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Effects of Intact Glucosinolates and Products Produced from Glucosinolates in Myrosinase-Catalyzed Hydrolysis on the Potato Cyst Nematode (*Globodera rostochiensis* Cv. Woll)

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The potato cyst nematode (*Globodera rostochiensis* cv. Woll) is responsible for large yield losses in the potato crop, and opportunities for reducing the attack of these plant nematode species are, therefore, important. This study has been devoted to the testing of the in vitro effects on the potato cyst nematode of eight glucosinolates [prop-2-enyl-, but-3-enyl-, (*R*)-4-methylsulfinylbut-3-enyl-, benzyl-, phenethyl-, 4-hydroxybenzyl-, (2*S*)-2-hydroxybut-3-enyl-, and (2*R*)-2-hydroxy-2-phenylethylglucosinolate] as well as the effects of the products of this myrosinase-catalyzed hydrolysis. The glucosinolates were used at three concentrations, 0.05, 0.3, and 1.0 mg/mL, in the presence or absence of the enzyme myrosinase. The effects of the compounds on the mortality were monitored every 8 h for a 72 h period. No effects were found for any of the intact glucosinolates. However, when active myrosinase was included with 1 mg/mL phenethylglucosinolate at pH 6.5, 100% mortality was observed within just 16 h. A similar effect was achieved at the same concentration of benzyl- and prop-2-enylglucosinolates in the myrosinase-containing solutions, although longer exposures were required (24 and 40 h, respectively). The main aglucone products released from the glucosinolates with pronounced effects on the nematodes were shown to be the corresponding isothiocyanates. The results suggest that mixtures of these specific glucosinolates and active myrosinase or autolysis of plant materials containing these enzymes and glucosinolates might be used to control the potato cyst nematode in the soil.

KEYWORDS: Glucosinolates; isothiocyanates; myrosinase; potato cyst nematode

INTRODUCTION

Glucosinolates (**Figure 1**) are a group of allelochemicals that occur in all plants of the order Capparales and in some few other plants (*1*−*4*). More than 130 structurally different glucosinolates have been identified (*3*, *4*) and may, according to the structure of the amino acid derived side chain (R), be divided into three subclasses comprising aliphatic, phenyl, and indol-3-ylmethylglucosinolates, respectively (*4*, *5*). However, it is also well-known that glucosinolates co-occur in the plant with the group of β-thioglucoside glucohydrolase isoenzymes (EC 3.2.3.1), generally known as myrosinase (*6*−*8*). When the tissue is damaged, glucosinolates are hydrolyzed to give a broad range of compounds, such as isothiocyanates, thiocyanates, nitriles, and oxazolidine-2-thiones, depending on the hydrolysis or autolysis conditions (*9*−*12*). These hydrolysis products have shown various bioactive effects, for instance, against some soil-borne diseases and nematodes (*5*, *13*). The effect of the glucosinolate derivative, prop-2-enylisothiocyanate, on the potato cyst nematode was first reported by Ellenby (*14*) and, later, methylisothiocyanate (Vapam) was found to be an effective nematocide (*15*), which is still used today. The high costs of this and other synthetic nematocides and their negative environmental impact prompted several studies, which suggest opportunities for new biotechnological processing to produce environmentally friendly products (*8*, *16*) and the use of nonhost plants containing glucosinolates in crop rotation, particularly rapeseed (*17*−*23*). From these studies it is clear that more specific information is needed concerning the effects of each individual glucosinolate and the concentrations at which they exert their influence. In vitro studies (*24*) have revealed the nematocidal effects of some glucosinolate breakdown products against the cyst nematode *Heterodera schachtii*, whereas no effects were achieved for the intact glucosinolates. Similar findings were reported in experiments with prop-2-enylglucosinolate by Donkin et al. (*25*) on the nematode *Caenorhabditis elegans* and by Pinto et al. (*26*) on *Globodera rostochiensis*.

Consideration of opportunities for protection against pest problems caused by the potato cyst nematode is important as potato is one of the major vegetable crops grown worldwide,

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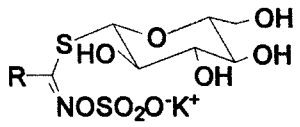

Structure of R-group:	Semi-systematic names	Trivial names
	Allylglucosinolate	Sinigrin
	But-3-enylglucosinolate	Gluconapin
	4-Methylsulfinylbut-3-enylglucosinolate	Glucoraphenin
	Benzylglucosinolate	Glucotropaeolin
	2-Phenethylglucosinolate	Gluconasturtiin
	4-Hydroxybenzylglucosinolate	Sinalbin
	(2S)-2-Hydroxybut-3-enylglucosinolate	Epi-progoitrin
	(2R)-2-Hydroxy-2-phenylethylglucosinolate	Epi-glucobarbarin

Figure 1. Structures of intact glucosinolates used for tests against the potato cyst nematode.

and severe yield losses have been reported in potato crops produced in soils infested with this nematode (*27*), particularly when no crop rotation is used. The suppressive effects of incorporated Brassicaceae residues on nematodes has been known since the beginning of the second half of the 20th century (*28*). Several of the commonly cultivated plants belonging to the Brassicaceae are hosts to one or more species of the nematode *Meloidogyne* (*29*). Results showed that collard (*Brassica oleracea* var. *acephala* cv. Georgia Southern) was the least susceptible followed by broccoli (*Brassica oleracea* var. *botrytis* cv. De Cicco). Isothiocyanates, apart from being recognized as one of the major inhibitors of microbial activity, have also shown insecticidal activity, especially the aromatic isothiocyanates, as has been reported in several studies (*5*). Recent in vitro research has revealed the susceptibility of the potato nematode (*G. rostochiensis*) to prop-2-enylisothiocyanate (*26*), and *Heterodera schachtii* has shown to be suppressed by glucosinolate hydrolysis products using an initial glucosinolate concentration of 0.5−5.0 mg/mL (*24*). Furthermore, green manure of *Brassica napus*, probably containing glucosinolates or glucosinolate hydrolysis products, was reported to reduce the number of nematodes (*Pratylenchus neglectus*) to 50% of the initial number (*30*). These results show the potential antinematocidal effects of some of the glucosinolate-derived hydrolysis products, but because of the limited number of glucosinolates studied, further research is required.

In this study we have performed a systematic investigation of the effects a number of common glucosinolates found in *Brassica* crops have against the potato cyst nematode. The objective has been the evaluation of in vitro effects on the activity of the potato cyst nematode (*G. rostochiensis* cv. Woll exerted by intact glucosinolates and the hydrolysis products released from the glucosinolates in myrosinase-catalyzed reactions, including isothiocyanates, oxazolidine-2-thiones, and alcohols.

MATERIALS AND METHODS

Glucosinolates and Other Chemicals. All glucosinolates have been isolated as potassium salts according to standard procedures described elsewhere (*2*). This procedure comprises an initial isolation of the total glucosinolate fraction in a mixture with other anions, using the described (*2*, *4*) anion exchange techniques. Separation and purification of the individual glucosinolates were subsequently performed by using the described (*2*) Sephadex G-10/G-25 (Fine), Polyclar AT, and SPE column chromatographic techniques and, finally, crystallization and recrystallization. The glucosinolates thus obtained and used in this project comprise prop-2-enylglucosinolate (sinigrin), but-3-enylglucosinolate (gluconapin), (*R*)-4-methylsulfinylbut-3-enylglucosinolate (glucoraphenin), benzylglucosinolate (glucotropaeolin), phenethylglucosinolate (gluconasturtiin), 4-hydroxybenzylglucosinolate (sinalbin), (2*S*)-2-hydroxybut-3-enylglucosinolate (epiprogoitrin), and (2*R*)-2-hydroxy-2-phenylethyl glucosinolate (epiglucobarbarin) (**Figure 1**). The identity and purity of the glucosinolates were determined by UV and NMR spectroscopy (*4*) and by micellar electrokinetic capillary chromatography (MECC) according to the procedures described in Sørensen et al. (*31*). All other chemicals were of analytical reagent grade.

Nematodes. Cysts of the potato nematode *G. rostochiensis* cv. Woll were collected in a naturally infested field near Vila Real, Portugal,

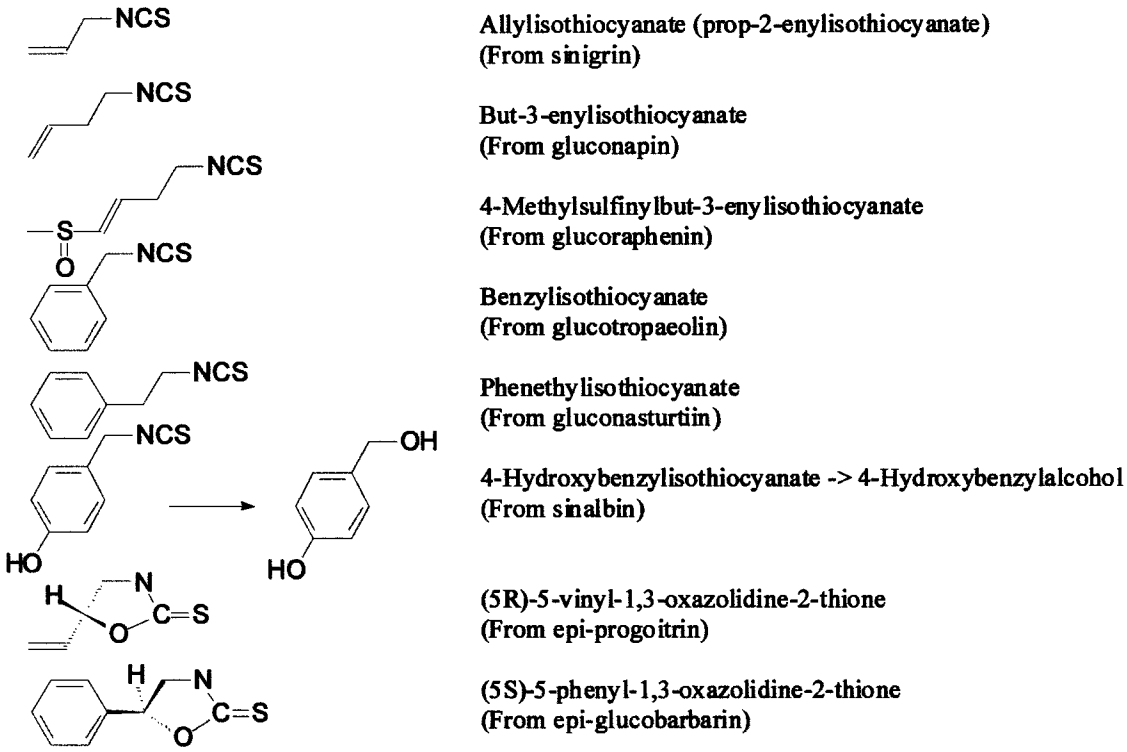

Figure 2. Structures of glucosinolate-derived hydrolysis products obtained.

and kept in a refrigerator at 4 °C. Second-stage juveniles were induced to hatch from the eggs within the cysts by placing the cysts in a small Petri dish for 3 days at room temperature (~20 °C), followed by 6 days in water and an extra 3 days in contact with potato root exudate by placing the cysts on sieves made of muslin and supported on glass blocks. During the initiation and release of juveniles from the cysts, these were kept in the darkness at room temperature (~20 °C).

Experiments. Tests were performed in Petri dishes (diameter = 5 cm) containing 2 mL of a solution of each of the glucosinolates (2 mL) either with or without co-addition of myrosinase. All experiments were carried out in 100 mM phosphate buffer, pH 6.5 (*31*). Phosphate buffer alone was used as a control. Each test was replicated five times using 15 second-stage juveniles per replicate. The Petri dishes were kept in darkness at room temperature (20 °C) throughout the experiment. The activity of the nematode juveniles was monitored every 8 h during a 72 h period. It was assumed that a nematode was dead when it was immobile in the solution and did not react to stimuli caused by pricking the body with a needle or if the larvae did not regain mobility when placed in water slightly heated by an incandescent light bulb. The mortality ratio was determined as defined by the Abbot (*32*) formula:

$$\text{mortality corrected} = \frac{\%\ \text{mortality induced} - \%\ \text{mortality control}}{100 - \%\ \text{mortality control}} \times 100$$

Analysis of Glucosinolates. Analyses of individual glucosinolates were performed by high-performance capillary electrophoresis (HPCE) using the described procedures based on MECC; cetyltrimethylammonium bromide was used as surfactant (*31*, *33*).

HPLC Analysis of Glucosinolate Degradation Products. Samples containing products produced during myrosinase-catalyzed hydrolysis of the tested glucosinolates were analyzed by reversed phase HPLC. The procedure is a modification of the previously described reversed phase HPLC and MECC methods, adapted to determinations of the glucosinolate degradation products (*4*, *9*, *11*, *31*, *34*). The Gilson HPLC system (Middleton, WI) consisted of two Gilson 306 pumps, a Gilson 811C mixer (1.5 mL), a Gilson 233 XL autosampler (20 μL sample loop), a Gilson 402 syringe pump, a Gilson 831 column oven, and a Gilson 119 UV−vis detector. Column back-pressure was measured by a Gilson 821 pressure regulator. The system was controlled by Unipoint system software v. 1.91 (Gilson).

Prior to HPLC analysis of degradation products, all samples were treated with NH_3 to transform isothiocyanates into thiourea derivatives. To 1 mL of each sample was added 1 mL of 2 M NH_3 in ethanol, and samples were mixed thoroughly. After 1 h at room temperature, samples were freeze-dried and analyzed by HPLC. All samples were analyzed using a YMC Basic HPLC column [250 × 4.6 mm (i.d.), 5 μm particles] and gradient elution. Eluent A was 25 mM NaH_2PO_4/Na_2HPO_4, pH 6.5, with 10% acetonitrile (ACN). Buffer B was 100% ACN. Buffers were degassed and subsequently filtered through a 0.40 μm filter prior to use. Initially, the eluent was 100% A/0% B. After 3 min, the eluent was linearly changed to 55% A/45% B in 12 min (3.75% B/min) and then returned to initial conditions. Flow rate was 1.5 mL min^{-1}, column temperature was held at 30 °C, and detections were performed at 230 and 240 nm.

RESULTS

Analysis of Glucosinolates and Degradation Products of Glucosinolates. The myrosinase-catalyzed hydrolysis of glucosinolates used in the tests of nematocidal activity toward the potato cyst nematode yielded a broad range of different products comprising both isothiocyanates, alcohols, and oxazolidine-2-thiones (**Figure 2**). No remaining intact glucosinolates, for example, sinigrin, gluconapin, glucoraphenin, glucotropaeolin, gluconasturtiin, epiprogoitrin, and epiglucobarbarin, could be detected following myrosinase reaction, thus indicating that the myrosinase-catalyzed hydrolysis reactions had proceeded to completion. Small amounts of nondegraded intact glucosinolate could be detected in the experiments utilizing sinalbin and its hydrolysis products. Generally, three different groups of degradation products from the selected glucosinolates could be detected. From sinigrin, gluconapin, glucoraphenin, glucotropaeolin, and gluconasturtiin, the respective isothiocyanates were identified by HPLC after transformation into their thiourea derivatives, which were formed upon addition of NH_3 in ethanol to the solutions. 4-Hydroxybenzyl alcohol was detected as the main degradation product from sinalbin, in agreement with the limited stability of the initially formed 4-hydroxybenzylisothiocyanate. Myrosinase-catalyzed hydrolysis of both epiprogoitrin

Table 1. Effect of the Eight Glucosinolates after Enzymatic Hydrolysis by Myrosinase To Release the Corresponding Isothiocyanate at Three Concentrations during Nine Sampling Times (Every 8 h)[a]

glucosinolate	concn (mg/mL)	8 h	16 h	24 h	32 h	40 h	48 h	56 h	64 h	72 h
	0.05	0	0	0	2.7	16	28	29.4	38.7	42.7
prop-2-enyl-	0.3	1.3	1.3	6.6	15.9	34.6	50.6	50.6	58.6	65.3
	1	6.7	20	29.3	56	100				
	0.05	0	0	2.7	2.7	2.7	13	10	13.2	14.2
but-3-enyl-	0.3	0	2.7	2.7	4	18.7	21.3	17.1	21.4	25.7
	1	4	6.7	13.3	28	30.7	36	37.1	41.4	44.3
	0.05	0	0	1.3	2.7	2.7	4	4	5.3	9.3
4-hydroxybenzyl-	0.3	0	0	0	0	2.7	2.7	5.3	8	12
	1	0	1.3	4	6.7	8	9.3	10.7	12	13.3
	0.05	0	0	0	0	0	0	1.3	1.3	1.3
4-methylsulfinylbut-3-enyl-	0.3	0	0	0	0	0	0	0	0	1.3
	1	1.3	1.3	2.7	2.7	2.7	2.7	2.7	4	4
	0.05	0	0	0	2.7	2.7	5.3	5.3	5.3	8
2-hydroxybut-3-enyl-	0.3	0	0	0	1.3	4	8	8	8	9.3
	1	0	2.7	5.3	9.3	12	12	12	13.3	13.3
	0.05	0	0	0	2.7	4	6.7	9.3	9.3	10.7
2-hydroxy-2-phenylethyl-	0.3	0	0	1.3	5.3	6.7	14.7	17.3	17.3	17.3
	1	0	1.3	9.3	10.7	13.3	16	18.7	22.7	22.7
	0.05	0	8	14.7	25.3	30.7	38.7	40	50.7	57.3
phenethyl-	0.3	1.3	18.7	38.7	46.7	54.7	57.3	64	65.3	70.7
	1	82.7	100							
	0.05	0	0	5	12	15	21	21	24	25
benzyl-	0.3	21.3	54.7	70.7	73.3	80	84	86.7	86.7	90.7
	1	82.7	93.3	100						

[a] Values are expressed in percentage of the cumulative mortality corrected according to Abbott's formula.

and epiglucobarbarin initially released the corresponding isothiocyanates, which were rapidly transformed into the oxazolidine-2-thiones (5*R*)-5-vinyl-1,3-oxazolidine-2-thione and (5*S*)-5-phenyl-1,3-oxazolidine-2-thione.

Effect of Intact and Enzyme-Hydrolyzed Glucosinolates. Intact glucosinolates induced no mortality on the second-stage juveniles of the potato cyst nematode *G. rostochiensis*. When myrosinase was co-added with each of the tested glucosinolates, a highly significant difference ($P < 0.001$) between glucosinolates and between concentrations was observed. The effect of each concentration was dependent on the glucosinolate as shown by the significant ($P < 0.001$) interaction glucosinolate × concentration. The cumulative mortality effects of each glucosinolate at the three tested concentrations (0.05, 0.3, and 1.0 mg/mL) are shown in **Table 1**. A 100% mortality was observed for phenethylisothiocyanate at a concentration of 1.0 mg/mL corresponding to 6 mM within just 16 h. Only this concentration resulted in a 100% mortality when the benzyl- and prop-2-enylglucosinolates were used and after larvae exposure of 16 and 40 h, respectively (**Table 1**). It is interesting to note that both phenethyl- and benzylisothiocyanate induced the same mortality within the first 8 h, but phenethylisothiocyanate was slightly more effective in the following period. Similarly, phenethylisothiocyanate was more effective than benzylisothiocyanate at a concentration of 0.05 mg/mL (0.3 mM), whereas the opposite occurred at 0.3 mg/mL. The lowest mortality effect was observed for 4-methylsulfinylbut-3-enyl-isothiocyanate at any of the tested concentrations. With the other glucosinolate hydrolysis products, the larvae mortality was <50% for the 72 h period even when the highest concentration was used.

DISCUSSION

To achieve mortality effect for any of the eight tested glucosinolates, it was necessary to co-add active myrosinase, as has been reported elsewhere (*24*−*26*). The mortality effect depended on the type of compound released during the myrosinase-catalyzed hydrolysis of the glucosinolates, of which the strongest effects were observed with phenethyl- and benzylglucosinolates, both yielding isothiocyanates as the initial quantitatively dominating product (*11*, *34*). Similar results have been reported by Lazzeri et al. (*24*), who found benzylisothiocyanate to have a nematocidal effect within 48 h of treatment of *Heterodera schachtii*. Now, it is, however, also shown that the homologous isothiocyanate, phenethylisothiocyanate, also demonstrates good antinematocidal effects.

At a concentration of 6 mM, phenethyl- and benzylisothiocyanate showed nearly identical antinematocidal effects during the first 8 h, but after 16 h, phenethylisothiocyanate was slightly more effective, whereas 0.3 mM phenethylisothiocyanate was more effective than benzylisothiocyanate; an opposite tendency at 2 mM was noted with 21.3% mortality within the first 8 h of benzylisothiocyanate treatment compared to 1.3% for phenethylisothiocyanate. After 16 h, >50% of the larvae were dead after being exposed to the benzylisothiocyanate, whereas only 18.7% mortality occurred in the presence of phenethylisothiocyanate. Benzylisothiocyanate was less effective in controlling the second-stage juveniles of *G. rostochiensis* than it was for juveniles of *H. schachtii* because a mortality of 100% was achieved for this nematode after 48 h at 3.3 mM (*24*). In our study the (2*S*)-2-hydroxybut-3-enylglucosinolate-derived compound, (5*R*)-5-vinyl-1,3-oxazolidine-2-thione, resulted in a nematode mortality as high as 13.3%, but greater effects are likely to occur when concentration increases, as has been shown in experiments with other nematodes (*25*).

In previous studies with *Brassica* crops (*5*) we found the highest phenethylglucosinolate concentration in the roots, thus leading to the suggestion of using these plants in association with potatoes, at least during the first growth stages to control the potato cyst nematode. Roots seem to be a poor host for some nematode species as reported by Johnson et al. (*21*), probably due to the high phenethylglucosinolate content.

Kirkegaard (*35*) and Potter (*30*) also suggested that root-type glucosinolates are more potent than leaf-type glucosinolates in soil biofumigation, mainly due to the effect from phenethylisothiocyanate. However, the plant root biomass represents only a small proportion of the total plant biomass, which depends on the *Brassica* species. Thus, if these plants are used in soil amendments, higher root biomass is required to increase the beneficial effects, unless the concentration in the roots can compensate for their lower biomass. Aerial plant parts containing glucosinolates and particularly prop-2-enyl- and/or benzylglucosinolates will induce, according to the results of this study, a synergetic effect on the control of this nematode depending on the glucosinolate concentration and biomass production. Environmental conditions during hydrolysis (soil pH, temperature, and humidity) might also affect the release of the desired isothiocyantes and other glucosinolate-derived compounds.

CONCLUSION

Intact glucosinolates have no mortality effect on the second-stage juveniles of the potato cyst nematode *G. rostochiensis*, but such an effect is achieved when the corresponding myrosinase-catalyzed hydrolysis products are released. The magnitude of the mortality is dependent on the type of glucosinolate, concentration, and time of exposure of the nematode to the isothiocyanate.

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