Allelochemicals Produced during Sinigrin Decomposition in Soil

Vladimir Borek, Matthew J. Morra,* Paul D. Brown, and Joseph P. McCaffrey

Department of Plant, Soil, and Entomological Sciences, University of Idaho, Moscow, Idaho 83844-2339

Although allelochemicals are produced from glucosinolates contained in *Brassica* spp. tissues, little is known concerning allelochemical formation and fate in soil. Enzymatic decomposition of sinigrin by myrosinase was determined in buffered and unbuffered solutions, soil extracts, and soils. Both allylnitrile and allyl isothiocyanate were identified as products in buffered solutions, with allylnitrile formation dominating at pH values below 4.0 and allyl isothiocyanate at higher pH values. The addition of Fe^{2+} to buffered solutions promoted the formation of allylnitrile, whereas Fe^{3+} inhibited the enzymatic reaction. Significant amounts of allylnitrile were formed in weakly buffered water extracts from soils but not in soils or ammonium acetate extracts obtained from those soils. The dominant product of sinigrin decomposition in soils and ammonium acetate extracts was allyl isothiocyanate regardless of soil characteristics. Because isothiocyanates have pesticidal activities and are dominant products formed from glucosinolates in soil, the use of *Brassica* spp. tissues to control soil-borne plant pests merits further attention.

INTRODUCTION

Glucosinolates constitute a class of approximately 100 organic anions possessing a β-D-thioglucose moiety, a sulfonated oxime, and a side chain that is aliphatic, aromatic, or heterocyclic (Chew, 1988) (Figure 1). Glucosinolates are produced by dicotyledonous plants, commonly by members of the order Capparales. The most economically important plants containing glucosinolates are in the Brassicaceae (Fenwick et al., 1983). Glucosinolates themselves possess limited biological activity until they are hydrolyzed by the endogenous enzyme myrosinase (β-thioglucoside glucohydrolase; EC 3.2.3.1). Hydrolytic reaction products for all glucosinolates include D-glucose and SO_4^{2-}. Formation of isothiocyanates, thiocyanates, nitriles, epithionitriles, oxazolidinethiones, amines, and other reaction products depends on side-chain structure and reaction conditions such as pH and iron concentration (Uda et al., 1986).

Interest in glucosinolates and their decomposition products has been generated because of the possibility of using them as natural pesticides. Many products of enzymatic glucosinolate hydrolysis are toxic compounds with antinutritional or allelochemic effects (Chew, 1988). Suppression of a variety of soil pests occurs after incorporation of cruciferous plant materials into soil (Lewis and Papavizas, 1971; Waddington, 1978; Parke and Rand, 1989; Choesin and Boerner, 1991; Lazzeri et al., 1993; Mojtahedi et al., 1991). This effect is attributed to the toxicity of glucosinolate decomposition products formed in the soil environment.

The chemistry of glucosinolates and the mechanism of their enzymatic decomposition were intensively studied during the past two decades (Björkman, 1976; Kjaer, 1976; Röbbelen and Thies, 1980; Larsen, 1981; Chew, 1988). However, little is known concerning the fate of glucosinolates in the soil environment except that isothiocyanates and ionic thiocyanate (SCN^-) are two degradation products (Brown et al., 1991). We used a commercially available glucosinolate, sinigrin (allyl glucosinolate, Figure 1), as a model compound to study glucosinolate decomposition in soil. Our objective was to identify products formed as a result of enzymatic hydrolysis and characterize the soil characteristics and variables controlling such formation.

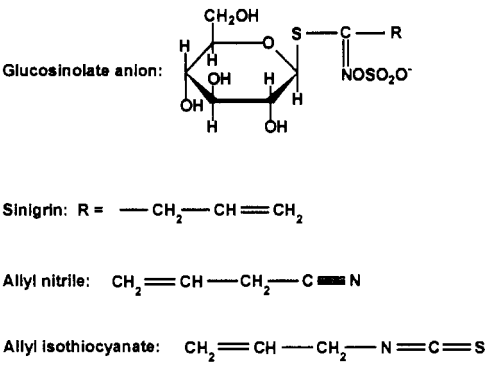

Figure 1. Molecular structures of glucosinolates, sinigrin, allylnitrile, and allyl isothiocyanate.

MATERIALS AND METHODS

Chemicals and Solutions. Myrosinase (Sigma, St. Louis, MO) at a concentration of 20 mg/mL was prepared in deionized water and shaken at 30 °C for 6 h in a water bath. Fresh 0.125 M solutions of sinigrin (Sigma) were prepared in deionized water before the start of each experiment. Citrate–phosphate buffers from pH 2.5 to 9.0 (0.5 pH increments) were prepared by mixing solutions of 0.1 M citric acid and 0.05 M disodium hydrogen phosphate in appropriate proportions to obtain the desired pH.

Soils. Surface samples (0–20 cm) were collected, air-dried, crushed, and sieved (2 mm). Soil samples were analyzed for pH (1:1 soil to water, combination glass electrode), organic C by a modified Walkley–Black method (Nelson and Sommers, 1982), total C and N by Dumas combustion (LECO CHN-600 determinator, St. Joseph, MI), particle size distribution by the hydrometer or pipet method (Gee and Bauder, 1986), exchangeable Fe by atomic absorption spectrophotometry (Sotera and Stux, 1979; Video 12 AA/AE spectrophotometer, Allied Analytical Systems, Andover, MA), and moisture content at a water potential of −0.033 MPa using a ceramic pressure plate (Klute, 1986). Selected soil characteristics are reported in Table 1.

Soil Extraction. Soil extracts were prepared by shaking soil samples with water or 1 M ammonium acetate (Thomas, 1982). To obtain a water extract, 100 g of air-dried soil was extracted for 30 min with 200 mL of water. The mixture was filtered through a 0.45-μm filter (Gelman, Supor 450) and brought to volume in a 200-mL volumetric flask. Ammonium acetate extracts were

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

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Table 1. Characteristics of Soils Used in Sinigrin Decomposition Experiments

soil			organic C,	total C,	total N,	clay,	sand,	water at	extractable
series	subgroup	pH	g/kg	g/kg	g/kg	g/kg	g/kg	0.033 MPa, %	Fe, μg/kg
Portneuf sil	Durixerollic Calciorthid	7.55	11.6	15.6	1.7	157	48	27.5	0.09
Latahco sil	Argiaquic Xeric Argialboll	6.10	41.0	41.0	3.9	159	122	39.2	0.13
Latahco sil	Argiaquic Xeric Argialboll	6.00	16.9	16.9	1.6	185	70	28.6	0.13
Andisol sil	Typic Vitricryand	6.25	15.0	15.0	1.8	117	190	58.0	0.73
Avonville sil	Andic Xerumbrept	5.65	39.0	39.0	3.9	96	265	46.8	0.09
Pancheri sil	Xerollic Calciorthid	8.30	1.8	18.7	0.7	101	289	29.5	0.09

obtained by shaking 10 g of air-dried soil with 50 mL of 1 M ammonium acetate for 30 min followed by 10 min of centrifugation at 550*g*. The extraction procedure was repeated, extracts were combined, and pH was titrated with 0.25 M ammonium hydroxide or 0.25 M acetic acid to soil pH as determined by combination glass electrode. The pH-adjusted extract was filtered through a 0.45-μm filter (Gelman, Supor 450) and brought to volume with 1 M ammonium acetate in a 100-mL volumetric flask.

Effect of pH, Fe^{2+}, and Fe^{3+}. Sinigrin decomposition was tested in citrate–phosphate buffer solutions ranging from pH 2.5 to 9.0. The effects of Fe^{2+} and Fe^{3+} ions were determined at pH 6.0 and 4.0. Assay replicates were prepared by adding 960 μL of buffer, 20 μL of myrosinase, and 20 μL of sinigrin solutions or 910 μL of buffer, 20 μL of myrosinase, 20 μL of sinigrin, and 50 μL of Fe^{2+} or Fe^{3+} solutions [$FeSO_4$ or $FeNH_4(SO_4)_2$; 0.2 M] to each test tube. Resulting concentrations were 2.5 mM sinigrin, 400 g/mL myrosinase, and 10 mM Fe^{2+} or Fe^{3+}. Test tubes were covered with Teflon-lined caps and incubated for 6 h in a water bath at 30 °C. Each treatment was replicated three times.

Sinigrin Decomposition in Soil Extracts. Each replicate contained 22.0 mL of a water extract or 2.2 mL of an ammonium acetate extract and 19.8 mL of deionized water (22.0 mL of deionized water or 0.1 M ammonium acetate, pH 7.0, for controls), 440 μL of sinigrin, and 440 μL of myrosinase solutions. Test tubes were covered with Teflon-lined caps and kept in a water bath at 25 °C. A 1.0-mL sample of each reaction mixture was removed at 3, 6, 12, 18, 24, 48, 72, 96, 120, 144, and 168 h.

Sinigrin Decomposition in Soil. Soils were incubated at moisture contents equivalent to those present at soil suctions of 0.033 MPa or under saturated conditions. Saturated soil was produced by adding 300% more water than present at soil suctions of 0.033 MPa. The desired volumes of deionized water (300–600 μL), 20 μL of sinigrin, and 20 μL of myrosinase solutions were added to each test tube. One gram of soil was immediately poured into each test tube, during which time the tubes were kept in an ice bath. Test tubes were covered by Teflon-lined caps and incubated in a water bath at 25 °C. Samples were analyzed for sinigrin decomposition products at 6, 12, 24, 48, 96, and 168 h. Each treatment was replicated three times.

Analytical Determination of Decomposition Products. Products formed during enzymatic decomposition of sinigrin by myrosinase were extracted into dichloromethane and determined by capillary GC. Each sample contained 1 mL of dichloromethane, 1 mL of 0.5 M disodium sulfate, and 2 μL of an internal standard solution (1 M phenyl isothiocyanate in dichloromethane). Samples were vigorously shaken for 5 min and sonified for 2 min (Bransonic 1200 ultrasonic cleaner, Danbury, CT). Separation of organic and water phases was accelerated by centrifuging samples at 550*g* for 5 min. Samples were extracted twice with 1 mL of dichloromethane. The organic phase was separated from the aqueous phase using disposable Pasteur pipets and dried overnight by addition of 300–500 mg of waterless disodium sulfate. Concentrations of allylnitrile and allyl isothiocyanate in dichloromethane extracts were determined by GC (HP 5890A, Hewlett-Packard, Avondale, PA), using a DB-5 capillary column (30 m × 320 m, 0.25-μm film, J&W Scientific, Folsom, CA) and a temperature program from 35 to 200 °C. Compounds were detected with a flame ionization detector and concentrations determined using phenyl isothiocyanate as an internal standard. Response factors of the analytes were linear in the concentration range 0.01–10 mM. Extraction efficiency and reproducibility of the method were verified by analysis of samples spiked with known amounts of allylnitrile and allyl isothiocyanate.

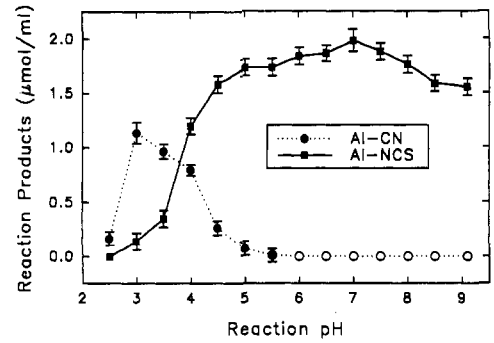

Figure 2. Allylnitrile and allyl isothiocyanate produced during sinigrin decomposition by myrosinase in buffered solutions. Open circles indicate that allylnitrile concentration was below the limits of detection. Means of three observations ± SEM are plotted.

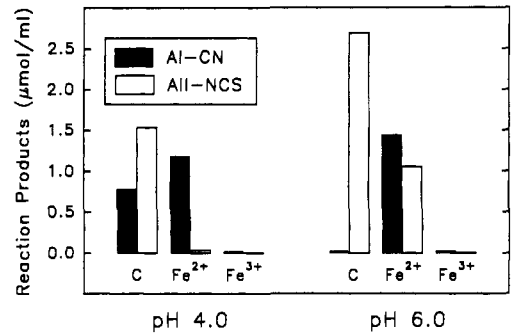

Figure 3. Sinigrin decomposition by myrosinase in deionized water control (C) or in the presence of Fe^{2+} or Fe^{3+}.

RESULTS

Effect of pH, Fe^{2+}, and Fe^{3+}. Enzymatic decomposition of sinigrin by myrosinase was strongly dependent on reaction pH (Figure 2). The major reaction product at pH 2.5 was allylnitrile. Allylnitrile production reached a maximum at pH 3.0 and declined from pH 3.0 to 6.0. At pH values above 6.0, allylnitrile was produced in trace amounts. Allyl isothiocyanate production increased in a nonlinear manner from pH 2.5 to approximately pH 5.0. Allyl isothiocyanate was the only sinigrin decomposition product at pH 6.0 and higher. The combined product yields of allyl isothiocyanate and allylnitrile between pH 3.5 and 9.0 represented 42–86% of the sinigrin originally present in the assay.

The presence of Fe^{2+} or Fe^{3+} at concentrations of 10 mM changed the distribution of reaction products (Figure 3). The enzymatic decomposition of sinigrin produced more allylnitrile than allyl isothiocyanate at both pH 4.0 and 6.0 when 10 mM Fe^{2+} was present. Under the same conditions, the presence of Fe^{3+} ions inhibited enzymatic decomposition of sinigrin by myrosinase. We found only small amounts of allylnitrile and no traces of allyl isothiocyanate in reaction mixtures containing Fe^{3+} ions.

Sinigrin Decomposition in Water and Water Extracts from Soil. Sinigrin decomposition by myrosinase was determined in water and ammonium acetate control solutions and in water and ammonium acetate extracts of

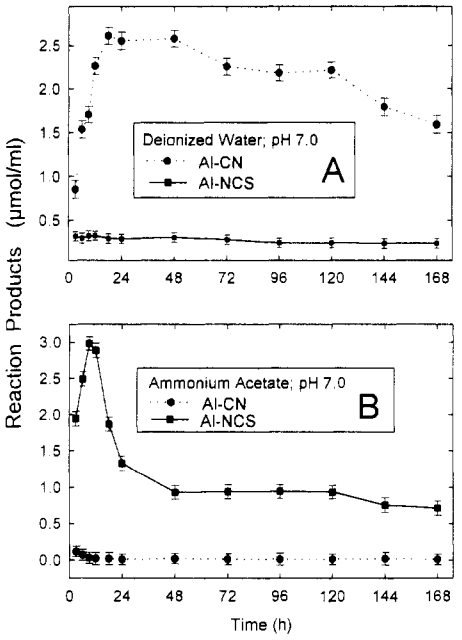

Figure 4. Decomposition products of sinigrin in water (A) or 0.1 M ammonium acetate (B). Means of three observations ± SEM are plotted.

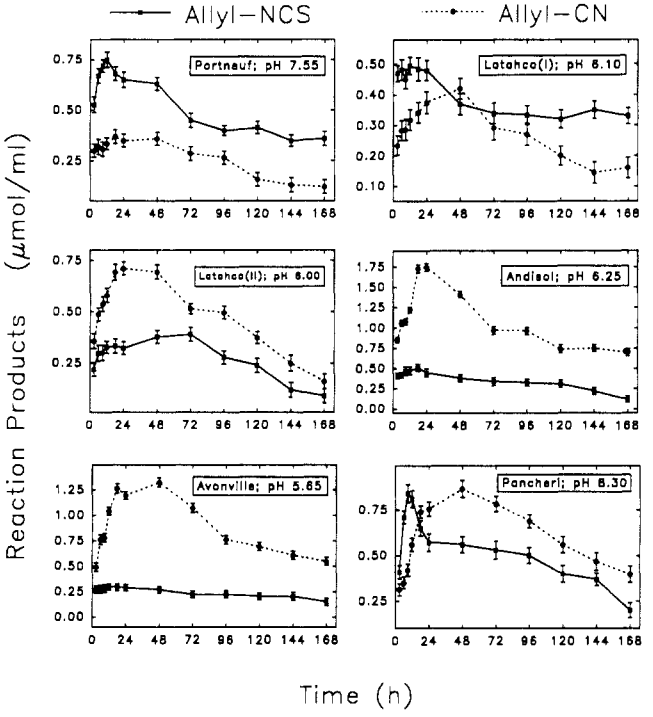

Figure 5. Decomposition products of sinigrin in water extracts from six soils. Means of three observations ± SEM are plotted.

six soils. Allylnitrile was the major reaction product in water (Figure 4A) and water extracts of Latahco(II), Andisol, and Avonville soils (Figure 5). Approximately equal proportions of allylnitrile and allyl isothiocyanate were produced in water extracts of Latahco(I) and Pancheri soils. Twice as much allyl isothiocyanate as compared to allylnitrile was produced in Portneuf soil. Concentrations of both reaction products increased, reached a maximum after 24 or 48 h, and decreased during the remainder of the incubation period. Maximum product formation occurred at 24 h in water controls and water extracts of Portneuf and Andisol soils and at 48 h in water extracts of Latahco(I), Latahco(II), Avonville, and Pancheri soils (Figure 5). Total product yields of the controls approx-

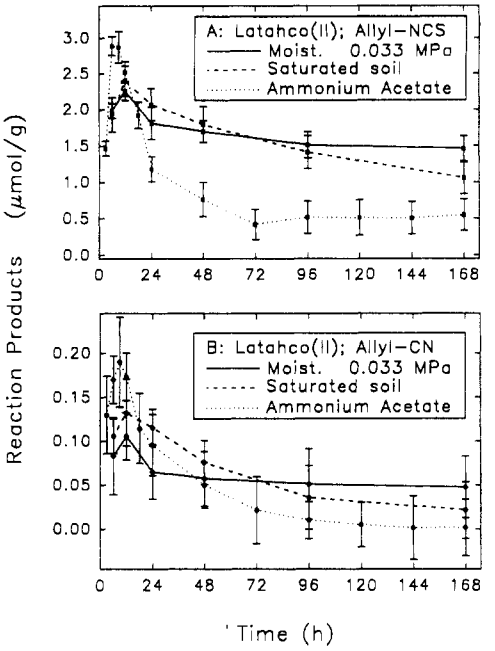

Figure 6. Decomposition products of sinigrin in ammonium acetate extracts from Latahco(II) soil and Latahco(II) soil incubated with moisture equivalent to that present at soil suctions of 0.033 MPa and under saturated conditions. Allyl isothiocyanate concentrations are shown in (A) and allylnitrile concentrations in (B) (note difference in Y-axis scales). Means of three observations ± SEM are plotted.

imated the theoretical 2.5 μmol/mL maximum (Figure 4), whereas reaction product yields in water extracts were lower (Figure 5).

Sinigrin Decomposition in Ammonium Acetate and Ammonium Acetate Soil Extracts. The decomposition of sinigrin in ammonium acetate control solutions (Figure 4B) and ammonium acetate soil extracts (Figure 6) was similar. Allyl isothiocyanate was the major reaction product, and allylnitrile appeared in the reaction mixture in trace amounts during the first 24 h of incubation. The decomposition of sinigrin in ammonium acetate extracts reached a maximum in less than 24 h, during which time allyl isothiocyanate and allylnitrile concentrations reached maxima and decreased.

Sinigrin Decomposition in Soil. Product distributions of sinigrin decomposition in Latahco(II) soil (Figure 6) were similar to decomposition products formed in ammonium acetate (Figure 4B). Allyl isothiocyanate was the major reaction product (Figure 6A), whereas allylnitrile was produced in trace amounts (Figure 6B). Product formation in saturated soils and those having moisture contents equivalent to soil suctions of 0.033 MPa reached a maximum in less than 24 h. All soils showed similar trends, and therefore only the data for Latahco(II) soil is presented (Figure 6).

DISCUSSION

We confirmed that enzymatic decomposition of sinigrin in water yields two main products, allylnitrile and allyl isothiocyanate (Figure 1). The production of cyanoepithiopropane or allyl thiocyanate was not observed during gas chromatographic analysis of the extracts. Cyanoepithiopropane formation is controlled by the presence of an epithiospecifier protein, whereas conditions required for allyl thiocyanate formation are not fully understood (MacLeod and Rossiter, 1985). The reasons for lack of detection include low yields, compound instabilities, or

lack of an epithiospecific protein in the commercially available myrosinase enzyme.

Total product yields and distributions as allylnitrile or allyl isothiocyanate were controlled by reaction pH and concentrations of ferrous/ferric ions in the reaction medium. Decomposition of sinigrin in acid solutions below approximately pH 4.0 yielded allylnitrile as the major reaction product. Nitrile production at low pH occurs because Lossen rearrangement of the proposed aglucone intermediate is inhibited (Gil and MacLeod, 1980). Decomposition of sinigrin in less acid solutions with pH values above 4.0 yielded allyl isothiocyanate as the major reaction product. The presence of ferrous ions in the reaction medium increased the yield of allylnitrile and decreased the yield of allyl isothiocyanate. Ferric ions significantly inhibited the reaction and nearly eliminated allylnitrile and allyl isothiocyanate formation. Our results of sinigrin degradation in aqueous solution are in agreement with similar studies of enzymatic degradation of not only sinigrin (Uda et al., 1986) but other glucosinolates including glucotropaeolin (benzyl glucosinolate) (Hasapis and MacLeod, 1982) and progoitrin [(2*R*)-2-hydroxybut-3-enyl glucosinolate) (MacLeod and Rossiter, 1987).

Sinigrin decomposition in water and ammonium acetate soil extracts was controlled by the same factors as determined for aqueous solutions. Acidity resulting from sulfate production (Gil and MacLeod, 1980) caused a decrease in pH of media with low buffering capacities, consequently increasing the proportion of allylnitrile. Ammonium acetate extracts buffered the reaction and, as compared to water extracts, increased the proportion of allyl isothiocyanate at the expense of allylnitrile.

In weakly buffered aqueous extracts, allylnitrile was as expected a major sinigrin decomposition product. As buffering capacities of the water extracts increased, the proportion of isothiocyanate produced also increased. Buffering capacity of the water extracts was a function of free carbonates and soil organic matter content. For example, both Portneuf and Pancheri soils have free carbonates as indicated by the difference in total carbon and organic carbon (Table 1). Greater amounts of isothiocyanate are expected in water extracts from these soils as compared to other soils having similar organic matter contents but lacking free carbonates. In addition, Portneuf soil has a much higher organic matter content than Pancheri, thus contributing soluble organic matter to the extract. Increased buffering capacity of the Portneuf water extract resulted in a greater proportion of isothiocyanate. Likewise, the Latahco(I) soil has a pH similar to that of Latahco(II) but more organic carbon (Table 1). Water extracts from Latahco(I) soil more effectively buffered the reaction, causing greater isothiocyanate production.

Regardless of differing pH characteristics, soils buffered the reaction fairly well and their pH was high enough that allyl isothiocyanate was the dominant product of sinigrin decomposition. Allyl isothiocyanate represented 95% of total measured product yields even in soil with a low pH (Avonville) and in soil with a predicted low buffering capacity (Andisol) (Table 1).

Other soil variables had a much smaller impact on sinigrin decomposition. Soil moisture content had a minimal impact on product formation rates and distributions. Although extractable iron concentrations varied among the soils (Table 1), it is likely that ferrous and ferric ions did not significantly alter reaction yields and product proportions because of their low concentrations in soils or interaction with soil organic matter.

Soil extracts permit the use of simpler and more precise analytical procedures to determine reaction products. We have shown that ammonium acetate extracts of soils can be used for qualitative studies of glucosinolate decomposition in the soil environment. This results mainly from the buffering capacity afforded by ammonium acetate and the fact that soils likewise buffer the reaction.

Evidence suggests that the enzymatic degradation products of glucosinolates are biologically active against a wide variety of organisms, especially herbivorous insects and fungi (Mason-Sedun et al., 1986; Bialy et al., 1990; Schung and Ceynowa, 1990; Williams et al., 1993). Conditions that control these allelochemic interactions in soil are not yet well understood, but organic isothiocyanates are thought to be of primary importance. Allyl isothiocyanate was indeed the predominant product formed from sinigrin decomposition in soil, regardless of soil characteristics. The effective use of glucosinolate-containing plant tissues to control soil-borne plant pests deserves further attention.

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