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Biofumigation for Control of Pale Potato Cyst Nematodes: Activity of Brassica Leaf Extracts and Green Manures on *Globodera pallida* in Vitro and in Soil

James S. Lord,[†] Luca Lazzeri,[§] Howard J. Atkinson,[†] and Peter E. Urwin*[,†]

[†]Plant Nematology Laboratory, Centre for Plant Sciences, University of Leeds, Leeds LS2 9JT, United Kingdom

[§]Council for Research in Agriculture, Centre of Research for Industrial Crops, Via di Corticella 133, 40129 Bologne, Italy

ABSTRACT: The effects of brassica green manures on *Globodera pallida* were assessed in vitro and in soil microcosms. Twelve of 22 brassica accessions significantly inhibited the motility of *G. pallida* infective juveniles in vitro. Green manures of selected brassicas were then incorporated into soil containing encysted eggs of *G. pallida*. Their effect on egg viability was estimated by quantifying nematode actin 1 mRNA by RT-qPCR. The leaf glucosinolate profiles of the plants were determined by high-performance liquid chromatography. Three *Brassica juncea* lines (Nemfix, Fumus, and ISCI99) containing high concentrations of 2-propenyl glucosinolate were the most effective, causing over 95% mortality of encysted eggs of *G. pallida* in polyethylene-covered soil. The toxic effects of green manures were greater in polyethylene-covered than in open soil. Toxicity in soil correlated with the concentration of isothiocyanate-producing glucosinolate but not total glucosinolate in green manures.

KEYWORDS: biofumigation, brassica, green manure, cover crop, soil amendment, glucosinolate, isothiocyanate, toxicity, potato cyst nematodes, *Globodera pallida*, disease control, mode of action

INTRODUCTION

Potato cyst nematodes (PCN), *Globodera pallida* and *Globodera rostochiensis*, are major pests of potato. They occur globally in many regions where potatoes are cultivated,[1,2] and new populations continue to be discovered in previously uninfested areas, including some U.S. states.[3,4] *G. pallida* is the more problematic of the two PCN species because potato germplasm resistant to it is severely limited and rarely offered in agronomically favored cultivars. In the United Kingdom, PCN are the second most economically important pest or disease of potato after late blight, costing an estimated £26 million year^{-1} in lost yield[5] and an additional £10 million year^{-1} in nematicides.[6] *G. pallida* is the predominant species of PCN in England and Wales, occurring in >90% of infested fields.[7]

Control of *G. pallida* usually relies on rotation and chemical nematicides, but two formerly widely applied nematicides, aldicarb and 1,3-dichloropropene, have now been withdrawn from use within the European Union (EU) (European Council Directive 91/414/EEC). Further EU restrictions (EC 1107/2009) could result in the withdrawal of some or all of the remaining approved nematicides, ethoprophos, fosthiazate, and oxamyl. The development of alternative methods to replace withdrawn nematicides is therefore a matter of priority for the EU potato industry and may benefit growers and consumers worldwide.

One potential approach is biofumigation, the suppression of soilborne pests and pathogens by incorporation of brassica green manures into soil. Field experiments have demonstrated the capacity of this approach to control plant-parasitic nematodes in certain cropping systems, including *Meloidogyne javanica* in vineyards[8] and *Meloidogyne incognita* in zucchini production.[9] However, the level of pest or pathogen suppression achieved with brassica green manures varies. In some cases, biofumigation has provided moderate to high levels of control,[8−16] but in other cases it has had little effect on target organisms.[17−22]

Understanding the mechanisms underlying biofumigation can aid its improvement. Brassicas contain a class of thioglucoside called glucosinolates,[23] which are hydrolyzed by specific endogenous thioglucosidases (myrosinases) to yield a variety of biologically active products, including nitriles, thiocyanates, and isothiocyanates.[24] The most toxic glucosinolate catabolites are isothiocyanates,[25−30] and the biocidal activity of brassica green manures is generally attributed to the production of these volatile toxins.[31−34] In addition to the glucosinolate hydrolysis products, decomposing brassica tissues produce other volatile sulfur-containing toxins, including methyl sulfide, dimethyl sulfide, dimethyl disulfide, carbon disulfide, and methanethiol,[31,35−38] which may play a role in biofumigation.[37,38] Methyl sulfide and dimethyl disulfide appeared to reduce the density of *Verticillium dahliae* in soil amended with brassica green manures,[38] and carbon disulfide acts synergistically with methyl isothiocyanate in toxicity to fungi.[39] Dimethyl disulfide and carbon disulfide are particularly toxic to nematodes.[40−42] Biological mechanisms have also been implicated in the suppression of certain fungal diseases after incorporation of brassica green manures.[43−45] *V. dahliae* disease severity on potato was negatively correlated with the proportion of *V. dahliae* antagonists within the Streptomycete community after incorporation of brassica or buckwheat green manures but not after fallow.[43] The suppressiveness of orchard soil to *Rhizoctonia solani* after treatment with *Brassica napus* seed meal

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appears to be due to the stimulation of *Streptomyces* spp., which induce resistance to *R. solani* in apple.[44,45]

Over 120 different glucosinolates occur within the Brassicaceae family.[23] Brassicas vary greatly in their glucosinolate profiles, both quantitatively and qualitatively, even among cultivars of a species,[46−48] and the nematicidal activities of different glucosinolate hydrolysis products vary greatly.[29,30,49−51] Therefore, green manures of different brassicas are expected to vary greatly in their nematicidal activities. The objectives of this study were to identify brassicas with activity toward *G. pallida* in vitro, to quantify any toxicity of green manures of these plants to encysted eggs of *G. pallida* in soil, and to examine the influence of glucosinolate profile on any toxicity observed.

MATERIALS AND METHODS

Toxicity of Brassica Leaf Extracts to Juveniles of *G. pallida* in Vitro. *Plants.* Brassicas were selected for screening either because they are marketed as biofumigant crops or because they are reported to contain high levels of effective glucosinolates. In addition, lines reported to contain very low levels of glucosinolate and the nonbrassica *Triticum aestivum* (wheat) were tested for comparison. *Brassica juncea* cv. Nemfix, *B. juncea* cv. Fumus, *B. juncea* cv. Arid, *Brassica napus* cv. Maxima Plus, *B. napus* cv. Nemcon, and *Raphanus sativus* cv. Weedcheck were provided by Dr. John Kirkegaard at CSIRO, Australia. *B. juncea* #6 plus a *Brassica carinata* cultivar and two cultivars of *Eruca sativa* (*E. sativa* #8 and #9) were supplied by Joordens Seeds Ltd., Denmark. *E. sativa* cv. Nemat was donated by Dr. Ekaterini Riga, Washington State University, and was originally developed at the Centre of Research for Industrial Crops, Italy. *B. rapa* variety MBG-BRS0132[48] was provided by Dr. Maria Cartea, CSIC, Spain. *Nasturtium officinale*, *Lepidium latifolium* (*L. latifolium* A), and *Cardaria draba* were obtained from the Jardin Botanique de Caen. Another *L. latifolium* accession (*L. latifolium* B) and *Barbarea vulgaris* were sourced from the Jardin Botanique de Bordeaux, and *Moricandia moricandioides*, *Sisymbrium austriacum*, and *Lepidium sativum* were sourced from the National Botanical Garden of Belgium. *Sinapis alba* was from the Botanische Garten der Universitat Bonn and *Hesperis matronalis* from Humbolt-Universitat zu Berlin. *T. aestivum* cv. Paragon was supplied by RAGT Seeds Ltd., U.K.

Ten individuals of each plant were grown in a glasshouse at 25−30 °C in a mixture of three parts peat-based compost to one part horticultural grade sharp sand (both from Sinclair Horticultural Ltd., Lincoln, U.K.) in 90 mm diameter pots in winter under natural light supplemented with 16 h day^{-1} illumination from a 400 W bulb (Hortilux Schreder, The Hague, The Netherlands) to provide a total of 150−250 mol of photons s^{-1} m^{-2} at the top of the foliage. The soil was kept moist by watering.

Preparation of Leaf Tissue Extracts. Leaves were harvested when plants began to flower or when 8 weeks old if flowers had not appeared by that time. For each cultivar, all of the leaves from at least five individuals were flash-frozen in liquid nitrogen, ground to a fine powder with a pestle and mortar, freeze-dried, and stored at −20 °C. Leaf extracts were prepared immediately prior to each assay by placing 30 mg of freeze-dried leaf tissue in a 15 mL centrifuge tube with 15 mL of sterile tap water and incubating for 200 min in a rotary incubator at 37 °C and 200 rpm. The extract was passed through a 0.20 μm filter (Sartorius Stedim, Aubagne, France) to remove insoluble debris and microorganisms.

Determination of the Toxicity of Brassica Leaf Extracts to G. pallida Juveniles. Cysts of *G. pallida* Pa2/3 were surface-sterilized[52] and incubated at 20 °C in potato root diffusate to stimulate hatch. Second-stage juveniles (J2) were collected every 2 days for use in toxicity assays. Aliquots of 250 ± 22 (standard deviation) J2 were transferred to 1.5 mL microcentrifuge tubes in ∼100 μL of potato root diffusate. Nine volumes of leaf extract or sterile tap water was added, and the tubes were incubated for 24 h in a rotary incubator at 19 °C and 200 rpm.

After 24 h, J2 were transferred to sand columns for separation of motile and immotile individuals. Sand columns consisted of 14 mm deep × 10 mm internal diameter polypropylene cylinders sealed at the bottom with 100 μm aperture diameter nylon mesh and filled with 0.30 ± 0.02 g of autoclaved silica sand of particle diameter 212−300 μm (BDH Laboratory Supplies, Poole, U.K.). Each column was placed into a well of a 48-well microtiter plate (Cellstar, Greiner Bio-One, Frickenhausen, Germany) and wetted with 600 μL of sterile tap water. The J2 from one microcentrifuge tube were added to the top of each column and incubated for 48 h at 20 °C in the dark. Motile J2 that moved through the columns accumulated in the microtiter plate wells. They were counted on a Peter's counting slide[53] under a dissecting microscope at 25× magnification.

The experiment was carried out in blocks containing 7 replicates each of four randomly selected plant extracts and 10 replicates of water alone. Each plant extract was tested in three blocks, giving a total of 21 replicates. For each sample, the proportion of J2 recovered through the sand column was estimated as the number of J2 recovered through the sand column divided by the mean number of J2 recovered through sand columns after treatment with water alone in that experimental block. Proportion data were arcsin square-root transformed and analyzed by two-way analysis of variance with treatment and experimental block as fixed factors and Games−Howell post hoc test using the statistical package SPSS 16.0 (IBM, Somers, NY).

Validation of an RT-qPCR Method for Quantifying Viable PCN Eggs. To study the effects of biofumigation on the viability of *G. pallida* eggs, a method was developed to quantify viable PCN eggs by quantifying actin 1 mRNA by RT-qPCR. This gene target was chosen as it is constitutively expressed.[54] An assay was performed to determine the accuracy of the method by measuring the relative abundance of viable eggs in samples containing known proportions of heat-killed and viable cysts. The duration of mRNA persistence in PCN eggs in soil after death was also assessed.

Determination of the Accuracy of RT-qPCR for Quantification of Viable PCN Eggs. Two batches of 100 mg of *G. rostochiensis* cysts were placed into microcentrifuge tubes containing sterile tap water. One batch was incubated at 65 °C overnight and the other at room temperature (∼23 °C). After 6 months of incubation at 4 °C, subsamples of each population were surface-sterilized[52] and incubated at 20 °C in potato root diffusate to stimulate hatch. Root diffusate was changed weekly for 6 weeks and the presence or absence of hatched J2 noted. Heat-treated and untreated cysts were picked with forceps and added to microcentrifuge tubes in the following proportions: 0:20, 5:15, 10:10, 15:5, and 20:0 heat-treated/untreated cysts, giving a total of 20 cysts per sample. There were three biological replicates per ratio of heat-treated/untreated cysts. Each sample of 20 cysts was used to generate one RNA sample and one cDNA sample, each of which was tested in triplicate PCR.

RNA was extracted from cysts using a Qiagen RNeasy Plant Mini Kit, according to the manufacturer's instructions for extracting RNA from animal tissue. An on-column DNase treatment using RNase-free DNase I (Qiagen, Crawley, U.K.) was incorporated, according to the manufacturer's instructions. RNA was stored at −80 °C until use. Complementary DNA (cDNA) was synthesized from RNA using SuperScript II Reverse Transcriptase (Invitrogen, Paisley, U.K.), according to the manufacturer's instructions, except that the duration of the RT enzyme incubation step at 42 °C was reduced to 20 min. One reverse transcription reaction was performed for each RNA sample concurrently with one control reaction to which no RT enzyme was added to check for the presence of contaminating genomic DNA.

Each cDNA and no RT enzyme control sample were diluted 20 times in sterile distilled water before use in qPCR. Each qPCR contained the

following reagents: 1× Bio-Rad iQ SYBR Green Supermix (Bior-Rad, Hemel Hempstead, U.K.); 300 nM forward primer GpACTF1 (5′-CCG CCG CCT CCT CCT CCT C-3′); 300 nM reverse primer GpACTR1 (5′-GCC GAC TCC ATG CCG ATG AAG-3′); and 5 μL per reaction of diluted cDNA or no RT control sample in a total reaction volume of 25 μL. In addition to the test samples, a 10-fold dilution series of cDNA of known concentration was included. This cDNA had been prepared using RNA extracted from untreated cysts, and its concentration had been determined spectrophotometrically using a Nanodrop ND-1000 (Labtech International, Ringmer, U.K.). Controls with water instead of cDNA were included. PCR amplification was performed in a Stratagene Mx3005P qPCR thermal cycler with the following thermal profile: predenaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s, followed by a final extension step at 72 °C for 60 s. The thermal cycler was set to measure SYBR Green fluorescence, and MxPro software was used to analyze fluorescence data. Threshold fluorescence values were calculated automatically. A standard curve was generated by plotting threshold cycle (C_t) values from the cDNA dilution series of known concentration against log-transformed cDNA concentration. This standard curve was used to determine the initial concentration of cDNA in the test samples from their C_t values. Means from triplicate PCR were taken as the cDNA concentrations of each biological replicate. The relationship between the proportion of viable cysts in a sample and the estimated content of actin 1 mRNA was analyzed by linear regression in using SPSS 16.0 (IBM).

Determination of the Duration of mRNA Persistence in Soil. Thirty-six batches of 5 mg of dry *G. pallida* Pa1 cysts were mixed into 7.0 g portions of sand-loam, consisting of one part sharp sand (Sinclair Horticultural Ltd.) to one part loam soil (Baileys of Norfolk), and transferred to 7 mL Bijou containers (VWR International, Lutterworth, U.K.). The caps were screwed on loosely, allowing gaseous exchange, and the containers were incubated at 10 °C. After 7 days, 1.2 mL of a solution containing 3.6% dimethyl sulfoxide (Sigma), 18% 2-propanol (Fisher Scientific, Loughborough, U.K.), and 14 mg mL^{-1} Basamid (containing 99% Dazomet and 1% inerts, BASF, Cheadle Hulme, U.K.) was added to 12 of the containers, which were sealed and incubated at 10 °C for 24 h. The other 24 containers received 1.2 mL of sterile tap water alone and were then sealed. Twelve of these were incubated at 10 °C and 12 at 65 °C.

After 24 h, three samples per treatment were immediately taken for extraction of cysts and the remainder were incubated at 10 °C. At 4, 9, and 21 days post-treatment, a further three samples per treatment were collected for extraction of cysts. At each time point, the contents of the containers were emptied into plastic weigh boats and allowed to dry for 6 h in a fume hood with airflow. Cysts were then extracted by flotation using a Fenwick can[53] and transferred to microcentrifuge tubes, all of the cysts from one Bijou container being transferred to one microcentrifuge tube. Cysts were dried by adding three strips of filter paper to each tube and leaving to dry overnight at 4 °C. RNA was extracted the following day as described above. RNA was stored at −80 °C until the RNA extractions from all time points had been performed. RT reactions and qPCR were performed as described above.

Toxicity of Brassica Leaf Extracts to *G. pallida* Eggs in Soil. Green manures of 12 plants were incorporated into soil artificially infested with cysts of *G. pallida*. After 74 days, the cysts were recovered, and the viability of the eggs they contained was estimated by quantifying mRNA transcripts from the nematode actin 1 gene by reverse-transcription quantitative PCR (RT-qPCR). Additional leaves of each brassica were flash-frozen in liquid nitrogen when green manure was produced. These samples were then freeze-dried and stored at −80 °C before being used for determination of glucosinolate profiles by HPLC.

Plants. Eleven of the plants from the in vitro screen described above were selected for determination of their activity toward encysted eggs of *G. pallida* in soil. These plants were *B. juncea* cv. Nemfix, *B. juncea* cv. Fumus, *B. juncea* cv. Arid, *R. sativus* cv. Weedcheck, *E. sativa* #8, *E. sativa* cv. Nemat, *B. rapa* variety MBG-BRS0132, *N. officinale*, *B. vulgaris*, *M. moricandioides*, and *T. aestivum* cv. Paragon. In addition, *B. juncea* cv. ISCI99, supplied by the Centre of Research for Industrial Crops, Italy, was also tested.

At least f50 plants of each cultivar were grown in a mixture of three parts peat-based compost to one part horticultural grade sharp sand (both from Sinclair Horticultural Ltd.) in 90 mm diameter pots. Plants were grown in a glasshouse (14−28 °C) during the summer (May 6−June 18, 2009) under natural light supplemented with 250 W metal halide bulbs 1.5 m above the bench for 4 h in the morning and 4 hours in the evening. Air was circulated with fans, and soil was kept moist by watering. At the time of green manure production, all brassicas except *B. vulgaris* had begun to flower.

Treatment of Soil Infested with Cysts of G. pallida with Green Manures. Cysts of *G. pallida* Pa2/3 that had passed through a 600 μm sieve but had been retained on a 500 μm sieve were added into a sand-loam mixture consisting of 1 part sharp sand to one part loam soil (Baileys of Norfolk, Norwich, U.K.), to provide a density of 182 viable eggs g^{-1} soil. Green manures were added to this soil after it had been stored at 20 °C for 3 days. The storage period allowed the cysts to equilibrate at the correct temperature having previously been kept at 4 °C. Leaf material (135 g) of each plant variety was homogenized in a high-power commercial blender (Waring, Torrington, CT) and mixed thoroughly into 2.57 kg of nematode-infested sand/loam. The soil was watered with 320 mL of tap water, and mock treatments received water alone. Sixteen replicate portions of 168 g of treated soil were immediately dispensed into 90 mm diameter pots lined with 500 gauge black polyethylene. The surface of the soil in each pot was covered with a layer of horticultural grit to reduce evaporation from the soil surface. For each green manure treatment, eight pots were left open and a further eight were covered with a double layer of polyethylene. Pots were kept in a glasshouse at 17.1 ± 3.2 (SD) °C for 74 days. One day after incorporation, four open and four covered pots were randomly selected and weighed. These pots were weighed every week to monitor evaporation, and sufficient water was added to all pots to compensate for this loss.

Determination of the Viability of Encysted Eggs of G. pallida Extracted from Soil Treated with Green Manures by Quantification of Actin 1 mRNA Using RT-qPCR. Soil was transferred to trays and air-dried for 14 days at 14.5 ± 2.4 °C 74 days after green manures were incorporated into infested soil. Dry soil was stored at 4 °C for 80−100 days until cysts were extracted by flotation using a Fenwick can[53] and transferred to microcentrifuge tubes. Each microcentrifuge tube contained all of the cysts from one pot, and these were used to prepare one RNA sample. There were five biological replicates per treatment. To control microbial contamination and to aid subsequent grinding, cyst samples were dried by adding strips of filter paper (Whatman U.K. Ltd., Maidstone, U.K.) to act as wicks and incubating them for 1 week at 4 °C in the dark. RNA was extracted, cDNA was synthesized, and qPCR was performed as described above. Means of three technical replicates were taken as the cDNA concentration for each biological replicate.

The mean cDNA concentrations in mock-treated samples from open containers and from covered containers were taken to be 100% relative viability for samples in open and covered containers, respectively. For each sample, the relative viability of eggs after treatment was estimated as the cDNA concentration in the sample divided by the mean cDNA concentration in the corresponding mock treatment. Proportion viability data were arcsin square-root transformed and analyzed by one-way analysis of variance with S−N−K post hoc test using SPSS 16.0 (IBM).

Glucosinolate Analysis by High-Performance Liquid Chromatography (HPLC). At the time of incorporation into soil, 100 g of leaf material was frozen in liquid nitrogen and stored at −80 °C. The frozen material was ground to a fine powder in a pestle and mortar and

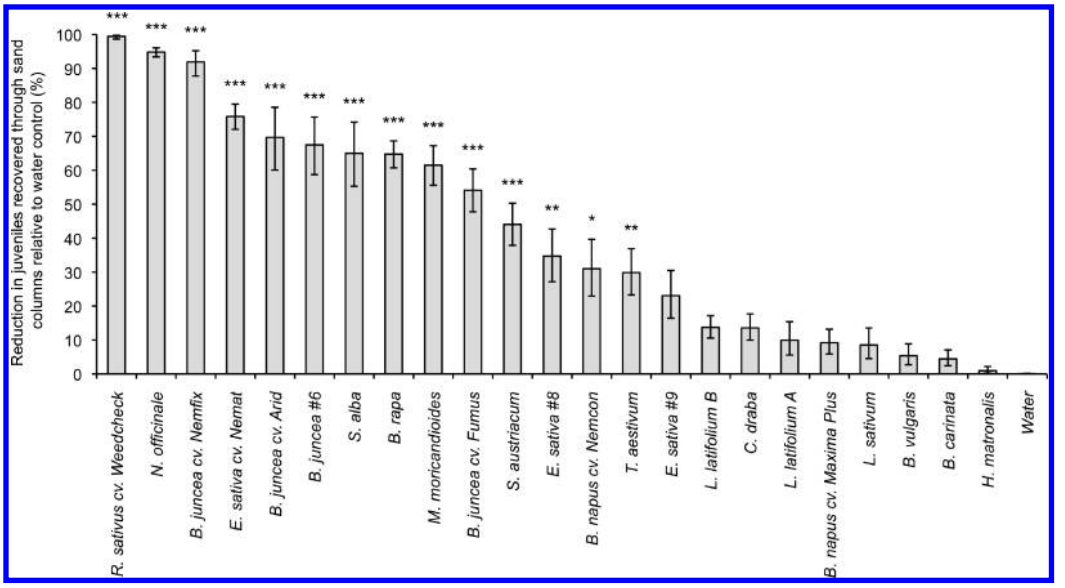

Figure 1. Percentage reduction of second-stage juveniles of *G. pallida* recovered through sand columns after 24 h of exposure to aqueous extracts from leaves of 22 brassica lines or wheat (1.8 mg of dry leaf tissue mL^{-1}) relative to water control. Data presented are back-transformed means ± standard errors of the mean. Significant differences from water control are highlighted with asterisks (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n \geq 20$, Games−Howell post hoc test).

then freeze-dried. The material was weighed before and after freeze-drying to calculate tissue moisture content. Dry leaf tissue of each brassica was used for quantification of individual glucosinolates by HPLC according to the ISO 9167 (1992) procedure[55] with some minor modifications[56] using a Hewlett-Packard chromatograph 1090 L equipped with a diode array detector and a 200 × 4.6 mm column (HP ODS Hypersil C18, 5 μm). Glucosinolate concentration per gram of dry tissue was multiplied by the mass of dry tissue incorporated into soil to calculate the amount of glucosinolate that had been incorporated. The amounts of both total glucosinolate incorporated and its isothiocyanate-producing components were plotted against percentage reduction in viability and analyzed by linear regression using SPSS 16.0 (IBM).

RESULTS

Toxicity of Brassica Leaf Extracts to *G. pallida* Juveniles in Vitro. The effects of brassica leaf extracts on motility of J2 *G. pallida*, measured as movement through sand columns, ranged from almost total inhibition of motility to no effect (Figure 1). Leaf extracts from the majority of the plants tested, including *T. aestivum* cv. Paragon (spring wheat), caused significant suppression relative to treatment with water alone. Cultivars of three species, *R. sativus* cv. Weedcheck (radish), *N. officinale* (cress), and *B. juncea* cv. Nemfix (Indian mustard), were particularly potent, causing 97, 93, and 89% inhibition, respectively. The following cultivars all caused between 55 and 75% suppression: *E. sativa* cv. Nemat (arugula), *B. rapa* (turnip), *S. alba* (white mustard), *M. moricandioides*, and all of the remaining *B. juncea* cultivars, including the low-glucosinolate cv. Arid. Two of the commercially marketed biofumigant crops, *E. sativa* #9 and *B. napus* cv. Nemcon (rape), performed poorly against *G. pallida*, causing 25 and 34% suppression, respectively. This was not significantly different from the effects of wheat.

Validation of an RT-qPCR Method for Quantifying Viable PCN Eggs. *Determination of the Accuracy of RT-qPCR for Quantification of Viable PCN Eggs.* A preliminary experiment, in which viable and heat-treated cysts were mixed in different proportions

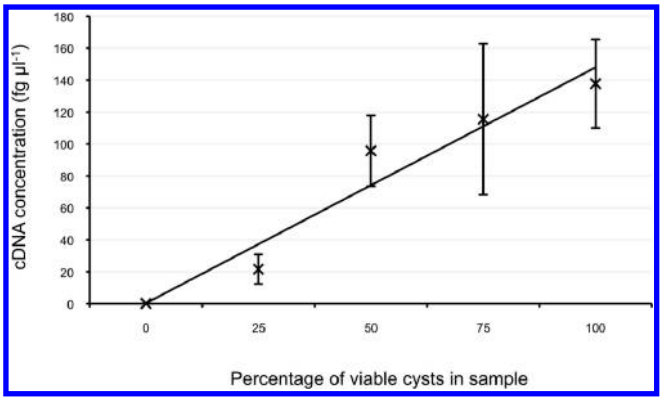

Figure 2. Determination of the accuracy of an RT-qPCR method for quantifying viable PCN eggs. A close relationship was observed between the proportion of viable cysts in mixtures of viable and nonviable, heat-killed *Globodera rostochiensis* cysts and the concentration of cDNA in preparations made from those cysts ($R^2 = 0.94$, $P < 0.01$). Data presented are means (± standard error of the mean) of three biological replicates.

and mRNA was quantified by RT-qPCR, showed that abundance of actin 1 mRNA correlated closely with the proportion of untreated, viable eggs within samples ($R^2 = 0.94$, $P < 0.01$) (Figure 2). The maximum difference between the true percentage of viable cysts in a sample and the percentage predicted by the regression line was 15%. Cysts heated to 65 °C for 24 h showed a complete lack of hatching even after 6 weeks of exposure to potato root diffusate, whereas mock-treated eggs hatched abundantly. Thus, the mock-treated cysts were confirmed as viable, whereas the heat treatment appeared to have caused 100% mortality.

Determination of the Duration of mRNA Persistence in Dead Globodera Eggs in Soil. Experiments were conducted to ascertain the duration of persistence of mRNA in *Globodera* eggs after death. By 1 day post treatment, mRNA levels in heat- or Dazomet-treated cysts were 3 orders of magnitude lower than

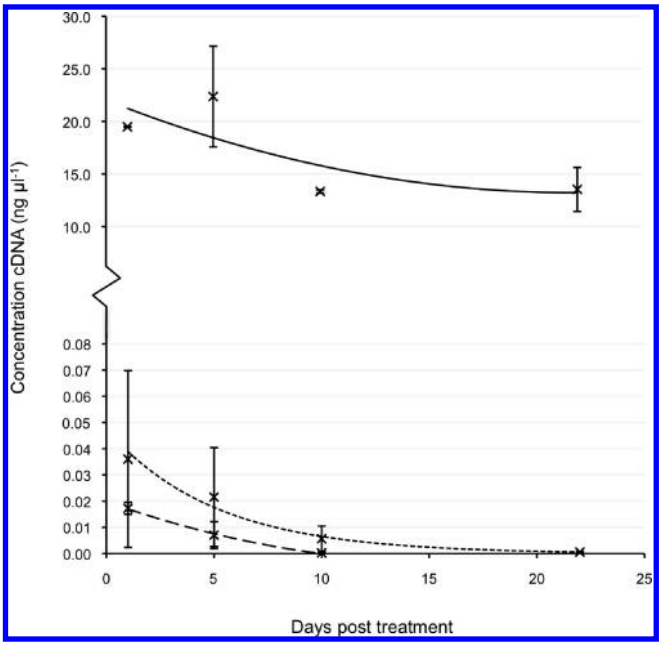

Figure 3. Rate of mRNA degradation in encysted eggs of *Globodera pallida* in soil at 10 °C following treatment with Dazomet (long dashed line), heat (short dashed line), or water (solid line). Note the scale change. Data presented are means (± standard error of the mean) of three biological replicates.

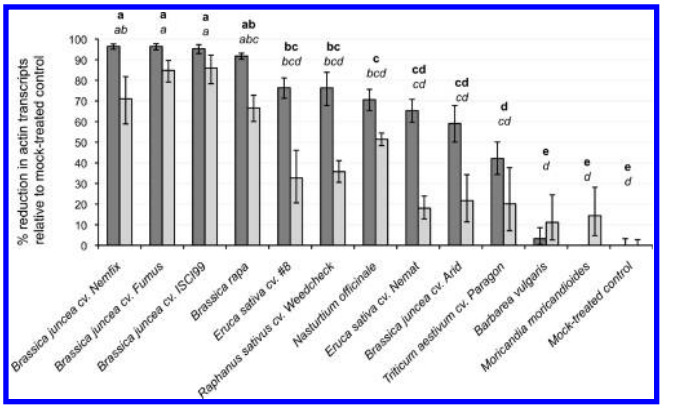

Figure 4. Results of a glasshouse trial to determine the effects of green manures on encysted eggs of *G. pallida* in soil. Columns indicate the percentage reduction in viability of encysted eggs of *G. pallida* in soil after treatment with green manures (5% w/w) in polyethylene-covered (dark columns) and open (light columns) containers relative to mock-treated controls. Viability was measured by quantifying the abundance of nematode actin 1 mRNA. Separate analyses of variance with S−N−K post hoc tests were performed on square-root-transformed data for open and covered soil treatments. Data presented are back-transformed means ± standard errors of the mean. n = 5. Bold letters indicate homogeneous subsets within covered soil treatments, and italicized letters indicate homogeneous subsets within open soil treatments.

those in untreated viable cysts (Figure 3). RNA abundance continued to decline exponentially after treatment with Dazomet or heat. By 10 days post treatment with Dazomet and 22 days post treatment with heat, mRNA was effectively undetectable.

Toxicity of Brassica Leaf Extracts to *G. pallida* Eggs in Soil and Relationship with Glucosinolate Content. Brassica green manures incorporated into soil at a rate of 5% (w/w) caused significant reductions in the viability of encysted eggs of *G. pallida* as measured by quantifying the abundance of nematode actin 1 mRNA (Figure 4). All but two of the green manures had a nematicidal effect in polyethylene-covered soil. The three *B. juncea* cultivars with high levels of 2-propenyl glucosinolate (Figure 5) had the greatest effect, causing >95% mortality to *G. pallida* eggs. *B. rapa*, which contained large quantities of 3-butenyl glucosinolate, caused >90% mortality. The next plants ranked by efficacy were *R. sativus* cv. Weedcheck and *E. sativa* cv. #8, which caused >75% mortality in covered soil. These plants both contained various glucosinolates with sulfur-containing side chains, with total glucosinolate concentrations approximately half of those in the more effective *Brassica* species. *E. sativa* cv. Nemat had a glucosinolate profile very similar to that of the other *E. sativa* cultivar and caused similar mortality. *N. officinale* caused 70% mortality in covered soil. This plant contained comparable total amounts of glucosinolate to the high-glucosinolate *Brassica* species but the predominant glucosinolate was 2-phenylethyl glucosinolate. The low-glucosinolate *B. juncea* cultivar, Arid, caused 60% mortality in covered soil, despite containing lower levels of glucosinolate than the ineffective *M. moricandioides*. The nonbrassica, wheat, although not as effective as most of the brassicas, still caused a significant 43% reduction in viability in covered containers. *B. vulgaris* showed no significant nematicidal activity, despite containing high concentrations of 2(*R*)-hydroxy-2-phenylethyl glucosinolate. The rank order of efficacy was similar in covered and

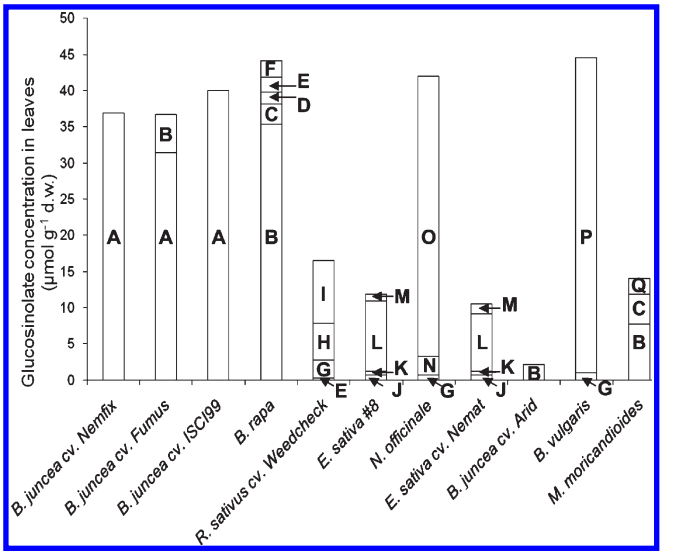

Figure 5. Glucosinolate profiles of brassicas at the time of their incorporation as green manures into soil in the glasshouse trial. Key to glucosinolates: A, 2-propenyl; B, 3-butenyl; C, (*R*)-2-hydroxy-3-butenyl; D, 4-pentenyl; E, 4-hydroxyindol-3-ylmethyl; F, indolyl; G, 1-methoxyindol-3-ylmethyl; H, 4-methylsulfinyl-3-butenyl; I, 4-methylthio-3-butenyl; J, 4-(methylsulfinyl)butyl; K, 4-(methylthio)butyl; L, 4-mercaptobutyl; M, unidentified 1; N, unidentified 2; O, 2-phenylethyl; P, 2(*R*)-hydroxy-2-phenylethyl; Q, 4-methoxyindol-3-ylmethyl.

open pots except that *N. officinale* ranked higher in open than in covered pots. Covering the soil enhanced the efficacy of all of the green manures that had a nematicidal effect.

The brassicas could be divided into two groups by glucosinolate content. There were six high-glucosinolate plants containing ≥35 μmol g^{-1} dw and five low-glucosinolate plants

containing ≤17 μmol g^{-1} dw (Figure 5). The three *B. juncea* cultivars from the high-glucosinolate group were highly toxic to *G. pallida* eggs (Figure 4). They shared similar glucosinolate profiles. *B. juncea* cv. ISCI99 and *B. juncea* cv. Nemfix each contained between 36 and 40 μmol g^{-1} dw 2-propenyl glucosinolate as the sole glucosinolate. *B. juncea* cv. Fumus contained 31.4 μmol g^{-1} dw 2-propenyl, as well as 5.4 μmol g^{-1} dw 3-butenyl glucosinolate. Another high-glucosinolate plant, *B. rapa*, contained predominantly 3-butenyl glucosinolate (80% of total) and about equal quantities of 4-pentenyl, (*R*)-2-hydroxy-3-butenyl, indolyl, and 4-hydroxyindol-3-ylmethyl glucosinolate. The other two high-glucosinolate plants were *N. officinale* and *B. vulgaris*, which contained 41.9 and 44.6 μmol g^{-1} dw total glucosinolates, respectively. *N. officinale* contained predominantly 2-phenylethyl glucosinolate (92%) with a small proportion of an unidentified glucosinolate (6%) and 4-methoxy-indol-3-ylmethyl glucosinolate (2%). *B. vulgaris* contained almost exclusively 2-(*R*)-hydroxy-2-phenyethyl glucosinolate (98%) with a small proportion of 4-methoxy-indol-3-ylmethyl glucosinolate (2%).

The brassicas with lower levels of total glucosinolate were *R. sativus* cv. Weedcheck, the two *E. sativa* cultivars, *B. juncea* cv. Arid. and *M. moricandioides*. *R. sativus* cv. Weedcheck contained a total of 16.5 μmol of glucosinolate g^{-1} dw, of which 53% was 4-methylthio-3-butenyl, 31% was 4-methylsulfinyl-3-butenyl, 15% was 4-methoxyindol-3-ylmethyl, and 2% was 4-hydroxyindol-3-ylmethyl glucosinolate. The two *E. sativa* cultivars had similar profiles. *E. sativa* #8 contained 11.9 μmol g^{-1} dw total glucosinolate, 12% more than *E. sativa* cv. Nemat. This difference could be accounted for by the lower concentration of the predominant glucosinolate in Nemat. This glucosinolate formed a dimer during HPLC and has been provisionally identified as 4-mercaptobutyl glucosinolate.[57] *E. sativa* #8 and *E. sativa* cv. Nemat contained, respectively, 82 and 75% 4-mercaptobutyl, 8 and 13% of a second unidentified glucosinolate different from that found in *N. officinale*, 6 and 7% 4-(methylsulfinyl)butyl, and 5% each 4-(methylthio)butyl glucosinolate. *B. juncea* cv. Arid contained 2.1 μmol g^{-1} dw total glucosinolate, of which 89% was 3-butenyl and 11% was 2-propenyl glucosinolate. *M. moricandioides* contained 14.1 μmol g^{-1} dw total glucosinolate, of which 55% was 3-butenyl, 29% was (*R*)-2-hydroxy-3-butenyl, and 16% was 1-methoxyindol-3-ylmethyl glucosinolate.

There were significant correlations between mortality in both the covered (R^2 = 0.839, P = 0.001) and open containers (R^2 = 0.843, P < 0.001) and the isothiocyanate-producing glucosinolate content of the brassica green manures (Figure 6). In open containers, the high R^2 value and a predicted intercept for the regression line close to the origin suggest that isothiocyanates were the sole cause of the observed mortality. In contrast, with covered containers, the intercept is much higher, around 60% when the two ineffective green manures (*B. vulgaris* and *M. moricandioides*) are excluded from the analysis. This suggests that some of the mortality caused by the effective green manures in covered containers was independent of isothiocyanate production. In contrast to the above results, there was no significant correlation between mortality of *G. pallida* eggs in either open or polyethylene-covered soil and the total glucosinolate content of brassica green manures (data not shown).

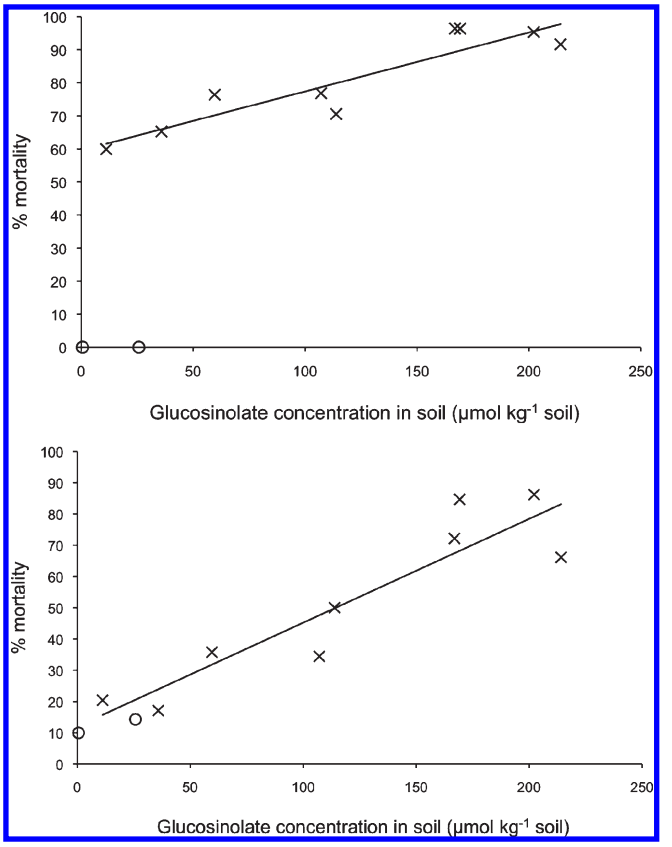

Figure 6. Correlations between total isothiocyanate-producing glucosinolate in plants and percentage mortality of *Globodera pallida* eggs after incorporation of brassica green manures into infested soil in (top) polyethylene-covered and (bottom) open containers. The plants that had no significant impact on the mortality of *G. pallida* eggs, *Barbarea vulgaris*, and *Moricandia moricandioides* (circles) have been excluded from the analysis. There were significant correlations with mortality in covered containers (R^2 = 0.839, P = 0.001) and open containers (R^2 = 0.843, P < 0.001).

DISCUSSION

An in vitro screen of 23 brassicas was used to identify varieties possessing activity toward *G. pallida*. Leaf extracts of 12 of the brassica varieties significantly inhibited motility of *G. pallida* infective juveniles (Figure 1). Three lines were particularly potent: *R. sativus* cv. Weedcheck, *N. officinale*, and *B. juncea* cv. Nemfix. Leaf extracts of wheat also displayed inhibitory activity but to a lesser extent than the brassicas.

To control *G. pallida* in the field, biofumigation should target encysted eggs and not just hatched juveniles. This is because PCN invade potato roots for several weeks after potatoes start growing,[58] the latest that a green manure could be incorporated is at the time of potato planting, and isothiocyanates have half-lives of tens of hours at the very most.[59,60] The vast majority of J2 invading potato roots would inevitably hatch after isothiocyanate concentrations had become negligible. The present study has demonstrated for the first time that brassica leaf tissue incorporated into soil can cause high levels of mortality to encysted eggs of *G. pallida*, thus demonstrating the potential of brassica rotation crops to control *G. pallida* in potato production. Over 95% mortality of encysted eggs was observed in soil treated with any one of several *B. juncea* cultivars and then covered with polyethylene. Mortality of up to 85% was observed when treated soil was left uncovered. This compares favorably with levels of control provided by synthetic fumigants in the field. For instance, metham sodium or 1,3-dichloropropene caused mean mortality

rates of 60 and 64%, respectively, in fields in northern England.[61] Fumigation with 1,3-dichloropropene in clay soils in The Netherlands caused from 48 to 72% mortality.[62] The rate of fresh brassica biomass applied in this work (5% by mass) is around the upper limit of the rate that could be applied using only plants grown within the treated field before incorporation. Field experiments are now required to determine levels of control achieved by the green manures in such conditions. An initial prediction is that population densities of *G. pallida* in the field could be reduced by 50% or more by biofumigation with *B. juncea*. This estimate is based upon the observed mortality caused by *B. juncea* in the present study, dose−response curves for inhibition of *G. pallida* J2 motility by 2-propenyl isothiocyanate (unpublished data), the vertical distribution of PCN cysts in potato fields,[62] a brassica dry matter content of 10% (unpublished data), and typical yields of 4 t dw ha^{-1} for the epigeal parts of *B. juncea* grown for 8−9 weeks in the field.[13,16,22] Considerably higher yields, around 10 t dw ha^{-1}, have been achieved,[63] and thus higher mortalities may be possible.

A close correlation was observed between the toxicity of brassica green manures to *G. pallida* and their isothiocyanate-producing glucosinolate content (Figure 6), implicating isothiocyanates as the main cause of toxicity. Both *B. vulgaris* and *M. moricandioides* lacked efficacy against *G. pallida* (Figure 4). These plants contained large quantities of indole glucosinolate or glucosinolates containing a hydroxyl group at the C-2 (Figure 5), neither of which form stable isothiocyanates.[64] In open soil, the concentration of isothiocyanate-liberating glucosinolate was the principal determinant of the nematicidal activity of brassica green manures (Figure 6, bottom panel). A reduction in the formation of new *G. rostochiensis* cysts was shown to be associated with the type and concentration of glucosinolates following treatment with plant extracts.[65] In covered soil, other factors may have been influential because there was a predicted baseline of around 60% mortality independent of glucosinolate content (Figure 6, Bottom). These findings are similar to those of Potter et al.,[66] who found a weak, nonsignificant correlation between leaf glucosinolate content and suppression of *Pratylenchus neglectus* ($R^2 = 0.48$, $P > 0.05$, $N = 6$) with an intercept at approximately 50%. In contrast, McLeod and Steele[67] found suppression of *M. javanica* to be unrelated to glucosinolate content, although in their study brassica leaf tissues were only coarsely chopped, rather than thoroughly pulverized, which would have impeded glucosinolate hydrolysis and thus isothiocyanate production.

B. juncea cv. Arid caused 60% mortality in covered soil, despite containing only 1.9 μmol g^{-1} dw 3-butenyl glucosinolate and 0.2 μmol g^{-1} dw 2-propenyl glucosinolate. *M. moricandioides* contained 4 times this concentration of 3-butenyl glucosinolate yet caused no significant mortality. This suggests that other products besides glucosinolate catabolites also contributed to the nematicidal activity of *B. juncea* in covered soil. As discussed earlier, decomposing brassica tissues can produce other volatile sulfur-containing toxins besides glucosinolate hydrolysis products. Emission of volatile sulfur-containing toxins from decomposing brassica tissues is favored under anaerobic conditions.[31,36] Such conditions can develop following incorporation of green manures into soil subsequently covered with plastic[11] and may have occurred in the present study due to the high moisture content of the soil in the covered containers. Despite attempts to control soil moisture content, the open soil dried between weekly watering events, whereas the covered soil remained wet at field capacity. When the coverings were removed, unpleasant odors characteristic of volatile sulfur compounds were observed, and it is possible that the greater efficacy of green manures in polyethylene-covered soil was due to the development of anaerobic conditions under which microbial activity generated volatile sulfur compounds. Trapping of isothiocyanates by the polyethylene is another potential explanation, but polyethylene is permeable to methyl isothiocyanate[68] and thus possibly also larger isothiocyanates. The higher soil water content of the covered soil would also help to retain the isothiocyanate via partitioning to the water phase and may increase chemical exposure.

The results of the present study show that brassica green manures have potential to contribute to the integrated management of *G. pallida*. The conditions employed in this experiment were designed to favor biofumigation; the brassica tissue was extremely thoroughly homogenized and incorporated at 5% (w/w) with excess water, the soil was a relatively light sandloam, and a covering of polyethylene was used. Further studies are required to quantify the effects of biofumigation on *G. pallida* under field conditions.

AUTHOR INFORMATION

Corresponding Author

*Phone: +44 113 343 2909. Fax: +44 113 343 2861. E-mail: P.E.Urwin@leeds.ac.uk.

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Text=Potato%20Cyst%20nematode%20control&SortString= Project Code&SortOrder=Asc&Paging=10#Description.

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