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Effects of Glucosinolates and Their Enzymatic Hydrolysis Products via Myrosinase on the Root-knot Nematode *Meloidogyne incognita* (Kofoid et White) Chitw.

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The root-knot nematode *Meloidogyne incognita* (Kofoid et White) Chitw. is responsible for large yield losses in several horticultural crops. Fumigation with chemicals has been efficient in fighting this soil pest, but it clearly shows a negative environmental impact. Thus, it is necessary to find an environmentally friendly alternative to control this nematode and meet the requirements imposed by world regulation to ban some chemical fumigants in the world after 2005. The glucosinolate−myrosinase system, typical of the Brassicaceae family, appears to be an important natural alternative for the control of several soilborne pests and pathogens. The aim of this study was to evaluate, in vitro, the biocidal activity of 11 glucosinolates and their degradation products on second-stage juveniles of the root-knot nematode *M. incognita* expressed by the nematicidal (LD_{50}) and immobilization effects, after 24 and 48 h. None of the intact glucosinolates had any biological effect. After myrosinase addition, their hydrolysis products (essentially isothiocyanates) resulted in highly different biocidal activities. Among the hydrolysis products of the tested glucosinolates, 2-phenylethyl, benzyl, 4-methylthiobutyl, and prop-2-enyl isothiocyanate showed the stronger activity, with an LD_{50} at concentrations of 11, 15, 21, and 34 μM, respectively. On the basis of the the in vitro test results, new genotypes of *Brassicaceae* had been selected for high content in the roots of the glucosinolates generating the more active isothiocyanates and their agronomic performances verified in view of a full-field application as catch crop plants. With this aim, the qualitative and quantitative glucosinolate contents in the roots of these potentially nematicidal plants are also reported and discussed.

KEYWORDS: Nematicidal; Brassicaceae; biocidal green manure; isothiocyanate

INTRODUCTION

In conventional agriculture, the production of several horticultural crops depends on the use of chemical fumigants such as methyl bromide to control a wide array of soilborne fungi, nematodes, insects, and weeds. However, in accordance with the Clean Air Act, the use of this fumigant will be banned in most developed nations by 2005 (*1*). This decision has prompted increased interest in alternatives to control soil pathogens with a low environmental impact, involving physical (solarization, steam disinfection, and others) or biological systems (biocontrol agents) (*2*, *3*). In recent years, there has been a growing interest in the potential of green manure, especially with Brassicaceae plants, in the control of several fungi (*4*), nematodes (*5*−*7*), and other minor pests (*8*, *9*). The biocidal activity of this group of plants is due to the presence, in the cells, of the glucosinolate−myrosinase system and to its capability of producing a number of biologically active compounds including isothiocyanates, nitriles, epithionitriles, and thiocyanates (*10*) (**Figure 1**).

The nature of glucosinolate degradation products (GLDPs) via myrosinase (MYR) depends on the structure of the glucosinolate (GL) side chain R and the reaction conditions (mainly pH and additional proteinic factors) (*11*, *12*), even if, in soil and in plants, isothiocyanates seem to prevail (*13*). In recent years, several in vitro test and in vivo trials have shown a wide biocidal activity of GLDPs on several nematode species such as *Meloidogyne chitwoodi* and *Meloidogyne hapla* (*14*), *Heterodera schachtii* (*5*), *Caenorhabditis elegans* (*15*), *Pratylenchus neglectus* (*16*), and *Globodera rostochiensis* (*4*, *17*). No data are reported in the literature on *Meloidogyne incognita*, although it represents worldwide, on sandy soils, a great problem for several horticultural crops with consequential economically important losses. It was thus important to test whether the GL−MYR system could be considered a practical option to control *M. incognita* and to confirm even on this nematode that the efficacy of the GL−MYR system changes with the class of the GLDPs (isothiocyanates are often the most active) and, within

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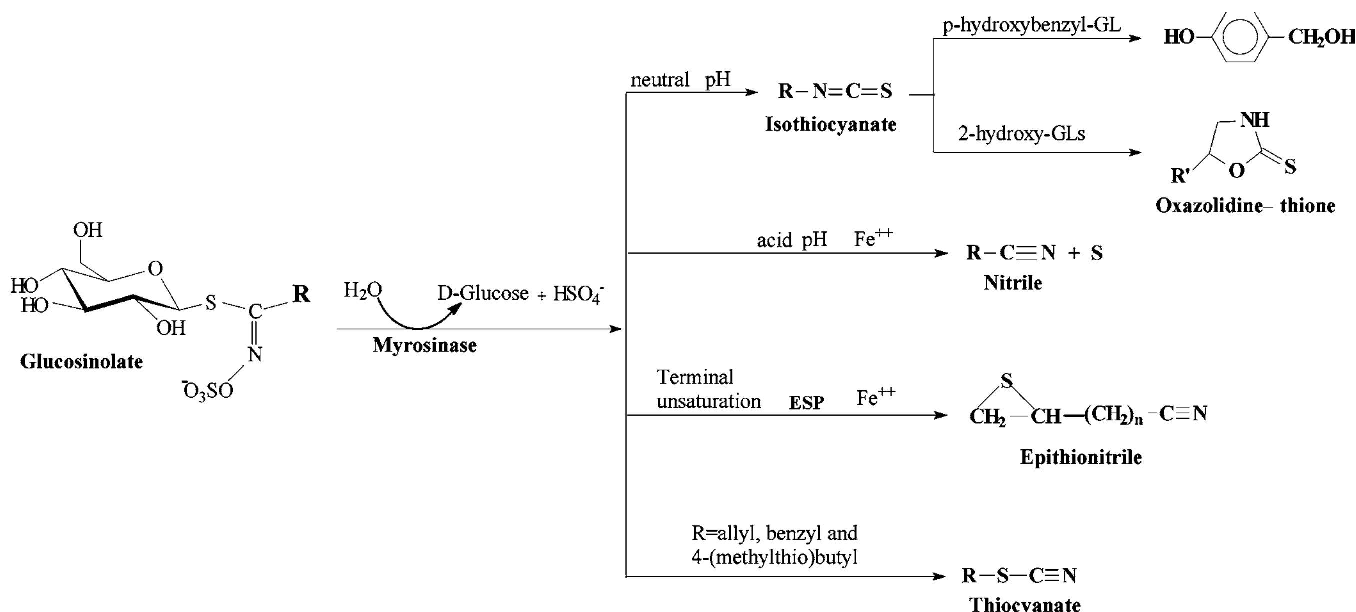

Figure 1. General scheme of glucosinolate hydrolysis via myrosinase.

Table 1. Tested Glucosinolates

trivial name	systematic name	abbreviation
sinigrin	prop-2-enyl-	SIN
gluconapin	but-3-enyl-	GNA
glucoconringiin	2-hydroxy-2-methylpropyl-	GCN
epi-progoitrin	(S)-2-hydroxybut-3-enyl-	*epi*-PRO
sinalbin	4-hydroxybenzyl-	SNB
glucoerucin	4-methylthiobutyl-	GER
glucoraphasatin	4-methylthiobut-3-enyl-	GRH
glucoraphenin	4-methylsulfinylbut-3-enyl-	GRE
glucoiberin	3-methylsulfinyl-propyl-	GIB
gluconasturtiin	2-phenylethyl-	GST
glucotropaeolin	benzyl-	GTL

the class, with the structure of the starting GL, its concentration, and the exposure time of the pathogen to these compounds (*5*).

Therefore, 11 GLs were used for several in vitro tests to verify their nematicidal and immobilization effects, with and without pure MYR addition, on second-stage juveniles (J2) of the root-knot nematode *M. incognita*. In addition, three plant selections containing high levels of the GL precursors of the isothiocyanates that in vitro had shown a stronger nematicidal activity were grown at field conditions, and the amounts of their root GLs were measured at flowering phase.

MATERIALS AND METHODS

Glucosinolates. Two alkenyl (prop-2-enyl and but-3-enyl), three hydroxy [2-hydroxy-2-methylpropyl, (*S*)-2-hydroxybut-3-enyl, and 4-hydroxybenzyl], four thiofunctionalized [4-methylthiobutyl, 4-methylthiobut-3-enyl, (*R*)-4-methylsulfinylbut-3-enyl, and (*R*)-3-methylsulfinylpropyl], and two aromatic (benzyl and 2-phenylethyl) GLs were isolated from Brassicaceae seeds and plants (**Table 1**) and purified essentially following the procedure proposed by Thies (*18*) and modified by Visentin et al. (*19*). The identity and purity of isolated GLs were checked by HPLC. The identity of the GLDPs produced via MYR was confirmed by GC-MS analysis.

Myrosinase. The enzyme MYR had been isolated and purified from *Sinapis alba* seeds according to the method proposed by Palmieri et al. (*20*). The final specific activity of the enzyme solution, evaluated by direct spectrophotometric assay (*21*), was 28 units mg^{-1}.

Glucosinolate Analysis. GLs were determined as desulfoglucosinolates, essentially following the Official Procedure of the European Community for rapeseed (*22*). HPLC analyses were carried out using an Agilent Technology chromatograph, model 1100, equipped with an Inertsil ODS-3 column, 250 × 3.0 mm, DP 5 μm (Varian, prepacked by GL Sciences) maintained at a temperature of 35 °C, and a diode array detector. A linear gradient of CH_3CN/H_2O from 1 to 22% in 22 min was used as eluant at a flow of 1 mL min^{-1}.

Glucosinolate Hydrolysis. GL solutions at the desired concentrations were prepared by diluting properly 5 μM stock solutions in 50 mM sodium phosphate buffer, pH 6.5. Afterward, 1 mL of each solution was moved in a glass cavity block (volume of cavity = 4 mL), and GL hydrolysis was initiated by adding 5−15 μL of MYR water solution (25 units mL^{-1}) depending on the GL concentration. After MYR addition, the glass cavity blocks, previously treated on the margin with silicone vacuum grease, were immediately covered by a sheet of glass to avoid any GLDP loss. Before the beginning of the trials, GL hydrolysis was checked, after 2 h, by analyzing the GLDPs by GC-MS following the procedures described below. To confirm the completeness of the reaction, the absence of intact GL in the same solutions was also checked, using the above-reported procedure.

Analysis of the Hydrolysis Derivative Products. Two hours after hydrolysis was begun, 1 mL of solution was shaken with 1 mL of CH_2Cl_2 to extract the GLDPs that were analyzed by GC-MS. A Hewlett-Packard GCD system model G1800A, equipped with a 30 m × 0.25 mm capillary column HP-5MS, 0.25 μm film thickness, was used. The flow rate of the carrier gas (He) was 1 mL min^{-1}, and samples were injected in splitless mode. The column temperature was 40 °C for the first 6 min and then linearly increased to 220 °C at a rate of 10 °C min^{-1}. Injector and detector temperatures were 220 and 280 °C, respectively. The MS spectra were scanned at 70 eV from 10 to 425 amu. Peaks were identified by using the NIST MS library (75K spectra) and/or by comparing the mass spectra with those reported in the literature (*23*) or those obtained from pure compounds.

Nematodes. A population of *M. incognita* was collected from a sandy soil close to Ferrara (Italy) and maintained on celery roots in a greenhouse. Egg masses were extracted from celery plants by shaking chopped galled roots in 1% sodium hypochlorite for 4 min (*24*). The eggs were placed in water and incubated at room temperature for 3 days; J2s were collected after egg hatching.

Immobilization and Nematicidal Effect. Ten J2s were placed in a glass cavity block (4 × 4 × 1.5 cm) with 1 mL of GL solution with and without the MYR addition, following the above-reported procedure. The glass cavity blocks were sealed and incubated at 22 ± 2 °C to assess the larvae immobilization and nematicidal activity after 24 and 48 h. Each GL was screened at least at three different concentrations, ranging from 0.0025 to 25 mM. In the experimental design, three repetitions were provided for each thesis, and each experiment was repeated twice. Phosphate buffer (50 mM, pH 6.5) was used as control.

Table 2. Main Cultivation Techniques in Spring Sowing of Four Brassicaceae Plants

species	inter-row (cm)	sowing type	seed (kg ha^{-1})	seed (no. m^{-2})	sowing depth (cm)
B. juncea sel. ISCI99	15	continuous row	7	300	2–3
E. sativa cv. Nemat	15	continuous row	5	300	2–3
R. sativus cv. Boss	15	continuous row	25	190	3–5
B. napus 00 cv. Simbol	15	continuous row	11	200	3–5

Larvae immobilization and nematicidal activity after treatments were determined by transferring the immobile nematodes, not reacting to mechanical stimuli, into distilled water to estimate those that did not regain mobility.

The immobile nematodes before the removal into distilled water were used to calculate ID_{50}, the dose that causes inactivity in 50% of the J2 population (immobilization effect); the J2s immobile after 24 h in distilled water were considered to be dead and used to calculate the LD_{50}, the dose that kills 50% of the J2 population (nematicidal effect).

Glucosinolate-Containing Plant Cultivation. In recent years, at the Research Institute for Industrial Crops of Bologna (Italy), some plant lines have been selected for their qualitative and quantitative GL content (*25*). Some of these selections (*Eruca sativa* cv. Nemat, *Brassica juncea* sel. ISCI 99) are characterized, in light of the in vitro tests reported in this paper, from a high content, in the roots, of the GLs precursors of the DPs with high activity against *M. incognita.* To verify the potential of these plants for large-scale cultivation as catch crops, they were cultivated in spring sowing (sowing time March 18, 2002) using as references a cultivar actually commercialized for its nematicidal effect (*Raphanus sativus* cv. Boss) and a Brassicaceae plant characterized by a low GL content (rapeseed 00 cv. Symbol). The trials were performed in the environment of Budrio (Bologna, Italy) located in the Po Valley (latitude 44° 32′ 13″ N, longitude 11° 29′ 40″ E, altitude 29 m asl), on a loamy-clayey soil (clay, 26%; silt, 54%; sand, 20%), using a randomized block design, with four replications in plots of 14 m^2. The main cultivation techniques used for each green manure species are reported in **Table 2**. All plots received 100 kg ha^{-1} of S (Tioscam 50%) as a presowing treatment.

At full flowering phase, the plant roots were harvested by excavating a big hole and removing the soil around them. Their length, weight, and water content were evaluated. In the meantime, a sample of five entire roots was weighed, immediately frozen in liquid nitrogen, stored at −20 °C, and subsequently freeze-dried using an Edwards Minifast Do. 1 freeze-dryer (Crawley, West Sussex, U.K.) (from −40 to 18 °C in 8 h with a vacuum of 10^{-1} mbar). The freeze-dried material was homogenized in a mortar and stored at −20 °C until GL analysis.

Statistical Analysis. A number of univariate one-way analysis of variance assays (ANOVA) were performed to highlight differences in GL root content. To account for heterogeneity in sample variance observed [$p < 0.05$ by Levene test (*26*)], an ANOVA procedure with rank-transformed data (*27*) was performed. Post-hoc mean comparisons had been carried out by applying a form of the Ryan−Einot−Gabriel−Welsch (REGW) test, based on studentized intervals (*28*). The range test (REGWQ) was chosen because it appears to be the most powerful step-down multiple-stage test in the current literature (*29*). It holds the maximum experimentwise error rate to the α level, thus ensuring a high protection level while discriminating among multiple means. REGWQ is becoming quite a bit more popular than alternative tests, as recommended by the most well-regarded statistical packages (e.g., SAS). Statistical analysis was performed using the ANOVA procedure of the package SPSS 11.0.1 (*30*).

The data concerning immobilization and nematicidal effects were corrected according to Abbott's formula (*31*) and processed in a linear regression analysis to calculate ID_{50} and LD_{50}.

RESULTS

Glucosinolate Hydrolysis. Two hours after MYR addition, the hydrolysis solutions did not contain any intact GLs. GC-MS analysis confirmed isothiocyanate as the main initial GLDP, but some of it was quickly converted into other compounds. In fact, SNB hydrolysis produced essentially hydroxybenzyl alcohol, whereas the hydrolysis of the two other hydroxy GLs (*epi*-PRO and GCN) produced, respectively, 5-vinyl-1,3-oxazolidine-2-thione and 5,5-dimethyloxazolidinethione (**Figure 1**). The isothiocyanates produced from the other GLs appeared to be stable under these experimental conditions also 48 h after hydrolysis. These results are in agreement with those obtained by Buskov et al. (7) in similar trials on *Globodera rostochiensis*.

Table 3. Immobilization and Nematicidal Effects (ID_{50} and LD_{50}) of Glucosinolate Degradation Products on J2s of *M. incognita*

glucosinolate precursor	immobilization effect (mM)		nematicidal effect (mM)	
	ID_{50} 24 h	ID_{50} 48 h	LD_{50} 24 h	LD_{50} 48 h
gluconasturtiin	0.013	0.010	0.013	0.011
glucotropaeolin	0.015	0.015	0.015	0.021
glucoerucin	0.021	0.021	0.021	
sinigrin	0.022	0.028	0.034	0.034
glucoraphasatin	0.053	0.051	0.059	0.058
gluconapin	0.062	0.032	0.077	0.074
glucoiberin	0.514	0.517	0.594	0.593
sinalbin	0.609	0.609	0.808	0.609
epi-progoitrin	0.065	0.065	0.944	0.707
glucoraphenin	0.951	0.766	1.292	0.937
glucoconringiin	2.047	2.500	5.690	5.404

Immobilization and Nematicidal Effect. Intact GLs did not induce any mortality on the J2s of the root-knot nematode *M. incognita* (data not shown). On the other hand, after MYR addition, the GLDPs of all the tested GLs produced immobilization (ID) and nematicidal (LD) effects according to the GL type, its concentration and the exposure time. In the tested GLDPs, ID_{50} ranged from 0.013 to 2.047 mM after 24 h and from 0.010 to 2.5 after 48 h, whereas LD_{50} ranged from 0.013 to 5.690 mM after 24 h and from 0.011 to 5.404 after 48 h (**Table 3**).

The immobilization effect is represented by the difference between ID_{50} and LD_{50}. When this difference tends to zero (viz. GST, GTL, and GER), the effect has to be considered as completely nematicidal, because all immobile larvae after treatment were dead. On the contrary, large amount of larvae that regain mobility after their removal into distilled water (resulting in large differences between ID_{50} and LD_{50}) indicated a strong immobilization but a weak nematicidal effect of the treatments (viz., *epi*-PRO and GCN).

Degradation products from GST, GTL, GER, SIN, and GRH showed a nematicidal activity from 10 to 400 times higher than the degradation products from the other GLs. LD_{50} was related with exposure time; usually LD_{50} after 48 h was lower than that after 24 h, except for GTL and GER degradation products.

In general, dead J2s showed a straight and rigid appearance and browning of internal organs. Only after GCN and GNA DP treatment dead larvae sometimes appear to be wound on themselves.

Glucosinolate-Containing Plants. *Eruca sativa* cv. Nemat, *Raphanus sativus* cv. Boss, and *Brassica juncea* sel. ISCI 99 showed a good adaptability to the Po Valley pedoclimatic conditions, even in spring sowing time, and did not require any

Table 4. Root Glucosinolate Content and Root Length of Three Biocidal Plants Compared to a Brassicacea (*B. napus* Cv. Symbol) Classified as Nematode Host

species	root length (cm)	GLs content (μmol g^{-1} of FM[a])	main GL type,[b] %	mol ha^{-1}
B. juncea sel. ISCI 99	17.4 ± 2.1 ab[c]	2.5 ± 0.2	GST, 59.5 SIN, 33.3	8.0 ± 1.7 a[c]
E. sativa cv. Nemat	10.5 ± 2.1 c	2.5 ± 0.2	GER, 72 GRA, 28	3.7 ± 1.2 b
R. sativus cv. Boss	15.3 ± 2.6 b	0.9 ± 0.3	GRH, 81 GRE, 19	3.7 ± 2.0 bc
B. napus 00 cv. Symbol	19.4 ± 2.4 a	0.4 ± 0.0	4-MOGBS, 25 PRO, 33	2.1 ± 0.5 c

[a] Fresh matter. [b] GRA, glucoraphanin; 4-MOGBS, 4-methoxyglucobrassicin; PRO, progoitrin. [c] Mean values in the same column with the same letter do not differ significantly for $P \leq 0.05$, according to LSD test.

pest or weed control treatments and irrigation (data not shown). These results confirm the observations registered in recent years in the trials conducted for plant selection.

The results of root GL content (**Table 4**) confirmed, even at full field level, the good content in the plant roots of highly active GLs. *B. juncea* sel. ISCI 99 contained a high level [2.5 μmol g^{-1} of fresh matter (FM)] of GST and SIN, whereas *E. sativa* cv. Nemat roots presented the same amount (2.5 μmol g^{-1} of FM) of GER and glucoraphanin (GRA) and *R. sativus* cv. Boss a medium level of GRH in roots (0.9 μmol g^{-1} of FM). It is interesting to observe that *Brassica napus* cv. Symbol contained a significantly lower GL content if compared to the other selections. If we consider also the ipogeal biomass, the GL yield (moles per hectare) of roots was higher for *B. juncea*, whereas *R. sativus* and *E. sativa* produced the same GL amounts. *B. napus* GL yield was significantly lower that the others. These results, even if referred to only one year, confirmed those obtained during the selection activity of these new varieties (data not shown) and will be presented in an extended version (Lazzeri et al., in preparation) that will comprise the study of several other parameters of these plants (biomass, humidity, length, and qualitative and quantitative GL content) for both epigeal and ipogeal parts.

DISCUSSION

These trials evidenced a clear in vitro nematicidal activity of GLDPs even on *M. incognita* and confirmed that intact GLs (without MYR addition) did not present any biocidal activity. GST, GER, GTL, SIN, and GRH DPs (all relatively hydrophobic isothiocyanates) showed higher nematicidal activities when compared to those reported in other trials on different nematodes (*5*). This difference could be due to the MYR addition to the GL solutions directly in the glass cavity blocks that surely limited nematicidal compounds loss during and after hydrolysis or even to a higher sensitivity of *M. incognita* larvae to isothiocyanates if compared to *Heterodera schachtii* or *Globodera rostochiensis* sensitivities.

In addition to the nematicidal effect, the results indicate also an immobilization effect of GLDPs on J2 larvae, a characteristic that could be important from an application point of view, because the immobile larvae are not able to penetrate the roots even if they cannot be considered as dead. This evidence could make theoretically interesting also *epi*-PRO DPs, which showed a low nematicidal activity but a high larval immobilization effect, similar to the most effective GLDPs.

The results of these trials seem to be an important starting point for studying the possibility of limiting *M. incognita* infestation by the use of plants selected for GL content of their roots. These plants could be used as biocidal catch crops, supposing that when *Meloidogyne* J2s penetrate the roots determine several cellular lesions where occurs the contact between root GLs and MYR. So, there is the production, in situ, of the corresponding GLDPs characterized from a clear nematicidal activity. In this way, nematodes live in a medium poisoned by GLDPs, and their development should stop few days after root penetration (*6*). Therefore, the nematodes do not produce any progeny, with a consequent decrease of the soil infestation level (*8*). In this regard, recent studies of nematode cycle on biocidal plant roots showed strong differences if compared to other plants that do not contain GLs and to host plants (Curto et al., in preparation).

In conclusion, the interest in natural biocidal compounds for plant defense in agriculture is very important not only in organic farming, but even as an environmentally friendly alternative to chemical fumigants such as methyl bromide. The biocidal activity of Brassicaceae selections seems to be clearly related to the presence of GLs and to the release of their degradation products during cultivation and after plants are chopped up. The results of this study clearly demonstrated the high immobilization and nematicidal activity toward the nematode *M. incognita* of some GLDPs, confirming the biological activity of these compounds evidenced in other studies on other nematodes or soilborne pathogens.

The roots of *E. sativa* cv. Nemat, *B. juncea* sel. ISCI 99, and *R. sativus* cv. Boss contain GLs producing the more active DPs, and these plants showed good agronomic performance even at a full field level. These characteristics, linked to the possibility of their cultivation in a period in which the nematode is in the soil upper part and is highly virulent, and to their root length that should permit a sufficient soil exploration, can be considered as an important starting point for the possible useful utilization of these plants as a new environmentally friendly defensive strategy for limiting *M. incognita* infestation.

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