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Effects of benzyl isothiocyanate on the reproduction of *Meloidogyne incognita* on *Glycine max* and *Capsicum annuum*

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Summary – Reproduction of *Meloidogyne incognita* on either *Capsicum annuum* or *Glycine max* was suppressed when infective second-stage juveniles (J2) were exposed to 0.03 mM benzyl isothiocyanate (BITC) for 2 h prior to inoculation of the host. Infectivity as rated by a gall index was significantly reduced on both *G. max* and *C. annuum*. In *C. annuum*, nematode egg masses were recovered from 48% of the plants inoculated with BITC-treated J2 compared with 98% for the controls, and egg mass scores were reduced. Egg mass production was reduced in *C. annuum* by 69% and mean total eggs/plant was reduced by 97% in *G. max*. When plants were inoculated with treated J2, two measures of plant health, root weight and shoot weight, were reduced in *C. annuum* but not in *G. max*. However, root and shoot weights were not correlated with egg production for either host plant, irrespective of treatment. There were strong interactions between egg production, as measured by mean total eggs/plant (*G. max*) or mean total eggs/egg mass (*C. annuum*), and hatching of J2 from eggs obtained from all combinations of plant host and J2 treatment. Hatch of J2 from eggs obtained from *G. max* was significantly lower when plants had been inoculated with BITC-treated J2 than when plants had been inoculated with control J2. Such effects were not observed with *C. annuum*. BITC may have important residual consequences on the progeny of *M. incognita* not directly exposed to the chemical.

Keywords – egg production, hatching, infectivity, nematode, pepper, plant health, soybean.

Glucosinolates comprise a large family of chemicals, widely distributed among the higher plants, that are metabolised through the action of β-thioglucoside hydrolases (myrosinase) into a variety of biologically active compounds upon plant tissue disruption (Fahey *et al.*, 2001; Buskov *et al.*, 2002; Grubb & Abel, 2006). These active compounds serve the plant in defence against soil-borne pathogens (Lazzeri *et al.*, 1993, 2004; Brown & Morra, 1997). Among these active compounds are the isothiocyanates (ITCs), potent biocides toxic to nematodes and of considerable interest as plant-parasitic nematode suppressive agents (Buskov *et al.*, 2002; Nagesh *et al.*, 2002; Zasada & Ferris, 2003; Yu *et al.*, 2005). A number of ITCs, including the aromatic benzyl isothiocyanate (BITC), are known to be lethal to *Caenorhabditis elegans* (Kermanshai *et al.*, 2001) and to the plant-parasitic nematodes *Globodera rostochiensis* (Buskov *et al.*, 2002), *Meloidogyne javanica* and *Tylenchulus semipenetrans* (Zasada & Ferris, 2003) at micromolar and millimolar doses. This high toxicity of ITCs to soil-borne pests contributes to the strategy known as biofumigation, in which plant material is incorporated into soil for the purpose of releasing ITCs as part of a non-synthetic chemical pest management approach (Brown & Morra, 1987; Kermanshai *et al.*, 2001; Gimsing *et al.*, 2007). However, complex interactions within the soil and the chemical nature of ITCs can make exposure of plant-parasitic nematodes to ITCs somewhat problematic, and understanding nematode population suppression at sub-lethal doses becomes important to ITC practicality (Brown & Morra, 1997; Gimsing *et al.*, 2007; Zasada *et al.*, 2009). Exposure of *Meloidogyne* spp. (root-knot nematodes) to micromolar doses of ITCs, including BITC, resulted in behavioural and reproductive effects (Zasada & Ferris, 2003; Zasada

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et al., 2009), including significant reductions of infectivity on soybean, *Glycine max* (Zasada *et al*., 2009). Here we present the first report of using a sub-lethal BITC dose to suppress *M. incognita* infectivity and egg production on pepper, *Capsicum annuum*. The effects are compared with expanded observations on *G. max* and suggest potential long-term effects on root-knot nematode populations.

Materials and methods

Nematode culture

Meloidogyne incognita (Salisbury Race-1) was raised on pepper (*C. annuum* cv. PA-136), a prolific host for *M. incognita* (Dukes *et al*., 1997). Plants were grown in sand-filled beakers using a constant moisture system (Sardanelli & Kenworthy, 1997), at 27°C and 16 h light/8 h dark photoperiod. Cultures were maintained by inoculating plants with five *M. incognita* egg masses each. Approximately 5 weeks after inoculation, plants were harvested and roots gently rinsed free of sand with tap water. Egg masses, collected under a dissecting microscope, were placed on modified Baermann funnels kept at 27°C and 16 h light/8 h dark photoperiod to hatch infective second-stage juvenile (J2). Freshly collected J2 were used in *M. incognita* plant infectivity and reproduction studies. Plants used for these studies were *C. annuum* and soybean (*G. max* cv. Kent), maintained as above except as noted below.

Nematode treatment and plant inoculation

Benzyl isothiocyanate (BITC; Sigma-Aldrich, St Louis, MO, USA) was prepared as a 100 mM stock solution in dimethylsulfoxide (DMSO; Sigma-Aldrich), and solutions for nematode treatment were diluted from this stock to a final working concentration of 0.03 mM BITC in 2% DMSO. The control treatment was 2% DMSO. For the treatment of J2, 10-20 ml of either 2% DMSO or 0.03 mM BITC in 2% DMSO was used to suspend freshly collected J2 (*ca* 3000 nematodes ml^{-1}), and the suspensions were incubated for 2 h at 25°C. Nematode behaviour was used as an indicator of the efficacy of the BITC preparation. To monitor behaviour, 10 μl aliquots of each suspension were transferred to a 96-well microtitre plate containing 100 μl of the corresponding BITC or DMSO solution per well. Nematode movement and behaviour during the 2 h exposure period were used to verify that J2 were affected by BITC as expected (characteristic reduction in head movement frequency and change from sinusoidal to rod-like appearance; Zasada *et al.*, 2009). After 2 h, host plants were inoculated with 500 μl of suspended J2 (*ca* 1500 nematodes/plant). Inoculated plants were maintained in 100 ml sand with rearing conditions as above, except that after 48 h all plant roots were gently rinsed free of infested sand and transplanted to 900 ml of fresh sand. Control treatments were replicated at least four times per experiment. However, since reproduction levels were low to zero on plants exposed to BITC-treated J2, a greater number of these replicates (2-4-fold more than for controls) were used for each experiment. Experiments were conducted five or six times for each host plant species, and data were pooled across experiments.

Harvesting and plant health

Five weeks after inoculation, *G. max* or *C. annuum* were carefully harvested by gently rinsing the root systems free of sand by submersion in a container of tap water. Intact root systems were assigned a gall index on a scale of 0 to 8 according to the method of Daulton and Nusbaum (1961), with heavily galled systems scored as 8 and root systems with no visible galling scored as 0. In addition, *C. annuum* was assigned egg mass scores according to a modification of the Daulton and Nusbaum (1961) scale in which egg masses were counted and assigned a value of 0 to 4. Roots with no egg masses were scored as 0 and roots with 1-4, 5-24, 25-100 or >100 egg masses were scored as 1, 2, 3 or 4, respectively. The fresh weights of both the root system and corresponding shoot were recorded for each plant.

Reproduction assays

For *G. max*, eggs were collected using destructive processing involving blending the root system in 0.3% sodium hypochlorite (Zasada *et al*., 2007). Aliquots of released eggs were counted to estimate total number of eggs per plant. Eggs were then placed on Baermann funnels filled with tap water at 27°C and 16 h light/8 h dark photoperiod to monitor hatching. J2 were collected daily and percent hatch ((J2 collected/eggs on funnel) × 100) calculated through 14 days. Hatch rates (percentage total hatch/day) for the progeny of either the BITC- or DMSO-treated J2 used to infect *G. max* were determined using eggs harvested at 5 weeks after inoculation, processed as above and loaded onto Baermann funnels for J2 collection. Four individual experiments (treatment, inoculation, harvest, collection) were set up over a 5-month period, and

4-5 individual plants (replicates) were harvested per experiment. Eggs obtained from replicate plants were loaded onto separate funnels and J2 were collected periodically over a 2-week period. The mean cumulative percent hatch at each collection was determined using a minimum of 16 replicates pooled over four experiments. Linear regression was used to compare hatch rates.

For *C. annuum*, egg masses were collected directly from roots using jeweller's forceps, and transferred to 96-well plates to monitor hatching. Each well contained a single egg mass in 100 μl tap water. Plates were incubated at 27°C, examined daily through 14 days, and total J2 counted each day. At the end of the experiment, each egg mass was treated with 0.3% sodium hypochlorite to release remaining eggs. All J2 and eggs were directly counted for each egg mass, and these totals were used to calculate daily and cumulative percent hatches.

Data analysis

Individual means were compared using Student's *t*-test with Mann-Whitney unequal sample size test, with *P* indicated for each comparison. Relationships between plant health measurement and infectivity, and between egg and J2 levels, were done using linear least-squares regression. Data were log-transformed for the regression analyses. Statistical analyses were done using JMP (SAS Institute, Cary, NC, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA) computer software programs.

Results

Plant health

Based upon fresh weights of harvested plants, treatment of J2 with 0.03 mM BITC prior to inoculation of *G. max* did not have an effect on root weight, relative to the control treatment of J2 with DMSO (Table 1). However, there was an effect on shoot weight. Mean shoot weight for *G. max* inoculated with BITC-treated J2 was 16% higher ($P < 0.05$) than for *G. max* inoculated with 2% DMSO-treated J2 (Table 1). By contrast, *C. annuum* root and shoot weights were reduced ($P < 0.001$) by 41.8% and 35.5%, respectively, in plants inoculated with BITC-treated J2 relative to controls (Table 2).

Table 1. Glycine max *plant health after exposure to* Meloidogyne incognita *infective juveniles treated with BITC.*

Measurement	2% DMSO	0.03 mM BITC in 2% DMSO
Root weight	4.31 ± 0.35 a (37)	3.69 ± 0.18 a (98)
Shoot weight	2.02 ± 0.15 a (29)	2.34 ± 0.08 b (58)

Means within measurements followed by different letters are significantly different ($P < 0.05$, Student's *t*-test). Number of plants sampled in parentheses.

Table 2. Capsicum annuum *plant health after exposure to* Meloidogyne incognita *infective juveniles treated with BITC.*

Measurement	2% DMSO	0.03 mM BITC in 2% DMSO
Root weight (g)	1.77 ± 1.17 a (49)	1.03 ± 0.07 b (185)
Shoot weight (g)	2.11 ± 0.14 a (49)	1.36 ± 0.07 b (185)

Means within measurements followed by different letters are significantly different ($P < 0.001$, Student's *t*-test). Number of plants sampled in parentheses.

Infectivity and reproduction

The mean gall index for *G. max* inoculated with BITC-treated J2 was 65.9% lower ($P < 0.001$) than the mean index for *G. max* inoculated with DMSO-treated J2 (Table 3). The mean number of eggs produced per plant by *G. max* inoculated with BITC-treated J2 was nearly 98% less ($P < 0.001$) than the mean for the control (Table 3). While egg masses from *G. max* could not be examined directly and thus eggs per egg mass not determined, the mean per plant ratio of total eggs to gall index was 22-fold greater for control plants than those inoculated with BITC-treated J2. This demonstrates that treating J2 with BITC prior to inoculation not only reduced gall numbers, but also reduced egg production per gall (and presumably per egg mass).

For *C. annuum*, most infectivity measurements were also significantly lower on plants inoculated with BITC-treated J2 compared with those inoculated with DMSO-treated J2 (Table 4). Galls were present in 100% of control plants and 77% of BITC-treated J2 inoculated plants. The mean gall index for control plants was 2.6-fold greater than that for all BITC-treated J2 inoculated plants (Table 4). When only plants with galls were used in the comparison, the control/treated gall ratio was lowered to 2.0-fold (Table 4). However, for each comparison the mean gall index was greater ($P < 0.001$) in control plants.

The mean egg mass score based upon all plants within a treatment was 3.2-fold higher in control *vs* BITC-treated J2 plants (Table 4). This was due primarily to the significant reduction in egg mass numbers in plants inoculated with BITC-treated J2. Whilst over 95% of control plants had visible egg masses, only 48% of plants inoculated with BITC-treated J2 produced egg masses. Comparing only those plants that produced egg masses, the mean egg mass rating for control plants remained significantly higher ($P < 0.001$) than for plants inoculated with BITC-treated J2 (Table 4), with a mean ratio of 1.6-fold, similar to that for gall indices. There was, however, no significant difference detected in the mean number of eggs per egg mass between treated and control samples (Table 4), indicating that reduced egg production was primarily dependent upon reduced egg mass numbers. There was no correlation between plant health (root weight, shoot weight) and egg production (Table 5) with any of the treatments, suggesting that host plant condition *per se* was not a factor in egg production measurements.

Table 3. *Infectivity of* Glycine max *by* Meloidogyne incognita *infective juveniles treated with BITC.*

Measurement	2% DMSO	0.03 mM BITC in 2% DMSO
Gall index	3.75 ± 0.17 a (29)	1.28 ± 0.12 b (58)
Eggs/plant	27 670 ± 6766 a (37)	632.2 ± 215.9 b (102)
Eggs/gall index	4650 ± 1329 a (28)	211.80 ± 76.07 b (47)

Means within measurements followed by different letters are significantly different ($P < 0.001$, Student's t-test). Number of plants sampled in parentheses.

Table 4. *Infectivity of* Capsicum annuum *by* Meloidogyne incognita *infective juveniles treated with BITC.*

Measurement	2% DMSO	0.03 mM BITC in 2% DMSO
Gall index	3.11 ± 0.15 a (49)	1.19 ± 0.06 b (185)
Gall index (>0)*	3.11 ± 0.15 a (49)	1.55 ± 0.05 b (143)
Egg mass score	2.33 ± 0.15 a (49)	0.72 ± 0.06 b (185)
Egg mass score (>0)	2.43 ± 0.14 a (47)	1.52 ± 0.05 b (88)
Eggs/egg mass	970.57 ± 43.83 a (34)	926.18 ± 24.13 a (60)

Means within measurements followed by different letters are significantly different ($P < 0.001$; Student's t-test). Number of plants sampled in parentheses.

* Data based only on plants with index or score values greater than zero.

JUVENILE PROGENY HATCHING

In the *G. max* studies, hatch rates (percentage total hatch/day) for progeny of either the BITC- or DMSO-treated J2 were each linear ($y = mx + b; r^2 = 0.97$) through 14 days (Fig. 1). However, clear differences in mean hatch level were observed as early as day 2 ($P < 0.01$), where percent hatch was 0.35 ± 0.15 for J2 progeny from eggs collected from *G. max* inoculated with BITC-treated J2, and 6.42 ± 1.64 for J2 progeny from eggs collected from *G. max* inoculated with DMSO-treated J2. The hatch rate (Fig. 1, slope m, % hatch/day) was 3.7-fold lower ($m = 0.66$) in progeny of the BITC-treated J2 than in progeny of the DMSO-treated J2 ($m = 2.44$). This rate

Table 5. *Correlation between* Meloidogyne incognita *egg production after exposure to 0.3 mM BITC prepared in 2% DMSO and a 2% DMSO control, and subsequent plant health of* Glycine max *and* Capsicum annuum *exposed to treated nematodes.*

Host	Treatment	N	Line	r^2
Root weight				
G. max	DMSO	35	$y = 0.12x - 3.47$	0.11
G. max	BITC	40	$y = 0.11x - 2.21$	0.12
C. annuum	DMSO	34	$y = 0.001x - 2.97$	0.0001
C. annuum	BITC	60	$y = 0.01x + 2.95$	0.003
Shoot weight				
G. max	DMSO	27	$y = -0.15x + 4.14$	0.03
G. max	BITC	23	$y = -0.23x + 2.96$	0.05
C. annuum	DMSO	34	$y = -0.02x + 3.01$	0.03
C. annuum	BITC	60	$y = 0.016x + 2.93$	0.02

Interactions of egg production with plant health were described by linear regression where y is the $\log_{10}$ of either the mean total eggs/plant (*G. max*) or the mean total eggs/egg mass (*C. annuum*) and x is root or shoot weight. N = number of individual plants.

difference resulted in a significantly lower ($P < 0.001$) mean cumulative percent hatch, at 14 days, from eggs produced from BITC-treated J2 ($8.62 \pm 1.87\%$) compared with that from eggs produced from DMSO-treated J2 ($33.52 \pm 3.61\%$). By contrast, no effect of BITC treatment on progeny hatch was observed with *C. annuum*. At 14 days, the mean cumulative percent hatch from eggs produced from BITC-treated J2 ($66.76 \pm 2.05\%$; $N = 69$) was not significantly different ($P > 0.05$) than the mean for controls ($71.57 \pm 2.56\%$; $N = 38$). Regardless of treatment, there were strong interactions between egg production and juvenile hatch with both host plant species (Table 6).

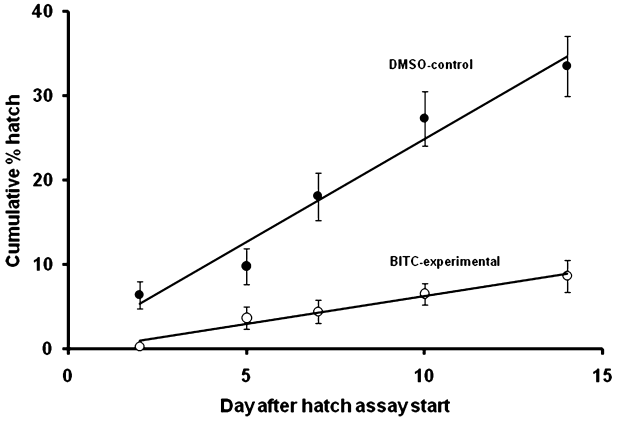

Fig. 1. *Residual effect of 0.03 mM BITC treatment of* Meloidogyne incognita *infective juveniles (J2) on the subsequent hatch behaviour of progeny J2 from females infecting* Glycine max. *Each data point is the mean cumulative percent hatch from eggs collected from a minimum of 16 replicates pooled over four experiments. Filled circles = DMSO-control (progeny hatch from eggs collected from* G. max *inoculated with DMSO-treated J2). Open circles = BITC-experimental (progeny hatch from eggs collected from* G. max *inoculated with BITC-treated J2). Linear regression (*$y = mx + b$*) was used to compare hatch rates. See text for details.*

Discussion

Exposure of *M. incognita* J2 to 0.03 mM BITC for 2 h resulted in significant decreases in both infectivity and reproduction on both *G. max* and *C. annuum*. These effects on *M. incognita* underscore the practical potential of ITCs as components of biofumigation strategies for plant-parasitic nematode control. The significant repressive effects were obtained by exposing J2 to a sub-lethal (Zasada *et al*., 2009) dose of BITC for a limited time. These two factors are of considerable importance given the limited bioavailability of ITCs introduced into the soil environment (Matthiessen & Shackleton, 2005; Gimsing *et al*., 2007).

There was no negative effect on *G. max* by the small amount of BITC transferred to the plants during inoculation (*ca* 15 μM final concentration), but both root and shoot weights were reduced in *C. annuum* exposed to BITC- *vs* DMSO-treated inoculum. Since only 5-week-old plants were harvested, later possible effects on plant health, flowering and seed production, for example, were not considered. However, the current observations with *C. annuum* and the known herbicidal effects of BITC (Norsworthy & Meehan, 2005) suggest that caution should be exercised in using ITCs for nematode suppression on some crops. Regardless of any possible effect BITC may have had on the plants directly, its reduction of *M. incognita* infectivity was striking. In fact, no interactions were detected between measurements of plant health and egg production, regardless of treatment. This demonstrates that any reduction in infectivity and reproduction was the direct result of the effects of BITC on the J2.

While BITC reduced J2 infectivity in each plant system, reduced infectivity alone might account for the reduction in egg production only on *C. annuum*, where eggs/egg mass were not affected by treatment but galling and egg mass production were. By contrast, the level of egg production on *G. max* was more than 20-fold lower in BITC-treated J2 infected plants *vs* DMSO-treated J2

Table 6. *Interaction of egg production with* Meloidogyne incognita *infective juvenile hatch.*

Host	Treatment	N	Line	r^2
G. max	DMSO	31	$y = 1.33x - 2.20$	0.83
G. max	BITC	15	$y = 0.99x - 1.26$	0.61
C. annuum	DMSO	69	$y = 1.23x - 0.88$	0.67
C. annuum	BITC	107	$y = 0.98x - 0.11$	0.75

$y = \log_{10}$ of the mean total J2/plant (*G. max*) or mean total J2/egg mass (*C. annuum*); $x = \log_{10}$ of the mean total eggs/plant or egg mass. N = number of plants (*G. max*) or egg masses (*C. annuum*).

infected (control) plants than would have been predicted by gall index alone. These observations suggest that some factor(s), in addition to reduced infection, might influence egg production, and that these factors could be host related.

One possibility may be that *M. incognita* J2 that successfully infected host plants after exposure to BITC represent a subset of the general population, perhaps a slower developing phenotype. In such a case, slower development would result in fewer egg masses or eggs relative to the control when both control and treated samples are harvested at the same time. Alternatively, while no interaction between plant health and egg production was observed, exposure of root systems to even micromolar concentrations of BITC may result in changes to plant physiology that, in turn, could affect nematode development. Also, possible sequestration of BITC by roots could provide a source of BITC affecting embryo development. This might be more of a factor with *M. incognita* grown on *G. max* where egg masses are predominantly internal to the root. The irreversible binding of ITCs to proteins and their induction of cell cycle arrest and apoptosis (Kawakishi & Kaneko, 1985; Mi *et al*., 2009) make this a possibility that needs further investigation.

With both treatments there was a strong correlation between egg production and J2 hatch. This was true for each host whether egg production was normal (DMSO control) or suppressed (BITC), and makes the observation on hatch effects rather intriguing. In *G. max* the difference in the rates of J2 hatch between those progeny derived from BITC-treated parental J2 and those derived from DMSO-treated parental J2 was both striking and unexpected. Possible explanations include the presence of different developmental states between the two populations of progeny, or perhaps differences in fitness of the hatched J2.

Since *G. max* root egg masses were mostly internal, it was necessary to use rather harsh mechanical and chemical conditions to collect eggs for the *G. max* hatch assay. Such conditions may have favoured the recovery of eggs containing more robust and well developed embryos, thereby favouring the control population. Also, the design of the assay depended upon the mobility of hatched J2 for collection in funnels, another demand on robustness. We attempted to harvest eggs from *C. annuum*, in which egg masses were mostly external, using the same aggressive methods, but hatch was zero for both treatments, suggesting all eggs were compromised.

We thus assessed hatching of J2 from *C. annuum* under much milder conditions using intact egg masses and observed essentially no difference in hatch between treatments. Under these conditions less stringent demands upon level of embryo development or J2 fitness might have allowed for increased hatch in the BITC-treated population. Another possibility is that *C. annuum* may be a more amenable host for *M. incognita* than *G. max*, obviating differences in developmental rates that might be more pronounced in *G. max*. Also, if the possible sequestration of BITC affects the predominantly internal *G. max* egg masses, it would have less effect on the predominantly external *C. annuum* egg masses.

While BITC clearly suppressed infectivity and egg production on both *G. max* and *C. annuum*, reduced egg production cannot be explained by reduced infectivity alone. Possible developmental effects on the *M. incognita* embryo need to be examined. In addition, at least in *G. max*, there was a clear depression of percentage hatch in progeny of BITC-treated J2, again suggesting developmental effects of the treatment, perhaps by population subset selection at infection. Epigenesis could provide an alternative explanation for the BITC-progeny hatch effect. Isothiocyanates are associated with epigenetic effects in animal cells (Beklemisheva *et al*., 2006; Ferguson, 2009), and BITC might contribute to a reduced hatch phenotype. Why depressed hatch was observed with *G. max* but not with *C. annuum* is unclear. However, even control nematodes express different phenotypes in these two hosts (*e.g*., external and internal egg masses). These possibilities are being explored with biochemical and molecular analyses of *M. incognita* physiology following exposure to ITCs.

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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