In Vitro Activity of Some Glucosinolates and Their Reaction Products toward a Population of the Nematode *Heterodera schachtii*

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An in vitro study was conducted of the nematocidal effect of some glucosinolates and the products of their myrosinase-mediated enzymatic hydrolysis on second-stage juveniles of the sugar beet cyst nematode *Heterodera schachtii*. The glucosinolates tested were purified from the seeds or from plant organs of some crucifers cited as hosts of the nematode: *Brassica napus*, *Lepidium sativum*, *Brassica rapa*, *Brassica carinata*, *Raphanus sativus*, and *Sinapis alba*. The glucosinolates were dissolved in various concentrations in both the absence and presence of the enzyme myrosinase, and their nematocidal effect in time on second-stage juveniles was then determined. All of the glucosinolates tested as such (i.e., in their native form) showed no nematocidal effect, whereas the products of the enzymatic hydrolysis at pH 7.0 (essentially isothiocyanate) of some of them (sinigrin, gluconapin, glucotropeolin, glucodehydroerucin, and the whole glucosinolate group extracted from rapeseed) demonstrated a mortality rate that varied as a function of both the concentration of product and the exposure time. The reaction products of glucoraphenin and sinalbin did not show any nematocidal activity at the concentrations tested.

INTRODUCTION

The cyst nematode *Heterodera schachtii* Schmidt is currently one of the worst enemies of sugar beet, especially in those areas where the all too frequent replanting (every 2–3 years) of this crop in the same soil favors the multiplication and spread of the nematode.

Chemical control, such as disinfestation with systemic nematocides, is not a valid solution to the problem because of both the high costs and the detrimental effects on the environment. On the other hand, the research conducted in recent years to develop a sugar-beet genotype that is resistant or at least tolerant has not given the desired results, although the dominance of the gene for resistance continues to prompt further studies in this direction (Heijbroek et al., 1983). There has thus been growing interest in comparing systems of agronomic pest control, e.g., growing sugar beet in 4- or 6-year cycles in rotation with nonhost plants and resistant catch crops (Tacconi and Olimpieri, 1983; Tacconi et al., 1990). Of the latter, the cultivation of certain cruciferous species as catch crops is particularly promising to maintain the degree of nematode infestation below the tolerance threshold, even though the species of this family are generally regarded as a classic hosts of the nematode *H. schachtii* (Steele, 1965). The different species used to date have shown various degrees of resistance: certain cultivars of *Raphanus sativus* spp. *oleiformis* have shown resistance that is excellent (e.g., Pegletta), good (Nemex), fair (Levana and Sereno), or absent (Silettina), while the Maxi cultivar of *Sinapis alba* has shown good resistance (Tacconi et al., 1990).

The biochemical basis of this resistance and the mechanisms by which it occurs are still not completely known, although some authors have hypothesized that it is related to necrosis of the syncytium caused by a hypersensitivity reaction of resistant plants (Wyss et al., 1984). It is therefore reasonable to suppose that some secondary compounds present in crucifers, i.e., glucosinolates and the products of their enzymatic hydrolysis, can, on the basis of their chemical structure and concentration in the roots, exert a nematocidal or antinutritional activity on the nematode, thereby causing the degradation of the syncytium after a few days and, therefore, the interruption of the life cycle of the phytophage.

Glucosinolates are glycosidic compounds present in various quantities and ratios in the plant organs and seeds of 11 families of dicotyledonous angiosperms, of which the crucifers are certainly the most numerous. The glucosinolates constitute a class of approximately 100 compounds with a common functional group and a variable side chain that can be aliphatic, aromatic, or heteroaromatic. With intact cells, these compounds are kept separate from the endogenous enzyme myrosinase, a β-thioglucoside glucohydrolase (EC 3.2.3.1) that catalyzes their hydrolysis with the resulting production of D-glucose, sulfate ion, and a series of compounds, viz., isothiocyanates, thiocyanates, and nitriles, according to the pH of the reaction (Figure 1).

Contact between glucosinolate and enzyme occurs at the wound inflicted by the phytophage at the time of attack (by means of the ovipositor or masticatory apparatus in the case of insects and with the stylet in the case of nematodes). This lesion causes, via enzymatic hydrolysis, the formation of compounds with effects that are sometimes toxic or, more generally, antinutritional (Kogan, 1986). This is one line of defense of the plant against these phytophages, although, in time, some of them, notably those attacking crucifers, have adapted and are even attracted by high concentrations of glucosinolates or their reaction products in the plant (Mitchell, 1977).

This paper is a continuation of previous research on the chemical and biological characterization of the glucosinolate/myrosinase system (Iori et al., 1988; Pessina et al., 1990; Hochkoeppler and Palmieri, 1992; Visentin et al., 1992) and, to the best of our knowledge, is the first

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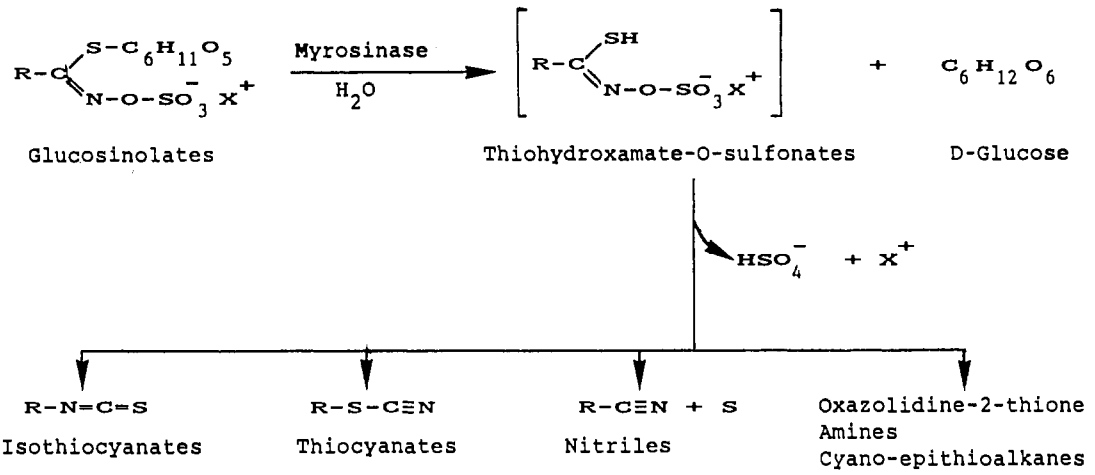

Figure 1. Scheme of the enzymatic hydrolysis of glucosinolates via myrosinase.

Table I. Origin, Structure, and Purity of the Glucosinolates Tested

species	cultivar	organ	major glucosinolate present	structure of side chain R	purity, %
B. napus	Jet Neuf	seed	progoitrin	2-hydroxy-3-butenyl	59.6
					17.2
S. alba	Maxi	seed	sinalbin	*p*-hydroxybenzyl	79.9
R. sativus	Pegletta	seed	glucoraphenin	4-methylsulfinyl-3-butyl	78.0
R. sativus	Pegletta	roots	dehydroerucin	4-methylthio-3-butenyl	89.0
B. rapa	Silla	seed	gluconapin	3-butenyl	80.0
L. sativum		seed	glucotropeolin	benzyl	66.6
B. carinata		seed	sinigrin	allyl	72.3

experiment aimed at explaining the role of this system in plant resistance to this nematode. We report an evaluation of the in vitro nematocidal action against second-stage juveniles of *H. schachtii* of seven glucosinolates and the products of their enzymatic hydrolysis as a function of their concentration and the exposure time.

MATERIALS AND METHODS

Glucosinolates. The glucosinolates sinigrin, gluconapin, glucoraphenin, dehydroerucin, glucotropeolin, sinalbin, and those from rapeseed were isolated and purified according to the method proposed by Thies (1988) with some important modifications reported by Visentin et al. (1992), starting from the seed or specified plant organ of some crucifers (Table I). The HPLC analyses of the desulfoderivates, conducted according to the method of Büchner (1986), showed the predominance of a single glucosinolate with a purity, solid weight, ranging from 66 to 89%, except for the rapeseed extracts, which contained a mixture of glucosinolates containing ca. 60% progoitrin, 20% gluconapin, and other minor glucosinolates: epiprogoitrin, 4-hydroxyglucobrassicin, gluconasturtin, gluconapoleipherin, and others (Figure 2).

Myrosinase. The enzyme myrosinase, isolated and purified from seeds of *S. alba* according to the method proposed by Palmieri et al. (1986), showed a specific activity of ca. 25 units/mg.

Enzymatic Hydrolysis. The glucosinolates were dissolved in 50 mM phosphate buffer (pH 7.0) and hydrolyzed with myrosinase in a screw-cap test tube immersed in water at 37 °C for 3 h. When analyzed with gas chromatography and mass spectroscopy, the hydrolysis products (ITC) proved to consist essentially of the corresponding isothiocyanates, although the presence of small quantities of nitriles and other minor products was detected. After the hydrolysis process, each solution was passed through a sterile filter (Acrodisk Millipore, Malsheim, France) with a pore size of 0.45 μm. The pH was determined at the end of the reaction and in all cases was in the 6.8–7.2 range.

Crude Extracts. The crude extracts used in the trials were prepared by homogenizing the seed in 50 mM phosphate buffer (pH 7.0) in the presence of the enzyme myrosinase with an Ultra-Turrax for 3 min. The homogenate was centrifuged and the supernatant was filtered first with paper and then with sterile filters. Small quantities of antibiotics (streptomycin, tetracycline,

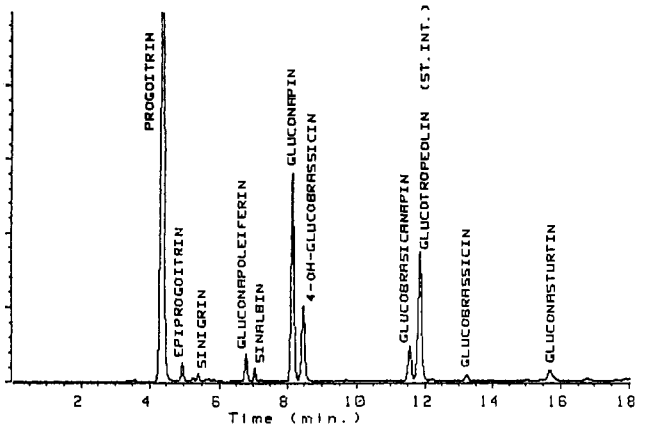

Figure 2. HPLC profile of an extract of rapeseed (*Brassica napus*).

nadilixic acid, konolicine) were added to retard fermentation phenomena which, by causing the formation of precipitates, would have hindered observation with the light microscope after 72, 96, and 114 h.

Nematodes. The second-stage juveniles of *H. schachtii* used in our trials came from cysts collected in a naturally infested field. The juveniles were induced to hatch from the eggs within the cysts by placing the cysts on filters in contact with a solution of $ZnCl_2$, and then they were kept refrigerated at 4 °C until use.

Nematode Mortality Rate. At the start of the experiment 50 second-stage juveniles were placed in a 5-cm Petri dish with 5 mL of test solution containing the glucosinolates at concentrations ranging from 0.005 to 1.0%. For the ITC test, 0.3 unit of enzyme was added to these solutions. The Petri dishes were kept at room temperature (20–25 °C) throughout the experiment. To evaluate the effect of the treatment, the nematodes were observed with a light microscope to determine the mortality rate of the second-stage juveniles after 24, 48, 72, 96, and (for tests at lower concentrations) 114 h. A nematode (second-stage juvenile) was considered dead if it was immobile in the solution, had a dark body even anteriorly, did not react to stimuli caused by pricking with a microscopic needle in various body parts, and did not regain mobility when placed in water slightly heated by an incandescent light bulb. As a further check of their vitality, the juveniles were placed in tap water for 48 h and their behavior

Table II. Nematocidal Effect on Second-Stage Juveniles of *H. schachtii* of Some Glucosinolates and Their Reaction Products at Different Concentrations as a Function of Treatment Time[a]

glucosinolate	concn, % w/v	myrosinase added	mortality, % ± SD				
			24 h	48 h	72 h	96 h	114 h
dehydroerucin	0.5	no	0	0	0	0	0
	0.5	yes	94	97 ± 0.6	97 ± 0.6		
	0.05	yes	3 ± 0.6	4 ± 0.6	4 ± 0.6	5 ± 1.0	
	0.005	yes	0	0	0	0	
glucosinolates from rapeseed	0.5	no	0	0	0	0	0
	0.5	yes	27 ± 1.5	98 ± 0.5	100		
	0.05	yes	0	0			0
	0.005	yes	0	0			0
gluconapin	0.5	no	0	0	0	0	0
	0.5	yes	93 ± 0.6	100	100		
	0.05	yes	0	0	0		100
	0.005	yes	0	0			0
glucoraphenin	0.5	no	0	0	0	0	0
	1.0	yes	3 ± 0.6	3 ± 0.6	4 ± 1.0	4 ± 1.0	
	0.05	yes	0	0	3 ± 1.1	3 ± 1.1	
	0.005	yes	0	0	0		
glucotropeolin	0.5	no	0	0	0	0	0
	0.5	yes	93 ± 3.8	100 ± 1.7	100		
	0.05	yes	0	0			52 ± 7.2
	0.005	yes	0	0			50 ± 6.0
sinalbin	0.5	no	0	0	0	0	0
	0.5	yes	5 ± 1.7	6 ± 1.7	8 ± 1.5		
	0.05	yes	8 ± 1.5	8 ± 1.5		9 ± 1.5	
	0.005	yes	3	3	3		
sinigrin	0.5	no	0	0	0	0	0
	0.5	yes	100	100			
	0.05	yes	7 ± 0.6	78 ± 2.6	93 ± 1.5	100	
	0.005	yes	0	0	2 ± 0.5	2 ± 0.5	

[a] The mortality reported has been corrected according to Abbott's formula.

observed once again to be certain that the substance had an irreversible effect.

The data were corrected according to Abbott's formula (Abbott, 1925):

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

The mortality of the control refers to a solution of 50 mM phosphate buffer (pH 7.0) to which 0.3 unit of the enzyme myrosinase was added for the ITC tests.

The concentrations reported refer to the starting glucosinolates, whereas for the ITC tests, due to their smaller molecular weights, the concentration (w/v) was lower—between 26 and 39% of the starting glucosinolate. All data are the means of at least three trials and are reported with their standard deviations (SD). Analytical grade reagents were used.

RESULTS

All of the glucosinolates (rapeseed glucosinolates, sinigrin, gluconapin, glucoraphenin, glucotropeolin, dehydroerucin, and sinalbin) tested as such at a concentration of 0.5% caused no mortality of the second-stage juveniles of the population of *H. schachtii* studied. In fact, it was noted that even after 96 h, the juveniles showed no symptoms of suffering and maintained normal color and motility.

On the contrary, the ITC obtained by enzymatic hydrolysis at pH 7 of the same glucosinolates produced a nematocidal effect that varied according to the nature of the compound, its concentration, and the exposure time (Table II). The ITC of rapeseed glucosinolates, gluconapin, glucotropeolin, and dehydroerucin, at an initial glucosinolate concentration of 0.5%, caused the death of nearly all of the juveniles after 48 h, whereas those of sinigrin demonstrated their effect after only 24 h. Some of these (gluconapin, glucotropeolin, and sinigrin) had a nematocidal effect even at a concentration of 0.05%, which was seen, however, after 96 h for the ITC of sinigrin and after 114 h for those of gluconapin. At this concentration and after 114 h, the ITC of glucotropeolin caused a mortality rate that just exceeded 50%. None of the compounds tested were active at concentrations below 0.05%.

Given the high toxicity of the ITC of sinigrin, a series of tests was conducted with crude extracts of seeds of *Brassica carinata*. A concentration of 0.05% sinigrin in the extract (present in quantities of ca. 120 μmol/g of whole seed) caused 100% mortality of the second-stage juveniles, as did the same concentration of the purified compounds, although for the crude extract the results were seen after a shorter exposure time (48 h) than with the pure product (data not reported).

DISCUSSION

An analysis of the data demonstrates an in vitro nematocidal action of the ITC obtained from the enzymatic hydrolysis of some glucosinolates toward *H. schachtii*. This effect is a function of the chemical composition of the side chain of the glucosinolate, its concentration, and the exposure times. This finding leads one to hypothesize that the resistance to nematode attack of plants containing glucosinolates that showed an in vitro nematocidal effect is related to the concentration of these compounds in the roots.

Research recently conducted in our laboratories has shown a diverse glucosinolate concentration in the roots of three varieties of radish (*R. sativus* ssp. *oleiformis*) with varying resistance to this nematode (unpublished data). The HPLC analyses conducted on the roots of plants in

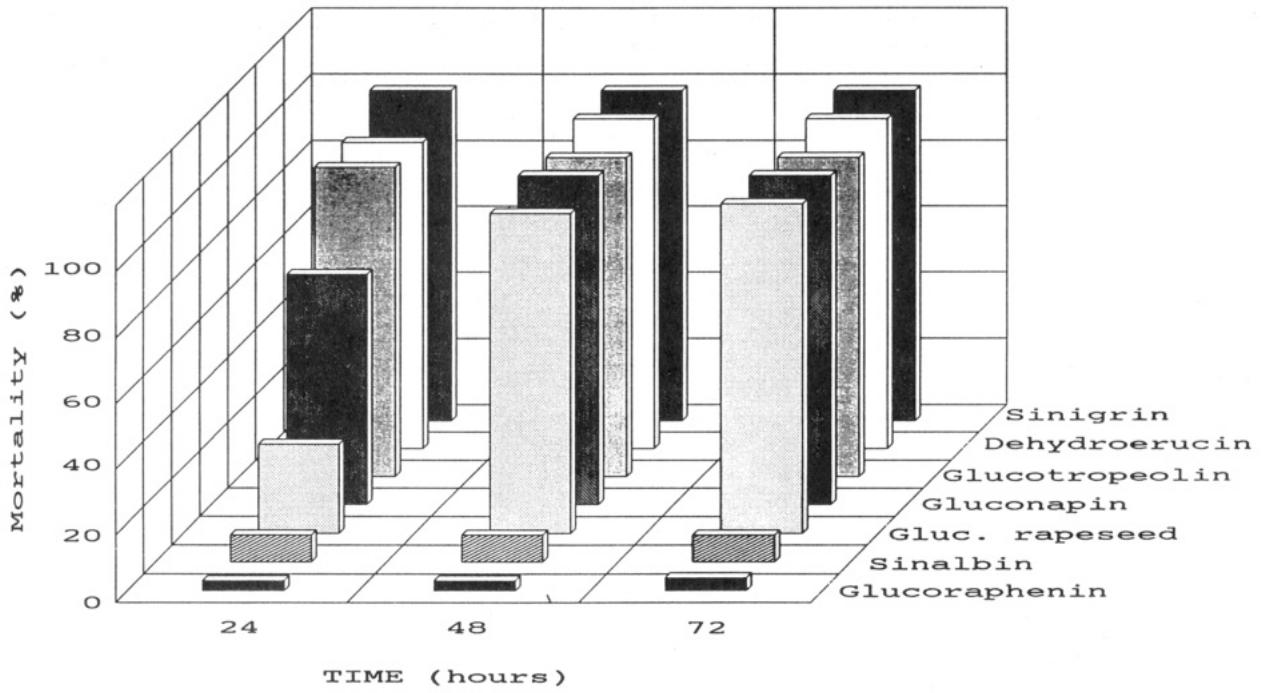

Figure 3. Different nematocidal effects of the reaction products via myrosinase of seven glucosinolates at a concentration of 0.5%.

the flowering stage grown in the province of Bologna, in fact, have shown that the cv. Pegletta contains 10 μmol of dehydroerucin/g of fresh root (ca. 0.45%), Nemex 6 μmol (ca. 0.27%), and Silettina 3 μmol (ca. 0.13%). These dehydroerucin concentrations correlate well with the varying resistance of these cultivars to nematode attack. In this regard, one can easily see that the initial concentration of 0.5% dehydroerucin, which, for the relative ITC gave an in vitro mortality rate of nearly 100%, is naturally present in the root of the cv. Pegletta when flowering. This leads one to believe that when the nematode attacks the roots of a resistant cultivar in certain growth stages, it finds a concentration of glucosinolate (and, therefore, of its ITC due to the effect of endogenous myrosinase) that makes its very survival impossible, resulting in the degradation of the newly formed syncytium a few days after the attack.

It is interesting to note the very slight nematocidal activity shown, even at 1%, of the reaction products of glucoraphenin (present in radish seed) and sinalbin (in mustard seed), although they differ, respectively, from dehydroerucin only by the oxidation of the sulfur atom of side chain R and from glucotropeolin by the presence of an oxyhydryl on the benzene ring. It is interesting that relatively slight structural differences cause profoundly different nematocidal effects, confirming that biological activity is a function not only of the concentration of the product but also of the chemical properties of side chain R (Figure 3).

At present it is not easy to explain the very meager nematocidal effect shown by the ITC of sinalbin, which is in contradiction to the fair resistance shown by the cv. Maxi of *S. alba* (Tacconi et al., 1990). *S. alba* seeds, in fact, have a good quantity of sinalbin, which is also the main root glucosinolate, although present in much smaller quantities.

Mention should be made of the data for the ITC from the hydrolysis of the glucosinolates from rapeseed. Since the HPLC analysis showed the presence of around 20% gluconapin (Figure 2), one is led to believe that the mortality of 0.5% found is due to the presence of ca. 0.1% gluconapin, which proved active even at a concentration of 0.05%. In this regard, the results are in agreement with those of other experiments (Mithen et al., 1986), where progoitrin demonstrated a smaller inhibitory activity toward some pathogens than other glucosinolates (e.g., sinigrin).

CONCLUSIONS

Until now, glucosinolates have been considered mainly as detrimental compounds with potentially toxic or antinutritional properties. The findings in our experiments appear to confirm, on the contrary, that many of them, together with the reaction products obtained with the enzyme myrosinase, may play an important role in the mechanisms of resistance toward many pathogenic parasites (Nault and Styer, 1972). In our case, the action of the glucosinolates and their ITC offers a hypothesis at the molecular level explaining the varying degrees of resistance to the nematode *H. schachtii* seen in some cultivars of *R. sativus* ssp. *oleiformis*. In fact, it appears that the various dehydroerucin contents in the roots determine to a large extent the degree of resistance in radish to *H. schachtii*. It is reasonable to assume that further genetic improvement of the level of this glucosinolate in the roots would provide even better nematode control. Similarly, given the strong nematocidal action of allyl isothiocyanate (the ITC of sinigrin) in vitro, we propose the catch-crop testing of plants with high sinigrin contents in the roots as a way of controlling the second-stage juveniles of *H. schachtii*. Work is underway in our institute to provide a definitive answer regarding the efficacy of new species as a means of controlling *H. schachtii*.

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