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Hatching of *Globodera pallida* is inhibited by 2-propenyl isothiocyanate *in vitro* but not by incorporation of *Brassica juncea* tissue in soil

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ABSTRACT

The aim of this study was to assess the feasibility of controlling the potato cyst nematode *Globodera pallida* through biofumigation with glucosinolate-rich *Brassica juncea* genotypes. The main glucosinolate of *B. juncea* is 2-propenyl glucosinolate which is the precursor of 2-propenyl isothiocyanate. Toxicity of 2-propenyl isothiocyanate to encysted *G. pallida* was tested *in vitro*. Fifty percent reduction in hatching was found within 2 h of exposure to 0.002% 2-propenyl isothiocyanate. Based on the *in vitro* results, we hypothesized that biofumigation with *B. juncea* would reduce hatching of *G. pallida in vivo* and higher 2-propenyl glucosinolate levels would have a stronger effect. Plant genotype, sulfur fertilization and insect herbivory affected 2-propenyl glucosinolate concentration of *B. juncea*. However, increasing 2-propenyl glucosinolate concentration of *B. juncea* did not affect *G. pallida* hatching after biofumigation. The absence of a biofumigation effect was most likely due to lower concentrations of 2-propenyl isothiocyanate *in vivo* compared to *in vitro* conditions. These results show that it is difficult to reach sufficiently high levels of toxicity to reduce hatching of *G. pallida* under realistic conditions.

1. Introduction

One of the major causes of yield reduction in potato cropping is the damage caused by potato cyst nematodes (*Globodera pallida* and *Globodera* rostochiensis) (Evans and Stone, 1977; Trudgill, 1986). Nematode infestation by *Globodera* spp. can reduce potato yield up to 80% (Singh et al., 2013). Juveniles of *Globodera* spp. invade potato roots, start feeding, and after the final moult produce eggs which remain inside their body. A female body swells and toughens, thereby forming a protective cyst. The juveniles inside the egg within the newly formed cyst are in diapause and hatch after exposure to specific root exudates. Even after many years in soil in the absence of host plants, cysts can contain viable eggs. Potato cyst nematodes can be controlled by crop rotation, application of nematicides, growth of resistant potato cultivars, or biofumigation (Been and Schomaker, 1999; Evans and Stone, 1977; Kerry et al., 2003; Lord et al., 2011).

Biofumigation is the incorporation of glucosinolate-rich plant material into the soil (Kirkegaard and Sarwar, 1998; Van Dam et al., 2009). Glucosinolates are sulfur-containing secondary metabolites of Brassicaceae that are involved in plant defence (Fahey et al., 2001). Upon tissue disruption, for example by insect herbivory, glucosinolates come into contact with the enzyme myrosinase and as a result toxic isothiocyanates are released (Ahuja et al., 2010). The release of isothiocyanate is the basis of biofumigation, although tissue disruption is achieved with machines rather than through herbivory (Morra and Kirkegaard, 2002).

The life cycles of potato crops and cyst nematodes are synchronized: second stage juveniles will only hatch from encysted eggs in the presence of root exudates of a suitable host. During all active life stages, potato cyst nematodes live in close contact with potato roots. Since biofumigation takes place either before or after the potato growth period, the majority of the nematode population will be present in the form of cysts (Evans and Stone, 1977).

Previous studies that evaluated biofumigation effects focus on exposure of juveniles, but not on encysted *Globodera* spp., to glucosinolates and their hydrolysed products (Buskov et al., 2002; Lazzeri et al, 1993; Serra et al., 2002; Warnke et al., 2008). The choice for juveniles instead of cysts seems mainly based on methodological considerations rather than representing the field situation (Motisi et al., 2010). Only a few studies have used encysted eggs for toxicity tests. The viability of encysted *G. pallida* juveniles was assessed by quantifying nematode actin-1 mRNA in a RT-qPCR assay (Lord et al., 2011). However, this molecular technique does not determine whether the unhatched juveniles would be able to hatch. Juveniles inside the cysts that cannot hatch are of little agronomical relevance (Kroese et al., 2011). Hatching

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was taken into account by Valdes et al. (2012); however, no significant effect was observed on encysted *G. rostochiensis* after biofumigation with *Sinapis alba*. Glucosinolate content of *S. alba* was not measured, which might have been too low to cause any effect on hatching of *G. rostochiensis* (Valdes et al., 2012).

Glucosinolate levels are determined by the interplay of many factors, including plant phenological stage (Rangkadilok et al., 2002), climatic factors (Josefsson and Appelqvist, 1968), insect herbivory and other forms of wounding (Bodnaryk, 1992), availability of nitrogen and sulfur (Gerandás et al., 2009) and plant genotype (Josefsson and Appelqvist, 1968; Kirkegaard and Sarwar, 1998). Contrasting findings have been reported: 2-propenylglucosinolate concentration increased in *Brassica juncea* after herbivory by larvae of the generalist *Spodoptera* spp (Mathur et al., 2011). However, a significant decrease was found in both the total glucosinolates and in 2-propenyl glucosinolate concentration after feeding by the specialist larvae of *Athalia rosae* (Müller and Sieling, 2006). Besides different insect species being used, Müller and Sieling (2006) used unfertilized soil, while Mathur et al. (2011) applied Hoagland solution in their experiment. This suggests that the direction of change for glucosinolate concentrations could be dependent on the insect species, but also on environmental conditions such as nutrient availability.

In this study, we tested the toxicity of 2-propenyl isothiocyanate on encysted *G. pallida in vitro*. 2-propenyl isothiocyanate is a hydrolysed product of 2-propenyl glucosinolate, the main glucosinolate of *B. juncea* (Sang et al., 1984). As far as we know, we are the first to report on hatching of encysted *G. pallida* after *in vitro* exposure, in aqueous and vapor phase, to 2-propenyl isothiocyanate. Furthermore, we studied *in vivo* the effects of sulfur fertilization and herbivory with *Pieris brassica* caterpillars on the 2-propenyl glucosinolate concentration of two *B. juncea* genotypes. *G. pallida* cysts were added to the pots before sowing, and after two months of growth *B. juncea* material was incorporated into its own soil (i.e., biofumigation). We hypothesized that increasing sulfur availability and controlled insect herbivory would increase the 2-propenyl glucosinolate concentration in *B. juncea*. Increased 2-propenyl glucosinolate concentration in the plant material was expected to reduce hatching of *G. pallida* through biofumigation. These experiments enabled us to assess whether the 2-propenyl glucosinolate concentration of *B. juncea* can be manipulated sufficiently to reduce hatching of encysted *G. pallida*.

2. Materials and methods

2.1. Cyst population and hatching assessment

The *Globodera pallida* population selected was Pa3 Rookmaker. Cysts were obtained from a lab population from Plant Research International (Wageningen, the Netherlands), and cysts were 2 years old. The Pa3 Rookmaker population is reported to be one of the most virulent populations in the Netherlands and is often used in research and breeding programs (Rouppe van der Voort et al., 1998).

The hatching agent used in this study consisted of root exudates of a mix of five (commercially available) potato cultivars grown in a pot with peat soil in a greenhouse. The pots were watered with excess water once per week, and the leachate was collected. The leachate was filtered through a coffee filter and stored at 7 °C. Before use, the leachate was filtered over consecutive sieves of 53, 20 and 5 μm to reduce microbial contamination.

Intact cysts were exposed to 2-propenyl isothiocyanate in the toxicity experiment and to compounds released through biofumigation of *B. juncea* (see below). After exposure, intact cysts were extracted and subsequently cleaned and soaked in water for 48 h. After that, cysts were carefully crushed in water using a plunger to liberate the individual eggs, the egg suspension was sieved (150 μm) to remove the cyst walls. The density of the egg suspension (eggs ml^{-1}) was determined by counting at least two independent subsamples. Egg suspensions were deposited on sieves (25 μm mesh) that were placed in glass cups containing 1.5 ml hatching agent (undiluted). Mesh size allowed the passage of active *G. pallida* juveniles, while retaining eggs on the sieve. The sieves were occasionally transferred to new glass cups with fresh hatching agent, to count the number of juveniles that had passed the sieve (Been and Schomaker, 2001). Hatching of the nematodes was determined after 7, 13, 20, 34, 55 and 105 days of exposure to the hatching agent in the *in vitro* experiment; and after 14 and 28 days in the *in vivo* experiment. Based on the *in vitro* results, there was no need for longer incubation of the cysts *in vivo*. Hatched juveniles were counted at increasing intervals, because hatching declined over time. The percentage of hatching was calculated by dividing the cumulative number of juveniles that had hatched from a single sieve by the estimated number of eggs. Both dead and living juveniles were counted as hatched. The number of eggs was calculated from the density of eggs in the suspension and the volume of egg suspension (ml) that had been applied to the sieve.

2.2. Toxicity experiment

Batches of approximately 200 *G. pallida* cysts (200 eggs per cyst) were prepared by weight (5.3 mg) and exposed to 2-propenyl isothiocyanate, either in solution, or in headspace. There were six replicates per combination of exposure time and exposure mode, and batches of cysts were assigned randomly. Exposure times to 2-propenyl isothiocyanate were 1, 2, 4, 8, 24 or 168 h, or 0 h in case of the control. Exposure mode was either by mixing cysts into solution (100 ml) in 500 ml bottles, or in headspace by placing the cysts inside a flat bottomed glass vial (14 ml) which was put inside the 500 ml bottle. We dissolved 0.2% 2-propenyl isothiocyanate (97% pure, Sigma-Aldrich), and 0.2% Tween-20 as emulsifier, in tap water as stock solution. Based on a pilot experiment, a concentration of 0.002% 2-propenyl isothiocyanate was used to conduct the toxicity experiment. From that pilot experiment, it appeared that the length of the soaking period of the cysts influenced hatching (data not shown). To standardize the time that cysts were dry (in headspace) or soaked (in solution), the cysts for all treatments were prepared simultaneously. Bottles of 500 ml were filled with 99 ml water, and batches of cysts were put either directly into the water, or into a glass vial which was carefully placed in the water. For the longest exposure time treatment (168 h), 1 ml stock solution was immediately added and gently mixed. The shorter exposure-time treatments were initially exposed to water (vapor) and the 2-propenyl isothiocyanate was added later. The deviation from the intended exposure time was less than 5 min and the cysts in the control were exposed to water only, in solution and in headspace, for the entire week. Cysts were removed from the bottles, washed to remove traces of 2-propenyl isothiocyanate, and dried, after which hatching was determined as described before.

2.3. Pot experiment

Sandy soil was collected from an arable field in the vicinity of Wageningen. The soil was selected for its low sulfate levels. Total sulfur was 150 mg kg^{-1} (near-infrared spectroscopy), mineral sulfur was 10 kg ha^{-1} (0–25 cm depth) and the organic matter content was 2.5%. Pots (diameter 20 cm; height 22 cm) were filled with 7 kg of field-moist soil (60% water holding capacity). Before filling the pots, about 400 cysts per pot were mixed through the soil for each pot.

We selected two *Brassica juncea* genotypes: ISCI 99 (labelled "ISCI" in this study, Research Institute for Industrial Crops in