Project title:	Novel approaches for the management of leaf and bud nematodes (*Aphelenchoides* species) in hardy nursery stock
Project number:	CP 104
Project leader:	Andy Evans, SRUC
Report:	Annual Report, September 2015
Previous report:	N/A
Key staff:	Idowu Rotifa
Location of project:	SRUC Edinburgh
Industry Representative:	Nick Reese, Jackdaws' Field Nursery, Horsham – West Sussex. RH13 6LL
Date project commenced:	14th October, 2013
Date project completed (or expected completion date):	30th September, 2016

[The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.]

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Idowu Rotifa

PhD Student

SRUC / University of Edinburgh

Signature ..... Date .....

Dr Andy Evans

Applied Practice Team Leader

SRUC Edinburgh

Signature ..... Date .....

Report authorised by:

[Name]

[Position]

[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

CONTENTS

GROWER SUMMARY

Headline

Three applications of HDC 71 significantly reduced nematode multiplication on Japanese anemone and weigela compared with a two application treatment and a control (no HDC 71 applied) treatment.

Background

Foliar nematodes, also called leaf and bud nematodes (LBN), *Aphelenchoides* species cause serious damage on many ornamental plants grown both outdoors and under protection throughout the United States, Canada, and Europe. They are a significant foliar pest of hardy nursery stock plants (over 700 host species have been recorded), whose feeding results in angular-shaped blotches on the leaves which are defined by the veins and often accompanied by leaf distortion. In the UK, *Aphelenchoides ritzemabosi* and *A. fragariae* are the two main foliar nematode species of economic importance.

The infestation usually starts at the base of the lower leaves where humidity is highest, and spreads upwards. LBN cause chlorotic lesions that can become necrotic. The lesions eventually turn blackish-brown and affected parts may shrivel. If buds or young leaves are infested, they may not develop properly and could become deformed. Flower development may also be affected. As ornamentals are sold for their aesthetic value, these plants are often unsaleable, making foliar nematode damage very costly for ornamental growers.

LBN problems have become important because of the withdrawal and subsequent loss of systemic nematicides, increased nursery production of vegetatively propagated plants, and the world-wide trade in plant material. A range of products for the control and management of LBN have been evaluated previously (as part of HNS 131, Horticultural Development Company), the results suggesting that Dynamec (abamectin) is ineffective against LBN and that Vydate 10G (oxamyl) was probably the most effective available product at the time.

Vydate 10G can be used on outdoor ornamental plants and also has an extension of authorisation for minor use (EAMU) on protected ornamental plants (*which expired on 30 June 2015*). However, oxamyl is not compatible with IPM programmes. Its use also requires precautions for operator and environmental protection, with a re-entry time to treated glasshouses and a harvest interval. In addition, its continued future availability is uncertain.

This project therefore aims to develop new approaches for the management of these nematodes in hardy nursery stock by evaluating individually, and in combination, the

efficacy of products derived from plant extracts and currently approved plant protection products to reduce nematode infestation in plants.

The project evaluated the application of products that act as elicitors of plant defences to determine whether they can confer levels of resistance to nematodes. Elicitors are natural and synthetic compounds that induce defence responses in plants triggered by the pathogen infection/pest infestation. These studies were carried out in the laboratory, glasshouse and are ongoing at grower's nurseries.

Summary

As a follow up to the laboratory bioassays experiment conducted earlier in the project, which identified several potential products for use in foliar nematode management studies, we developed a method of nematode inoculation of nematode-free plants. The reason for this was to develop a robust inoculation method which could then be used for screening potential control products. In addition, studies were carried out on two elicitor products coded as HDC 71 and HDC 72 on two plant species, namely weigela and Japanese anemone. The outcome led to a further study whereby HDC 71 alone was investigated within different application programmes.

Results show that leaf inoculation led to significant nematode invasion and subsequent multiplication in the leaf was about 80% higher than multiplication rates under normal growing conditions. Also, the preliminary glasshouse trial with different treatment programmes (carried out to investigate the effect of HDC 71 on *A. fragariae* multiplication in Japanese anemone), showed that HDC 71 significantly reduced the multiplication of the nematode population by up to 60% when compared with a control treatment (no HDC 71) and that a three application programme was better than two. The quality and saleability of plants can therefore be extended by reducing the development of visual symptoms on the plant. However, it is expected that HDC 71 would not be used as a sole treatment but in combination with other treatments.

The promising products previously identified in laboratory bioassays and the elicitor (HDC 71) are currently undergoing preliminary field trials at two grower nurseries. Susceptible host plants being investigated include: *Astrantia*, *Bergenia*, *Brunnera macrophylla*, *Buddleja*, *Cistus*, *Dryopteris affinis*, *Gunnera mannicata* and Japanese anemone.

Products are being tested individually and in combination as an integrated management approach to assess their efficacy under commercial field conditions.

Financial Benefits

Although an accurate estimate of financial benefit cannot be given yet, more than half of the plants treated with HDC 71 were seen to be of commercially viable quality compared with the untreated. Discussions with growers suggest that they could save between £2,500 – £15,000 per annum depending upon the plants grown and size of the nursery, despite the additional cost of HDC 71, if used.

Action Points

Cultural control methods are an important component of the management of LBN within integrated pest management (IPM) programmes. The most effective of these methods is the adoption of high levels of crop hygiene, as foliar nematodes can survive for several years in infested dried leaf debris.

Control programmes should include:

- the removal and destruction of infested plants and debris
- avoidance of replanting in contaminated land
- sterilisation of pots and equipment prior to re-use
- if possible, minimising the use of overhead irrigation and misting systems which create ideal conditions for nematode infection
- use of oxamyl where permitted and appropriate.

SCIENCE SECTION

Introduction

Foliar nematodes (*Aphelenchoides* spp.), also called leaf and bud nematodes (LBN), are microscopic roundworms that live in leaf tissue and cause significant injury to many ornamental plants (Winslow, 1960). They are a significant foliar pest of hardy nursery stock plants (over 700 host species) whose feeding results in angular-shaped blotches on the leaves which are delineated by the veins and often accompanied by leaf distortion (Kohl, 2010 *et al*; Kohl, 2011). In the UK, *Aphelenchoides ritzemabosi* and *A. fragariae* are the two main foliar nematode species of economic importance.

In the past, chemical treatments such as aldicarb, diazinon, parathion and oxamyl have been used for effective management of foliar nematodes (Johnson & Grill, 1975). However, due to regulatory issues and environmental concerns, most of these chemicals are no longer available to growers today. Modern chemical control methods have variable results, depending on the plant being treated (Bennison, 2007; Young, 2000). Chemical treatments tested may be successful at killing nematodes in a water suspension, but then fail to control nematodes when applied to infected leaves (Jagdale & Grewal, 2002; Jagdale & Grewal, 2004). Also, there has been general concept that plants can actively defend themselves or have resistance induced against virulent pathogens including nematode (Walters *et al*. 2007).

After the initial bioassay test on some promising products, experiments were conducted to: (1) investigate the efficacy of direct inoculation method on both Weigela and Japanese anemone in a glasshouse test; (2) assess the effect of two elicitor products HDC 71 and HDC 72 for their potential to induce plant defences, thereby conferring levels of resistance to multiplication of foliar nematode (*A. fragariae*) on Weigela and Japanese anemone plants in glasshouses; and (3) study the application of the elicitor – HDC 71 – with three treatment programmes to manage foliar nematode (*A. fragariae*) on Japanese anemone in a glasshouse test.

Materials and methods

Experiment 1: To investigate the efficacy of the direct inoculation method of Aphelenchoides fragariae on leaves of Weigela and Japanese anemone plants in glasshouse.

The objective of this experiment was to evaluate the efficacy of directly inoculating *A. fragariae* on leaves as a suitable standard technique for use in further tests.

1.1 Materials

Nematodes: The nematodes (*Aphelenchoides* spp.) were isolated from infected evergreen fern (*Woodwardia fimbrata*). *A. fragariae* was identified by morphology features and confirmed by molecular identification through the use of PCR techniques and sequencing to confirm the nematode species. The leaves were cut into 1 cm² pieces and soaked in tap water for 24 hours at room temperature. The nematodes that emerged from the leaf pieces were recovered using nested sieves of 20 mesh (850 µm) & 500 mesh (25 µm), and collected in a beaker. The suspension was left for 2 hours, and excess water was reduced on top of the suspension. Nematode concentration was adjusted to the required concentration of mixed stage individuals per millilitre by counting with a light microscope. The nematodes were used within 2-3 days for laboratory experiments.

Plants: Nematode free plants (Weigela and Japanese anemone) were used in this test. The plants were grown in a glasshouse in individual two litre pots until they had at least six leaves. Six plants were used for each species.

1.2 *Method*

According to the method described by Zhen *et al*, (2012), three randomly selected leaves per plant were used for this study. Leaves were injured by making 10 perforations with a sharp needle scattered between veins at the upper side of the leaf. Leaves were wrapped with wet tissue paper (Kimpwipes, 11 by 21 cm; Kimberly–Clark). An aliquot (3 ml) suspension containing 200 live nematodes was carefully dispensed on the wet tissue paper. The plants were covered with black plastic bags after inoculation in order to maintain moist conditions. The bags and tissue paper were removed after 72 hours. All plants were completely randomised and kept in a glasshouse condition of 25 ± 2°C. Nematode multiplication was observed at 3, 5 and 8 weeks after inoculation when leaves were extracted using the extraction method outlined above in section 1.1 to assess nematode multiplication.

Data analysis: Nematode reproduction values from this test were subjected to an analysis of variance (ANOVA) using a General Linear Models Procedure (Minitab 15). Significant differences between treatments were determined with Tukey's multiple range test at $P<0.05$. Variables used were duration (weeks) versus nematode counts.

Experiment 2: Investigate two elicitor products HDC 71 and HDC 72 for their potential to induce plant defences against the multiplication of foliar nematode (A. fragariae) on Weigela and Japanese anemone plants under glasshouse conditions.

An experiment was conducted to investigate the efficacy of two elicitor products namely (HDC 71) and (HDC 72) on inducing plant defences against the foliar nematode *A. fragariae*. It was hypothesised that the elicitor products could trigger plant defences and increase plant resistance levels against the multiplication of *A*. *fragariae*.

2.1 Materials

Nematodes: The nematode species (*A. fragariae*) used in this test was extracted as described in section 1.1 above.

Elicitors: HDC 71 and HDC 72 were supplied by Syngenta Agrochemical Company, UK.

Plants: Nematode-free plants (Weigela and Japanese anemone) were used as previously described in experiment 1 above. Fifteen plants were used for each species.

2.2 *Method*

Glasshouse trials were conducted to evaluate the efficacy of two elicitor products on foliar nematode. The trials were carried out in 2 litre pots containing Weigela and Japanese anemone plants. The three treatments were: (i) HDC 71 + nematode (ii) HDC 72 + nematode, and (iii) Nematode only (Control). All of the treatments were arranged in a randomised design with five replicates per treatment. Based on manufacturer's instructions, both elicitor products were dissolved in 1 litre of water. An adjuvant called Tween was added at rate of 100 µl per 100 ml of water to both elicitor and control treatments. Elicitor products were sprayed as a foliar application on plants until run off. Control treatments had Tween added into water (100 µl per 100 ml of water) and sprayed on plants. Plants were left for 48 h before three randomly selected leaves per plant were inoculated with nematodes as described in the previous section (1.2). Each leaf was inoculated with 200 live nematodes. The plants were left in the glasshouse condition for 8 weeks. Leaf sampling for nematode reproduction was carried out at 3, 5 and 8 weeks after inoculation (see section 1.1 for methodology).

Data analysis was carried out using arcsine-transformed values of percentage nematode reproduction and subjected to analysis of variance (ANOVA) using a General Linear Models Procedure (Minitab 15). Significant differences between treatments were determined with Tukey's multiple range test at $P<0.05$. Variables considered include treatments, time (week) and nematode count.

Experiment 3: Application of the elicitor HDC 71 with different treatment programmes to manage foliar nematode (A. fragariae) on Japanese anemone in a glasshouse test

3.1 Materials

Plants and nematodes used were from the same source as described from the 2 previous experiments (Sections 1 and 2).

3.2 Methods

This experiment had five treatments, with each replicated five times. Plants were arranged in a randomised design. The two factors considered were nematode and HDC 71. Treatments (Trt) were:

- Trt 1 = HDC 71 (+ nematode) at week 1 (x 1 application)
- Trt 2 = HDC 71 (+ nematode) at week 1 and 3 (x 2 applications)
- Trt 3 = HDC 71 (+ nematode) at week 1 and 5 (x 2 applications)
- Trt 4 = HDC 71 (+ nematode) at week 1, 3 and 5 (x 3 applications)
- Trt 5 = Nematode only - Control

Treatments with HDC 71 and control had additions of Tween (adjuvant) in a water solution (100 µl per 100 ml of water). All treatments were applied as a foliar spray on plants till run off. Two days after the first HDC 71 application in Week 1, 200 live nematodes were inoculated directly onto three randomly selected leaves per plant (as described in Experiment 1, Section 1.1). Plant maintenance and sampling were the same as described in section 1.2.

Data analysis: as in the previous experiments, arcsine-transformed values of percentage nematode reproduction from this study were subjected to analysis of variance (ANOVA) using a General Linear Models Procedure (Minitab 15). Significant differences between treatments were determined with Tukey's multiple range test at $P<0.05$.

Results

Experiment 1

Figure 1 shows a 270% increase in nematode multiplication on Japanese anemone at week 3, which increased to 995% at week 8. There was a significant difference between week 3 and week 5, and between week 3 and 8. There was no statistical difference in nematode reproduction between week 5 and week 8 at $P<0.05$, despite the highest multiplication percentage value (1034%) being observed at week 5.

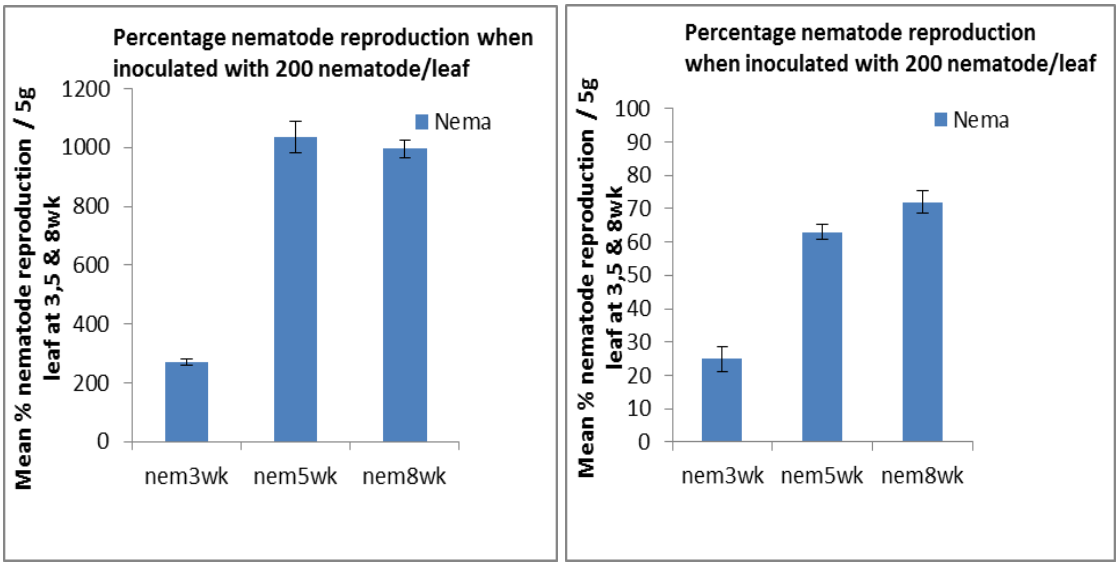

Figure 1 & 2. Results of the mean percentage nematode multiplication (± SEM) over 8 weeks when inoculated with 200 nematodes / leaf of Japanese anemone (figure 1) and Weigela (figure 2) plants. Nem3wk = nematode reproduction at 3 weeks, nem5wk = 5 weeks, nem8wk = 8 weeks.

Similar results were noticed on Weigela plants with multiplication increasing from week 3 to week 8 (Figure 2). The highest percentage multiplication (72%) was recorded at week 8 followed by 63% at week 5, and the lowest (25%) at week 3. Reproduction was significantly different ($P<0.05$) between weeks 3, 5 and 8 respectively. There was an increase in multiplication over time, which was seen for both plant species throughout the study. This confirmed that direct inoculation of nematodes onto leaves leads to considerable nematode multiplication for both plant species over time.

Experiment 2

HDC 72 had a highest mean percentage reproduction rate (2706%) at week 3 on Japanese anemone (Fig. 4), but dropped to the lowest value (343.5%) at week 5 between the three treatments (Table 1.). HDC 71 had a small increase in nematode multiplication on Japanese anemone (Fig. 4) and had the lowest overall nematode population (324%) compared with HDC 72 (343.5%) during the study. The control treatment was statistically higher (P<0.05) in nematode reproduction when compared with both HDC 71 and HDC 72. The control was also observed to have a steady percentage increase in nematode multiplication throughout the duration of the study in both plant species. Although the control had a low reproduction rate on Weigela, ranging from 25%, 63.4% and 72.1% at week 3, 5 and 8 respectively (Fig. 3), the highest values of 540.5%, 1992% and 2082% were obtained on the control treatment during the same duration on Japanese anemone (Fig. 4).

Table 1. Test of two elicitor products on the reproduction of *A. fragariae* in a glasshouse when inoculated with 200 nematodes / leaf on Weigela and Japanese anemone. Data are percentage mean values of nematode reproduction of five replicates when inoculated with 200 nematodes / leaf (N = nematodes)

		Mean Nematode Reproduction (%)	
		at 3, 5 & 8 week after inoculation	
Duration	Treatment	Weigela	Anemone
3 week	HDC 72+N	7.5	2706
	HDC 71 +N	24.8	324
	N	25.2	540.5
5 week	HDC 72+N	78.1	343.5
	HDC 71 +N	57.6	550
	N	63.4	2082
8 week	HDC 72+N	59.3	876
	HDC 71+N	30.8	751
	N	72.1	1992.5

Experiment 3

Figure 5 shows that three application doses of HDC 71 significantly reduced nematode multiplication (treatment 4, P <0.05), compared with other treatments, especially the control. Counts obtained at week 5 from all HDC 71 treatments showed a significantly lower nematode population during the study compared with the control (P <0.05); treatment 4 had

the least mean value (49) at week 5, while treatment 1 had the highest mean value (591) at week 8 among other HDC 71 treatments. The highest nematode population mean (2580.3) during the study was recorded in the control treatment at week 8

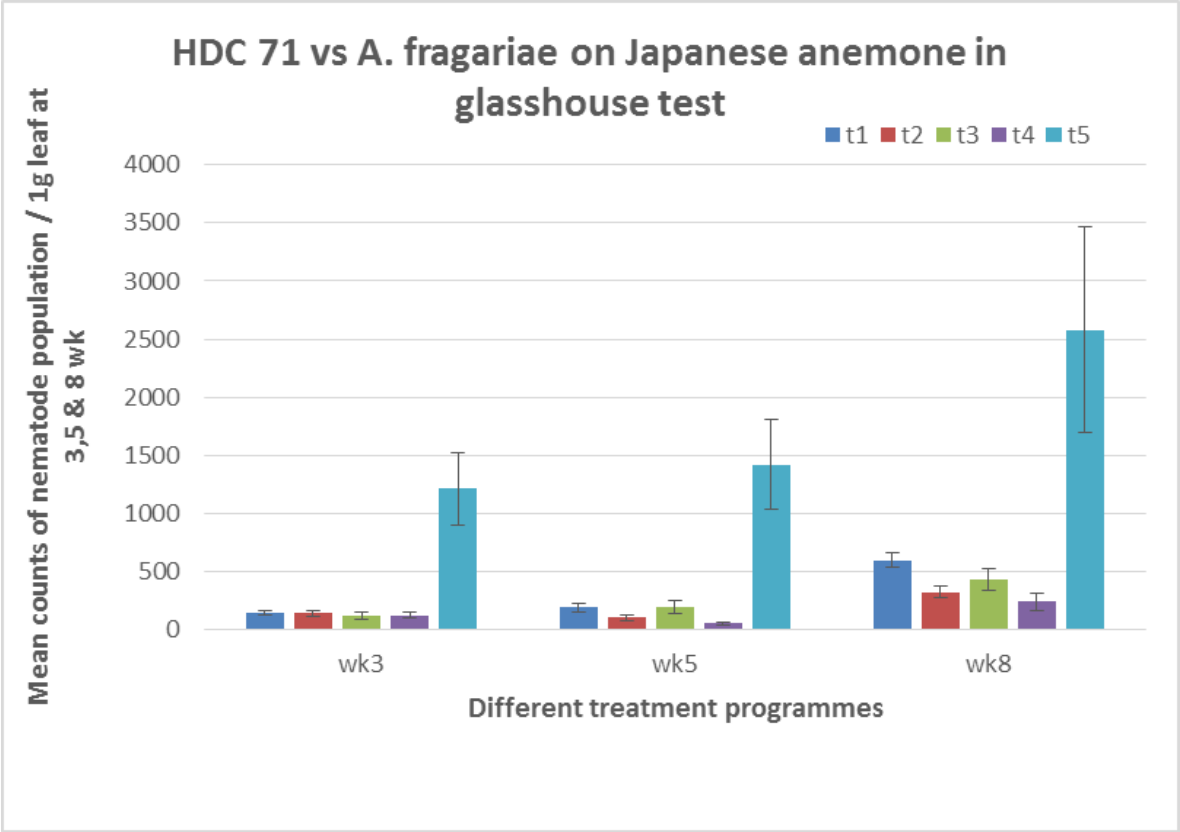

Figure 5. Mean counts of nematode population after an initial inoculation of 200 nematodes per leaf, on Japanese anemone at 3, 5 and 8 weeks with differing HDC 71 application programmes: t1 = HDC71 (+ nematode) at week 1; t2 = HDC71 (+ nematode) at week 1 and 3; t3 = HDC71 (+ nematode) at week 1 and 5; t4 = HDC71 (+ nematode) at week 1, 3 and 5; t5 = Nematode only. Error bars are standard error of the mean (±SE) of five replicates. Values are means of 5 replicates per treatment.

Discussion

Experiment 1 showed that nematodes multiplied progressively with time on both plant species, with the highest nematode reproduction obtained on Japanese anemone. The results also confirmed that the direct inoculation method is effective, and suitable to be used in further experiments. Similar results were reported by Zhen *et al* (2012), on the direct inoculation method of nematodes when used for nematode resistance screening on hosta cultivars. Although, there could be some challenges in term of leaf penetration by nematodes during the direct inoculation process, those nematodes that successfully penetrated the leaf stomata will multiply as they feed on the cell contents of host leaves. If

the direct inoculation method is applied on other plant species, we should bear in mind that the level of nematode multiplication will differ between plant species, as seen here with Japanese anemone and Weigela.

Experiment 2 followed on from this by evaluating the effect of two elicitor products against nematode multiplication on both Weigela and Japanese anemone plants when inoculated with 200 nematodes per leaf. In this experiment, the results were similar to Experiment 1, with lower nematode multiplication on Weigela plants against the nematode population recorded on Japanese anemone. Effect of low multiplication on Weigela or other plants may be due to the texture of leaves which could affect penetration of nematodes into leaf stomata according to Kohl *et al*. (2010). The leaves of both plant species show symptoms when foliar nematodes are present. Although there are no previous reports on the use of HDC 71 to control *A*. *fragariae*, past research on the use of elicitors confirm that as induced resistance is a host response, disease / pest control will be affected by many factors, including abiotic environment, host cultivar and the extent to which plant resistance in the field are already induced (Walters and Fountaine 2009; Reglinski *et al*. 2007). HDC 71 appears more reliable in this study in term of nematode reproduction management than HDC 72.

During Experiment 3, HDC 71 was investigated as a promising product to be used as an individual treatment, and potentially in combination with other products as alternatives to Vydate 10G for nematode management. With the results highlighted in all HDC 71 treatments (t1 – t4) during this study, HDC 71 demonstrated high potential for significant reduction of nematode population when compared with the control treatment. Previous research with elicitors has shown the successful use of BABA (beta-amino-butyric acid), which is known to induce resistance against pathogens in various systems, including tomato, potato, grapevines, and pea (Cohen *et al*., 1999; Jakab *et al*., 2001). In a glasshouse experiment, the use of the elicitor HDC 71 to control *Rhynchosporium commune* on spring barley gave 70% reduction in infection (Walters *et al*. 2014). Molinari and Baser (2010) reported that three doses of HDC 71 significantly decreased root–knot nematode reproduction on tomato by up to 74%. The HDC 71 treatment could therefore aid in management of nematode reproduction on Japanese anemone as well as other hardy nursery stock plants. Overall management of foliar nematodes in herbaceous and perennial ornamentals in nurseries is difficult since most nematicides are ineffective, especially when populations are high (Lamondia, 1999 & Warfield et al., 2003). The use of an elicitor along with other pesticide products in both the field and grower's nurseries could be effective control against foliar nematodes.

Conclusions

The findings from the experiments so far demonstrate a reliable and robust method of nematode inoculation on plants, which could be used during product bioassays. It was also found that applying elicitor products to Weigela and Japanese anemone plants in the glasshouse can significantly reduce nematode multiplication.

Knowledge and Technology Transfer

Description	***Date***
Presentation: 2014 at Advances in Nematology Annual Conference, London	16/12/2014
Presentation of Managing Leaf & Bud Nematode at HDC- HPTDG Technical Meeting, London	11/2/2015
Poster Presentation at Postgraduate Conference at SRUC, Edinburgh	19-20/3/15
Presentation at Agri-Science Young Researchers Conference by Syngenta Agrochemical Company, Bracknell UK	8/7/2015
Poster at AHDB - Horticulture Annual Studentships Conference `	16-17/9/2015
Presentation at Society of Chemistry & Industry (SCI), David Miller Awards and Horticulture Group AGM, University of Reading, UK	18/9/2015

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Appendices

Appendix 1 - Future work and milestones

Future Work

1. The potential products from the laboratory bioassays (±HDC 71) are currently undergoing preliminary tests at two nurseries. This initial curative test is to determine if the products have any effect on already infected plants by foliar nematode (*A*. *fragariae*) under a field conditions. This on-going test involves products such as HDC 71 as a single product and in combination with other products. The treatments currently used on grower's trials include (a) Movento (b) Dynamec (c) HDC 71 (d) Movento + HDC 71 (e) Dynamec + HDC 71 and (f) Control (Water). These treatments are being tested on such as *Gunnera mannicata*, *Cistus*, *Bergenia*, *Brunnera macrophylla*, *Dryopteris affinis*, Japanese anemone and *Budlleja* plants.

The initial nematode population was determined on the nematode infected plants before application of treatments. The final nematode population will be analysed after the duration of the trials.

2. The next study is to evaluate the efficacy of products from the contact bioassay experiment and in combination with HDC 71 on Japanese anemone in the glasshouse. This study will investigate efficacy of the products using foliar and soil treatments. This is to test

products' ability in preventing leaf nematode infection from soil, and also to manage the multiplication of nematodes on already infected plants.

- Soil treatment: the study will use Japanese anemone and the proposed treatments would include water (control), Vydate 10G (industry standard), HDCI 068, HDCI 070, HDCI 084 and HDCI 088. Nematodes would be applied to the soil first, and the products would be applied 24 h later. Nematodes will be extracted from soil and leaves using the Baermann funnel method. Nematode population in the soil and leaves will be assessed prior to treatment and at 8 weeks after treatment.

- As in the above study, products are to be tested in preventative and curative control methods.

  - ✓ *Preventative:* Systemic products from the contact bioassay test which include Movento, Dynamec, HDCI 069 and Water (control) are to be used. This study would investigate the preventative efficacy of these products against nematode multiplication on plants. Products will be applied as foliar sprays, while direct nematode inoculation on leaves will be carried out two days later.

  - ✓ *Curative efficacy:* This will involve inoculation of plant with nematodes on leaves, and after 7 days, the products will be applied as foliar spray on plants. This is to investigate if products could prevent multiplication and further infestation of nematode on leaves after nematodes may have penetrated into the stomata of leaves. Nematode population in the leaves will be assessed at 3, 5 and 8 weeks after products' treatment.

3. The treatment list would be refined, and the range of plants expanded and evaluated in second phase of grower's trials.

Milestones

Project Milestones from September 2015 onwards

Milestones	***Months / Year***
Poster Presentation: HDC Studentship Conference	September 2015
Conclude initial phase of products test on two grower's nurseries	October 2015
Presentation at AAB Nematology meeting in London	December 2015
Glasshouse trials to identify potential treatments for next field trials with growers	Feb 2016
Develop rating key for leaf nematode damage on Weigela and Japanese anemone	May 2016
Refine treatments and expand range of plants to be evaluated in 2nd grower trials	June 2016
Complete grower's trials	August 2016
Produce refined guidelines for leaf nematode management for hardy nursery stock	September 2016
Submit PhD thesis	October 2016