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Behavioural response of *Meloidogyne incognita* to benzyl isothiocyanate

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Summary – The breakdown of brassicaceous plant material produces nematotoxic isothiocyanates (ITCs). However, after the incorporation of brassicaceous plant material into soil, many nematodes are likely to be exposed only to sublethal concentrations of these compounds. Although unknown, the effect of these low ITC concentrations on the behaviour of plant-parasitic nematodes could play a role in nematode suppression. To address this question, the behaviour of infective second-stage juveniles (J2) of *Meloidogyne incognita* was evaluated following *in vitro* exposure to sublethal concentrations of benzyl isothiocyanate (BITC). Behaviour was qualitatively and quantitatively affected. Overall nematode activity in treatment groups scored visually, and individual nematode movement frequencies, quantified by video assay, were each significantly reduced within 2 h of exposure to 0.01 mM BITC. All responses were dose dependent. Infectivity of BITC-treated J2 of *M. incognita* on soybean (*Glycine max*) was measured directly by root staining and gall rating, and indirectly by egg production. All experiments showed significantly reduced infectivity after treatment of J2 with 0.01 mM BITC. In addition, egg production was almost completely eliminated (<5% of control) by 0.03 mM BITC. The correlation between the effect of BITC concentrations on J2 activity and infectivity was positive, with decreased J2 activity and infectivity resulting in decreased egg production. BITC concentrations that do not cause mortality significantly affect the behaviour of *M. incognita* J2, indicating that sublethal concentrations contribute to the overall nematode suppression by brassicaceous green manures.

Keywords – behaviour, brassica, cover crop, isothiocyanate, nematode control, root-knot nematode.

Brassica cover crops, green manures and seed meals have been used to suppress a variety of soil-borne pathogens (Shetty *et al.*, 2000; Mazzola *et al.*, 2001) including plant-parasitic nematodes (Mojtahedi *et al.*, 1991; Walker, 1997; Potter *et al.*, 1998; Walker & Morey, 1999; Mazzola *et al.*, 2001). Plants in the family Brassicaceae produce glucosinolates, and when brassicaceous plant material is incorporated into soil, glucosinolates are hydrolysed by plant myrosinase enzymes into a variety of volatile hydrolysis products (Fahey *et al.*, 2001). The most widely implicated hydrolysis products responsible for pest suppression are isothiocyanates (ITCs) (Brown & Morra, 1997). Isothiocyanates vary in both volatility (Chew, 1988) and nematode suppressive ability (Buskov *et al.*, 2002; Zasada & Ferris, 2003). They act as general biocides whose activity results from irreversible interactions with proteins (Kawakishi & Kaneko, 1985).

Most research on the efficacy of brassicaceous amendments has focused on determining the ITC concentrations required for nematode mortality. Allyl, benzyl and 2-phenylethyl ITCs each caused at least 50% mortality in both *Meloidogyne javanica* and *Tylenchulus semipenetrans* after 48 h exposure to <100 μM (Zasada & Ferris, 2003). The same ITCs caused 100% mortality in *Globodera rostochiensis* exposed to >1 mM from 16-40 h (Buskov *et al.*, 2002). In *Caenorhabditis elegans*, 15-45 μM benzyl isothiocyanate (BITC) caused 90% mortality within 13 h (Kermanshai *et al.*, 2001). Although these *in vitro* assays identified varied ITC concentrations and exposure times that were lethal to nematodes, obtaining such concentrations in soil and maintaining them over time may not be practical. ITC volatility, limited half-life in soil, and the complex interactions of ITCs with soil (Brown & Morra, 1997; Matthiessen & Shackleton, 2005; Gimsing *et al.*, 2007) decrease the chances

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that plant-parasitic nematodes would actually be subject to lethal ITC concentrations and exposure times. Knowledge of the effects of sublethal ITC concentrations on nematode biology and nematode suppression has tremendous importance for the science of nematode management. Therefore, we examined the response of infective second-stage juveniles (J2) of *M. incognita* to BITC, one of the more nematotoxic ITCs known (Kermanshai *et al.*, 2001; Buskov *et al.*, 2002; Zasada & Ferris, 2003). The main goals of the research were to identify the effects of sublethal concentrations of BITC on *M. incognita* J2 behaviour, and to determine if these concentrations affect infectivity and reproduction on a host.

Materials and methods

Nematodes

Meloidogyne incognita (Salisbury Race-1) was reared on pepper (*Capsicum annuum* cv. Yolo Wonder) grown in sand-filled beakers using a constant moisture system (Sardanelli & Kenworthy, 1997) at 27°C and 16 h : 8 h light : dark photoperiod. Cultures were inoculated with five *M. incognita* egg masses per plant. Approximately 5 weeks after inoculation, plants were harvested and roots were separated and rinsed with water. Roots were then placed on modified Baermann funnels at 27°C to hatch J2. Freshly hatched J2 were collected daily for use in assays.

Treatment solutions

Benzyl isothiocyanate (BITC; Sigma-Aldrich, St Louis, MO, USA) was prepared as a 100 mM stock solution in dimethylsulphoxide (DMSO; Sigma-Aldrich). Solutions for nematode treatment were diluted from this stock to final BITC concentrations of 0.01, 0.02 and 0.03 mM, all in 2% DMSO. A 2% DMSO control was included in all experiments. Treatment solutions were divided into aliquots for use in behavioural bioassays and root infectivity bioassays.

Behavioural bioassays

J2 of *M. incognita* were distributed into wells of a 96-well assay plate (Corning, Corning, NY, USA). Approximately 40-60 J2 were added to each well in a final volume of 200 μl of the appropriate treatment or control solution. Five replicate wells were used for each treatment, and assay plates were incubated at room temperature (25°C). J2 behaviour was observed at 40× using an inverted compound microscope (Nikon Instruments, Melville, NY, USA) fitted with a digital video camera and Nikon Elements image capture and analysis software (NIS Elements AR, Nikon). Two behavioural assays, based upon J2 movement, were conducted hourly from time 0 to 5 h.

Visual Assessment Assay (VAA)

Overall J2 activity was subjectively assessed in each well by observing general movement and morphology of all individuals as a group, and then assigning a group score based upon an arbitrary seven-step activity scale of 0 to 3. A maximum score of 3 was assigned to wells in which all J2 exhibited constant and vigorous sinusoidal movement as typically observed in the DMSO control. Intermediate scores of 2.5, 2, 1.5, 1 and 0.5 were assigned to wells in which J2 movement was progressively slower than nematodes in the DMSO control, with a score of 0.5 assigned to wells in which a mixture of slow moving and inactive J2 were present. A score of zero was assigned to wells containing non-motile nematodes with most J2 appearing straight. The mean VAA score for five replicate wells was treated as a single observation, and mean VAA scores from replicate assays conducted with separate J2 collections were combined to provide treatment means for statistical analysis, n = 4-7.

Video Recording Assay (VRA)

For each treatment in each assay, a single well was chosen for video file recording of J2 activity. Each file was reviewed by superimposing a 100 μm^2 grid on the video image, and quantifying the movement of J2 relative to the grid during a 25 s video recording. Two measurements, frequency and area, were done on a minimum of five J2 per treatment. Frequency was defined as the number of times the head of a J2 moved from one grid square to another grid square (square visits) during the 25 s observation. Multiple visits to the same square were counted as individual visits. Frequency was used to assess the level of activity of the J2. Area was defined as the total number of unique grid squares visited by the head during the recording period. In these measurements, different grid squares were counted only once. Area was used to assess locomotory behaviour of the J2. For the video frequency and area measurements, the score for each worm was considered a single observation, n = 20-30. In selected experiments, frequency and area measurements were done on tail movement for the same worms examined for head movement.

Root infection assay

Plant

Three seeds of soybean (*Glycine max*) cv. Essex were planted into cells of Top 3601 flats (T.O. Plastics, Clearwater, MN, USA) containing sand (Kolorscape all-purpose, Old Castle Retail, Atlanta, GA, USA) that was autoclaved and sieved (10-mesh) prior to planting. Five days after planting, emergent plants were culled to two per cell, and 2 days later (7 days after planting) plants were inoculated with 2% DMSO- or BITC-treated *M. incognita* J2. Plants were maintained in a glasshouse with 16 h : 8 h light : dark photoperiod. Plants were not fertilised.

Nematode treatment, inoculation and behaviour

Approximately 10 000 J2 of *M. incognita* were incubated in either 2% DMSO or in BITC dilutions at 25°C. After 2 h, 750 μl aliquots were removed and used to inoculate plants at a rate of 2000 J2 ml^{-1}. Inoculation was done by pipetting the aliquot directly into sand adjacent to each soybean plant within a cell. Parallel aliquots (30 μl each) were removed from the incubation and transferred to wells of a 96-well plate to observe behaviour (VAA and VRA as described above) in final volumes of 200 μl of appropriate treatment solution. Nematodes inoculated to soybean roots were allowed to invade for 48 h, after which one plant was removed for the measurement of infectivity level by staining J2 within the root (see below). The second plant was transferred to fresh sand and used to assess reproductive viability after infection (*i.e.*, root galling and egg production measurements, see below). All treatments were replicated five times within each experiment, and the experiment was conducted three times (n = 15).

Infectivity

Root systems used to evaluate infectivity were stained with acid fuschin, using methods modified from Byrd *et al*. (1983) and Mahalingam *et al*. (1998). The entire root system was placed in 0.6% NaClO and gently agitated for 3 min. The solution was decanted and the root rinsed and soaked in 40 ml water for 10 min. The rinsate was decanted, and 20 ml of fresh water was added to the root and brought just to the boiling point. To this hot solution 500 μl of acid fuschin (0.015 g (ml water)$^{-1}$) and 500 μl glacial acetic acid were added and brought just to the boiling point twice. The stained root was then cooled to room temperature and left to soak overnight. The staining solution was gently rinsed from the tube with water until the water washes were clear. Twenty ml of clearing reagent (equal volumes lactic acid, glycerol, distilled water) were then added and the root autoclaved for 10 min. Once cooled to room temperature, lateral roots were mounted on a slide and J2 were counted using an Olympus BX-51 microscope (Olympus, Center Valley, PA, USA) at 40× magnification. Roots from one plant member of each treatment pair (five root systems/treatment/experiment), collected 48 h after inoculation, were stained to observe and count the number of infective J2 relative to root length and wet weight.

Reproductive level

Following the 48 h infectivity period, one member plant was transferred to fresh sand. Prior to transplanting, the roots were rinsed with water to remove sand and any nematodes or residual treatment solution. Plants were maintained in a glasshouse with 16 h : 8 h light : dark photoperiod and 24-27 : 20-24°C day : night temperature for 5-6 weeks and did not receive supplemental fertiliser. This corresponds to approximately one *M. incognita* generation. At harvest, nematode reproduction was assessed starting with intact roots. Gall ratings of 0-8 were assigned to intact root systems according to the method of Daulton and Nusbaum (1961), with a 0 rating assigned for no galling and an 8 (maximum) rating assigned to heavily galled roots. Root length and wet weight were recorded, and the entire root system was destructively processed to extract *M. incognita* eggs (Zasada *et al*., 2007).

Data analysis

Data were log transformed ($\log_{10}(x+1)$) prior to analyses when necessary to meet the assumptions of the models; non-transformed data are presented unless otherwise noted. A variance-weighted ANOVA was used and significant differences between treatments were determined. Means were compared with Tukey's adjustment for multiple comparisons ($P < 0.05$). The relationships between *M. incognita* J2 activity, infectivity and egg production to each other were determined using linear least-squares regression models. All data were analysed using the computer software JMP (SAS Institute, Cary, NC, USA).

Results

Effect of BITC on activity of *Meloidogyne incognita*

The highest concentration of BITC (0.03 mM) had an immediate effect on behaviour resulting in reduced ne-

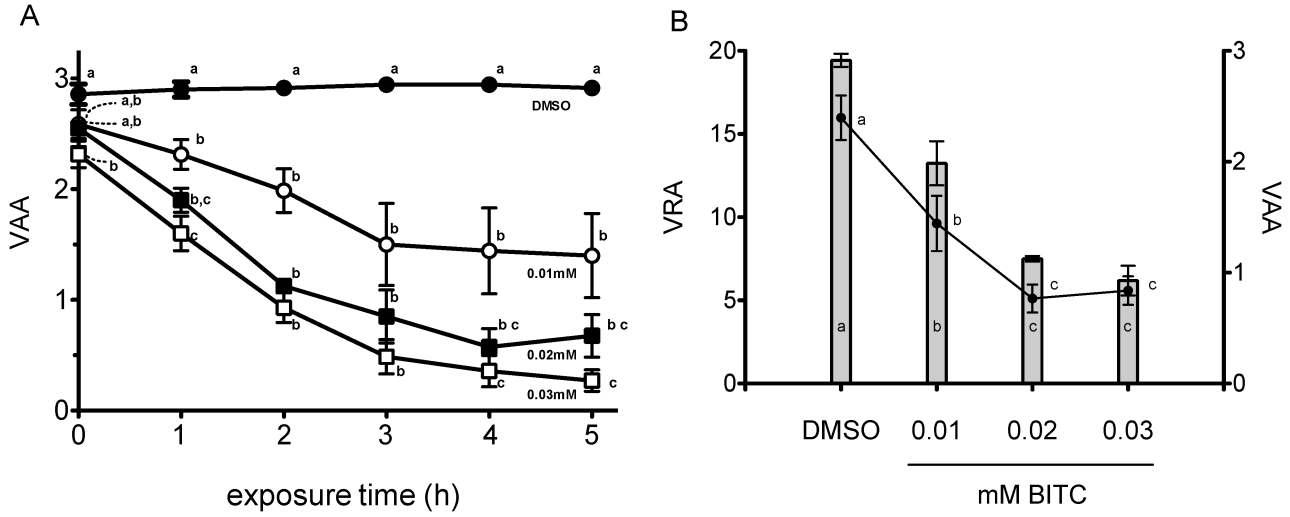

Fig. 1. *A: Visual assessment assay (VAA) scores over time after exposure of infective second-stage juveniles (J2) of* Meloidogyne incognita *to a 2% DMSO control and a range of benzyl isothiocyanate (BITC) concentrations prepared in 2% DMSO. Data are expressed as mean ± SEM of n = 25-35. Bars with different letters within a time are significantly different (*P *< 0.05) according to Tukey's adjustment for multiple comparisons. Treatments: control (filled circles), 0.01 mM BITC (open circles), 0.02 mM BITC (filled squares), 0.03 mM BITC (open squares); B: VAA and video recording assay (VRA) scores 2 h after exposure of J2 to a 2% DMSO control and a range of BITC concentrations prepared in 2% DMSO. Data are expressed as mean ± SEM of n = 13-15. Bars or points with different letters are significantly different (*P *< 0.05) according to Tukey's adjustment for multiple comparisons.*

matode movement (VAA) compared to the DMSO control (Fig. 1A). After 1 h, the VAA scores of BITC-treated J2 were lower than for DMSO-treated J2, and remained depressed through 5 h of observation (Fig. 1A). Nematode activity in the DMSO control remained unchanged throughout the experiment, while J2 activity was reduced more than 90% in the highest BITC concentration. Although some J2 remained active after 5 h exposure to 0.03 mM BITC, the majority of J2 were straight and motionless. To evaluate further the sub-lethal effects of BITC on nematode behaviour we closely examined the concentration and timing results (Fig. 1A) to select optimum conditions for further experiments. All of the BITC concentrations significantly ($P < 0.05$) reduced J2 activity after 2 h relative to the DMSO control, but with little mortality. Therefore, in a second set of experiments, the effect of 2 h exposure was again assessed visually (VAA) and compared with the effect on head movement frequency (VRA). There was a relationship between the two forms of measurement (Fig. 1B). Both VAA and VRA were significantly ($P < 0.05$) reduced at 0.01 mM BITC (VAA 60% of control; VRA 68% of control), and were further reduced at 0.02 mM (each <40% of control). No additional effects were noted at 0.03 mM. Thus, two different measures demonstrated that the activity of J2 could be reduced by exposure to sublethal BITC concentrations applied for a limited period. We then applied these conditions to studies of infectivity.

Effect of BITC on infectivity of *Meloidogyne incognita*

Infectivity of J2 of *M. incognita* was significantly ($P < 0.05$) reduced by all BITC concentrations compared to the DMSO control when soybean roots were directly examined for the presence of J2 (Fig. 2). Based upon root length (Fig. 2, white bars), 0.01 mM BITC reduced infectivity (J2 (cm root)$^{-1}$) by more than 80% ($P < 0.05$) and by more than 98% ($P < 0.05$) at 0.03 mM BITC. Infectivity based upon root weight (Fig. 2, shaded bars) was reduced by nearly 95% ($P < 0.05$) at 0.01 mM and more than 98% at 0.03 mM BITC.

Effect of BITC on reproductive capacity of *Meloidogyne incognita*

Based on gall indexing and egg count, there was a significant reduction ($P < 0.05$) in reproductive capacity of *M. incognita* after exposure of J2 to sublethal concentrations of BITC (Fig. 3A, B). The gall index assessment was based upon visual inspection and scoring,

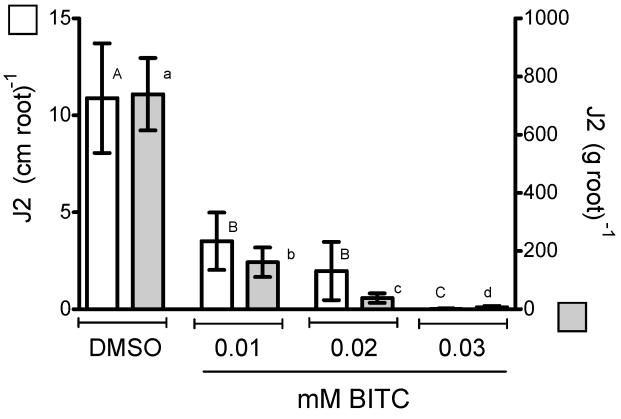

Fig. 2. *Infectivity levels of infective second-stage juveniles (J2) of* Meloidogyne incognita *on soybean (*Glycine max*) in response to exposure to benzyl isothiocyanate (BITC) concentrations prepared in 2% DMSO and to a 2% DMSO control. Data (J2 (cm root)$^{-1}$, light bars; J2 (g root)$^{-1}$, dark bars) are expressed as mean ± SEM of n = 13-15. Means of same shaded bars with different letters are significantly different (*P *< 0.05) according to Tukey's adjustment for multiple comparisons.*

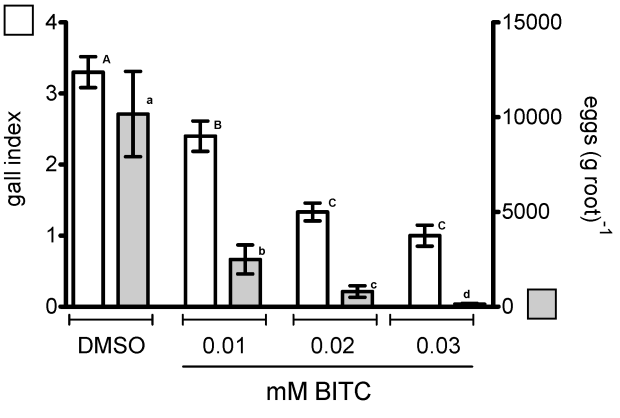

Fig. 3. *Reproduction levels of* Meloidogyne incognita *on soybean (*Glycine max*); second-stage juveniles (J2) of* M. incognita *were exposed to a range of benzyl isothiocyanate (BITC) concentrations prepared in 2% DMSO and to a 2% DMSO control for 2 h before being inoculated into the rhizosphere. Gall ratings (light bars) using the Daulton and Nusbaum scale (1961) and mean number of* M. incognita *eggs (g root)$^{-1}$ (dark bars). Data are expressed as mean ± SEM of n = 13-15. Means of same shaded bars with different letters are significantly different (*P *< 0.05) according to Tukey's adjustment for multiple comparisons.*

and revealed a gradual response to treatment from 0.01 mM (73% of control gall index) through to 0.03 mM (30% of control) (Fig. 3A). In contrast, egg (g root)$^{-1}$ were reduced dramatically at 0.01 mM BITC (<25% of control; $P < 0.05$), and by more than 98% after exposure to 0.03 mM BITC (Fig. 3B). It is notable, however, that reproduction was not completely eliminated at any BITC dose.

Correlation of behaviour and subsequent infectivity of *Meloidogyne incognita*

The behavioural assays varied in their correlation with J2 infectivity and subsequent egg production (Table 1). In general, none of the behavioural assay measurements were strongly correlated to the number of J2 that were able to infect roots on a length basis (J2 (cm root)$^{-1}$). Conversely, the number of J2 that infected roots on a weight basis (J2 (g root)$^{-1}$) was the most closely correlated with behaviour assay measurements ($R^2 > 0.41$), with VAA being the most closely correlated to the number of J2 that entered roots. The total area which BITC-treated nematodes moved based upon VRA assays was the most strongly correlated with the subsequent reproductive potential of BITC-treated infective J2. The number of J2 that penetrated roots (J2 (g root)$^{-1}$) was related to egg production (Fig. 4). These data suggest that those J2 that were able to penetrate and invade roots were able to establish a feeding site and produce eggs.

Discussion

Sublethal concentrations of the brassicaceous plant metabolite BITC depressed activity of J2 of *M. incognita* and reduced their reproductive capacity. In addition, this study demonstrated that there was a direct correlation between infectivity of BITC-treated J2 of *M. incognita* and subsequent egg production. These effects were manifested at low concentrations of BITC and a relatively brief BITC exposure period.

Most studies have focused solely on nematode mortality as a measure of ITC effectiveness. Lazzeri *et al.* (1993) produced mortality in *Heterodera schachtii* after 24-48 h exposure to enzyme-treated glucotropeolin, the glucosinolate precursor to BITC. A concentration necessary to kill 50% of a *M. incognita* population (LC_{50}) of 0.15 mM glucotropeolin was determined after 24 h exposure (Lazzeri *et al.*, 2004). Buskov *et al.* (2002) reported *in vitro* mortality of >80% of *G. rostochiensis* exposed to 2 mM glucotropeolin plus myrosinase for 48 h. After 24 h exposure, LC_{50} values of 1.2 mM sinigrin plus myrosinase, or 0.5 mM pure 2-propenyl ITC, were obtained *in vitro* for *C. elegans* (Donkin *et al.*, 1995). In all of these studies the actual effective ITC concentrations were

Table 1. *Correlation between activity of infective second-stage juveniles (J2) of* Meloidogyne incognita *after exposure to benzyl isothiocyanate (BITC) concentrations prepared in 2% DMSO and a 2% DMSO control and subsequent infectivity and egg production of BITC-treated J2 on soybean (*Glycine max*).*

Behavioural assay measures[1,2]	Root infection assay measures[2,3]	n	Equation of line	R^2
Total frequency[4]	J2 (cm root)$^{-1}$	60	$y = 0.74x - 0.32$	0.34
	J2 (g root)$^{-1}$	40	$y = 1.75x - 0.14$	0.41
	eggs (g root)$^{-1}$	40	$y = 1.49x + 1.10$	0.26
Total area[4]	J2 (cm root)$^{-1}$	60	$y = 0.72x - 0.16$	0.27
	J2 (g root)$^{-1}$	40	$y = 2.59x - 0.59$	0.52
	eggs (g root)$^{-1}$	40	$y = 2.65x + 0.35$	0.49
Visual assessment assay[5]	J2 (cm root)$^{-1}$	60	$y = 0.40x - 0.24$	0.47
	J2 (g root)$^{-1}$	40	$y = 1.00x - 0.07$	0.60
	eggs (g root)$^{-1}$	40	$y = 0.81x + 1.23$	0.36

[1] Infective J2 activity was assayed after 2 h exposure to 0.01, 0.02 and 0.03 mM BITC prepared in 2% DMSO or to 2% DMSO.
[2] Data were log transformed ($\log_{10}(x + 1)$) when necessary. Transformed data are presented in line equations.
[3] Infectivity was measured as the number of J2 detected in roots 5 weeks after inoculation relative to root length or root weight.
[4] Head and tail movement frequency or area were summed for each individual J2.
[5] Individual plate wells were visually scored for overall activity (VAA) on a 0-3 scale.

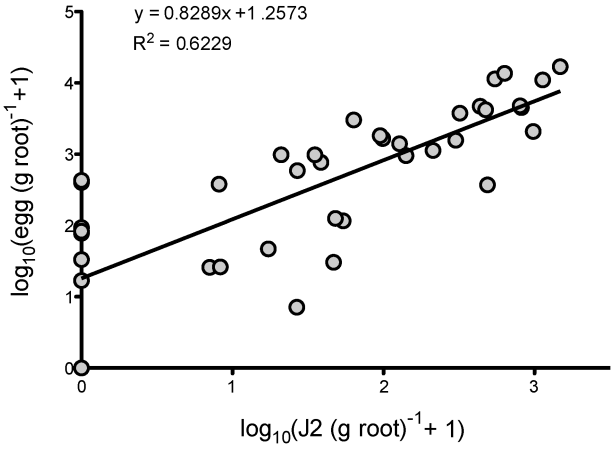

Fig. 4. *Relationship between benzyl isothiocyanate (BITC)-treated second-stage juveniles (J2) of* Meloidogyne incognita *found inside soybean (*Glycine max*) roots 48 h after inoculation into the root zone and egg production of J2 in soybean after 5 weeks. Data were log transformed ($\log_{10}(x + 1)$) prior to analysis; transformed data are presented and n = 40.*

probably lower than as predicted from their corresponding glucosinolate precursors. Indeed, pure compounds exhibit nematode suppressive effects at significantly lower concentrations. Zasada and Ferris (2003) reported 50% mortality of *M. javanica* exposed to 0.04 mM BITC for 48 h in sand, and 90% at 0.06 mM. *Caenorhabditis elegans* exposed to pure BITC at 0.015 to 0.045 mM *in vitro* exhibited 90% mortality after 4-5 h (Kermanshai *et al*., 2001).

This research demonstrates that short exposure times and low doses of BITC may have significant suppressive effects on *M. incognita*. This has two important implications. First, maintaining concentrations of ITCs in soil that result in nematode mortality may be hard to achieve in some environments compared to maintaining sublethal doses. Gimsing *et al*. (2007) reported that soil-dependent half-lives of benzyl glucosinolate and BITC can be as short as 18 and <8 h, respectively. In addition, loss of BITC in soil may be accelerated by other factors including sorption and volatilization. Isothiocyanates are hydrophobic and are adsorbed mainly by organic matter in soil. The toxicity of ITCs in soils rich in organic matter was lower than in those with low organic matter (Brown & Morra, 1997; Matthiessen & Shackleton, 2005). In non-sterilised soil, 2-propenyl and benzyl ITCs had half-lives between 0.9 and 2.8 h (Gimsing *et al.*, 2007). Such factors reduce the availability of ITCs in soil, and limit the chance of nematodes being exposed to lethal concentrations. Therefore, it appears that understanding the sublethal effects of ITCs may play a significant role in developing effective biofumigation tactics for control of plant-parasitic nematodes.

Another goal of our research was the development of experimental methods for use in evaluating the effect of a stressor (*e.g*., BITC) on behaviour of *M. incognita* J2. This is one of the first studies to examine how sublethal concentrations of plant-derived chemicals can influence ne-

matode behaviour, and supplements information on lethal concentrations. Observations of J2 movement and overall activity in the presence of stressors *in vitro* can provide reliable indicators for designing suppression experiments to address practical issues associated with glasshouse and field treatments. For example, if sublethal concentrations of BITC have significant effects on nematode behaviour, infectivity and reproduction, how can brassica green manures and seed meals be applied to provide consistent and predictable suppression of plant-parasitic nematodes?

One plausible explanation for the inconsistent suppression of plant-parasitic nematodes by brassicaceous material is that the distribution of isothiocyanates in soil after incorporation is not even, with some nematodes never being exposed to the compound. The challenge is to develop application technologies that ensure an even distribution of plant material in the zone of incorporation. Steps have been taken in this direction (Morra & Kirkegaard, 2002), but each system will differ (*i.e.*, soil type, brassica material, moisture) and will have to be considered individually.

This research demonstrates that using mortality as the sole indicator of the effectiveness of a compound to control nematodes may not be sufficient to evaluate potential efficacy. For BITC, sublethal doses had a significant impact on *M. incognita* J2 behaviour and subsequent ability to infect and reproduce on a host. This type of research can lead to a better understanding of how biofumigation works and the development of more efficacious techniques for its application.

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