrolysis products in soil extracts indicates that roots may play a more prominent role than shoots in contributing allelochemicals to the soil environment. The exact cause for this phenomenon is unknown, but several possibilities exist. First, release of hydrolysis products from shoot tissue may be incomplete as a result of product interaction with other tissue constituents. Isothiocyanate functional groups are relatively reactive, interacting with nucleophilic compounds to form dithiocarbamic esters with $-SH$ groups, thiourea derivatives with $-NH_2$ groups, and N-monosubstituent dithiocarbamic esters with $-OH$

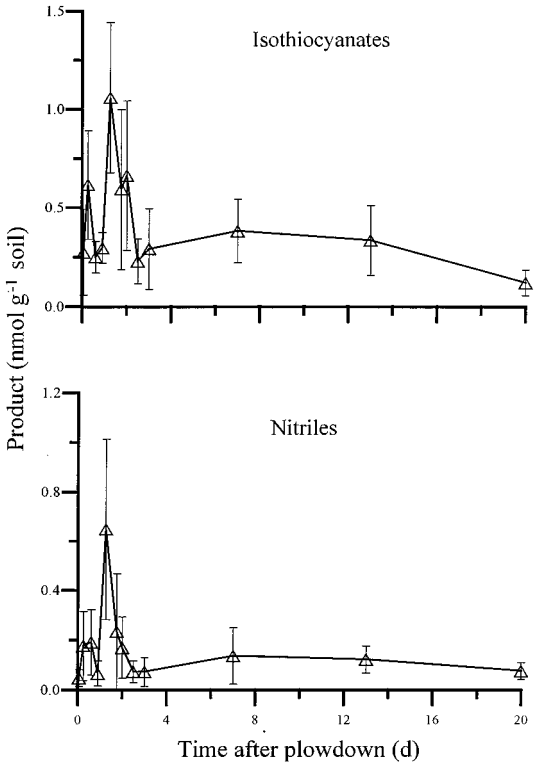

Figure 5. Appearance of glucosinolate degradation products in soil following the incorporation of rapeseed green manures. Samples across both soil depths and obtained from fields cropped with both cultivars were pooled to obtain the data shown. Vertical bars represent standard errors for each respective mean.

groups (Duus, 1979). For example, the efficiency of 3-butenyl isothiocyanate release from leaf and stem tissue was 8% of the amount predicted on the basis of the concentration of the respective glucosinolate (Brown and Morra, 1996). In contrast, only 0.7% of the predicted amount of 3-butenyl isothiocyanate was released from defatted seed meal, probably as a result of its increased protein content (Brown and Morra, 1995). The efficiency of release from root tissues has not yet been compared with that from above-ground tissues, but differences seem likely. Second, measured hydrolysis products may reflect continual release during plant growth from rapeseed roots, whereas above-ground tissues make a one-time contribution only at the time of incorporation. However, elevated concentrations of potential allelochemicals derived from rapeseed roots were not observed at the initial soil sampling times (Figure 5). Contributions prior to incorporation are thus possible, but any hydrolysis products contributed from such a mechanism did not accumulate to a detectable concentration. Finally, soil residence times vary among the hydrolysis products. Because the isothiocyanate functional group is so reactive, sorption occurs to soil constituents. Differences in soil residence times of various isothiocyanates are not likely controlled solely by this functional group but by differences in aqueous solubility, sorption, and volatility. The overall impact of each parameter on soil residence times and extractability of the individual isothiocyanates has not yet been determined but possibly controls extractable residue concentrations. Although the exact cause for the observed results cannot be determined from the current data, it can be concluded that any predictions of pest control based only on glucosinolate concentrations in above-ground tissues may be misguided. In fact, it appears that concentrations of glucosinolates in the roots were most important and that glucosinolates in above-ground tissue made only a minor contribution to the total allelochemical pool.

Concentrations of all compounds in all soils were very low, seldom exceeding 1 nmol g^{-1} (dry weight) of soil. Comparisons made with commercial fumigants indicate that isothiocyanate concentrations measured here are below those recommended for pest control. Soil fumigation with methyl isothiocyanate generated in situ by the hydrolysis of sodium *N*-methyldithiocarbamate can be used for comparison. The amount of isothiocyanate recommended for soil sterilization can be calculated by assuming a 15.2-cm depth of incorporation, soil bulk density of 1.4 g cm^{-3}, and 100% conversion from the dithiocarbamate to methyl isothiocyanate. The calculated values range from 517 to 1294 nmol of methyl isothiocyanate g^{-1} of soil depending on the crop and specific plant pest. Complete hydrolysis of the major isothiocyanate-forming glucosinolates (i.e., 3-butenyl, 4-pentenyl, 4-methylthiobutyl, 2-phenylethyl, and 5-methylthiopentyl) in root and above-ground tissues would result in 30 nmol of isothiocyanate g^{-1} of soil, assuming the same incorporation depth and soil bulk density as above. Thus, even total release would result in a contribution significantly below recommended synthetic pesticide rates. It should be remembered, though, that each sample represents a moment in time. The concentration at a particular instant may be less important than the total amount of a substance diffusing through the soil during the growth period. This is an important consideration given the fact that rapeseed roots possibly contribute hydrolysis products to the soil during an extended period.

Isothiocyanate concentrations, except 3-butenyl, increased and decreased in a similar fashion with time, resulting in a significant correlation ($p = 0.05$). No clear trend was observed for the production of 3-butenyl isothiocyanate. The abundances of most compounds peaked at the fifth sampling (Figure 5), 30 h after incorporation or a little later. Concentrations then trailed off erratically to the 12th sampling, 20 days after incorporation, by which time they were generally below the limit of detection. The change of concentration with time reflects (1) the rate of glucosinolate degradation and (2) the rate of dissipation of the degradation products under the edaphic and meteorological conditions of the time and place where the study was conducted. Papavizas and Lewis (1971) observed that the effectiveness of cruciferous soil amendments as fungicides diminishes at low temperature and excessive moisture. They suggest that low temperature slows the enzymatic degradation of glucosinolates and that excessive moisture can inhibit release of volatile sulfur compounds. Absolute changes in hydrolysis product concentrations in soil will thus be a function of soil and environmental characteristics.

Conclusions. While the entire plant was turned under, and many different glucosinolates are to be found in the different parts of the plant, our extractions were dominated by the degradation products of only one—2-phenylethyl glucosinolate. Little of this glucosinolate is present in *B. napus* shoots. Although above-ground tissues contain a larger pool of glucosinolates, release of significant amounts of their respective hydrolysis products to the soil environment at the time of incor-

poration was not observed. Breeders and others who concern themselves with glucosinolate levels in the Brassicaceae are accustomed to looking at the seeds, or perhaps the shoots, and not worrying so much about the roots. We would suggest that those interested in developing *B. napus* as a natural soil fumigant should give consideration to the roots as a primary source of allelochemicals for soil fumigation.

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