Project title:	Managing ornamental plants sustainably (MOPS)
Project number:	CP 124
Work package title:	Development of a rapid molecular diagnostic technique for the detection of leaf and bud nematodes
Work package leader:	Jude Bennison
Report:	Annual report, February 2016
Previous report:	None
Key staff:	Jude Bennison, ADAS Gemma Hough, ADAS (until October 2015) Ben Maddison, ADAS
Location of work:	ADAS Boxworth
Date work commenced:	1 May 2015
Date work completed (or expected completion date):	31 December 2015

AHDB, operating through its HDC division seeks to ensure that the information contained within this document is accurate at the time of printing. No warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

Copyright, Agriculture and Horticulture Development Board 2016. All rights reserved.

No part of this publication may be reproduced in any material form (including by photocopy or storage in any medium by electronic means) or any copy or adaptation stored, published or distributed (by physical, electronic or other means) without the prior permission in writing of the Agriculture and Horticulture Development Board, other than by reproduction in an unmodified form for the sole purpose of use as an information resource when the Agriculture and Horticulture Development Board or HDC is clearly acknowledged as the source, or in accordance with the provisions of the Copyright, Designs and Patents Act 1988. All rights reserved.

AHDB (logo) is a registered trademark of the Agriculture and Horticulture Development Board.

HDC is a registered trademark of the Agriculture and Horticulture Development Board, for use by its HDC division.

All other trademarks, logos and brand names contained in this publication are the trademarks of their respective holders. No rights are granted without the prior written permission of the relevant owners.

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.

Jude Bennison
Entomologist
ADAS UK Ltd

Signature ..   Date 15 February 2016

Report authorised by:

John Atwood
Project Leader
ADAS UK Ltd

Signature   Date 15 February 2016

© Agriculture and Horticulture Development Board 2016. All rights reserved

Contents

Growers Summary **1**
Headline 1
Background and expected deliverables 1
Summary of the work and main conclusions 1
Conclusions and action points 3

Science Section **4**
Introduction 4
Materials and methods 4
Primer design 4
Sourcing nematodes 5
DNA extraction 5
Setting up the reactions 6
Primer design 6
Testing the primer design for detection of *A. fragariae* 6
Testing the sensitivity of LAMP 7
Results and Discussion 7
LAMP profile 7
Testing the sensitivity of LAMP 9
Conclusions 9
Acknowledgements 10
References 10

© Agriculture and Horticulture Development Board 2016. All rights reserved

GROWERS SUMMARY

Headline

- A rapid molecular method was developed to detect DNA from *Aphelenchoides fragariae* extracted from infested leaves. The method could be further developed to offer growers a diagnostic service for confirming leaf and bud nematodes in plant material.

Background and expected deliverables

Leaf and bud nematodes, *Aphelenchoides* spp. are common, persistent and damaging pests of a range of economically important HNS plants. In the UK, both *A. ritzemabosi* and *A. fragariae* can occur. Feeding damage results in angular-shaped dark patches on the leaves, delineated by leaf veins and leaf distortion also often occurs. These damage symptoms can make infested plants unmarketable. However, growers are not always confident in recognising the damage symptoms, particularly in the early stages of infestation and on some host plants. The damage symptoms can also be confused with those of downy mildew on some host plants. There are now very few nematologists in the UK who are able to confirm *Aphelenchoides* spp. by microscopy. A novel reliable method for confirming infestation with leaf and bud nematodes would give growers the confidence to plan integrated control methods for this pest.

In AHDB Horticulture-funded project FV 415, ADAS worked with a Dutch company to validate a molecular PCR (Polymerase Chain Reaction) method for detection of stem nematode (*Ditylenchus dipsaci*) in soil in order to predict the risk of damage to onions and leeks. The method was effective at detecting even a single stem nematode in nematode species mixes extracted from soil and offers a rapid and reliable potential commercial test as an alternative to the current UK diagnostic service which relies on microscopic examination by a restricted number of skilled nematologists.

The work in this project aimed to develop a similar molecular method for reliable detection of leaf and bud nematodes that could potentially be offered as a diagnostic tool for UK growers.

© Agriculture and Horticulture Development Board 2016. All rights reserved

Materials and Methods

Target DNA in *Aphelenchoides fragariae* and *A. ritzemabosi* was identified on the National Center for Biotechnology Information database. Significantly more published DNA sequences were available for *A. fragariae* than for *A. ritzemabosi* therefore this species was chosen to develop the molecular diagnostic technique. DNA sequences for *A. fragariae* were designed using the Primer Explorer website and the primers were purchased from a commercial supplier.

Leaves infested with *A. fragariae* were obtained from Scotland's Rural College (SRUC) and *A. fragariae* were extracted using a standard laboratory technique. DNA was extracted from both *A. fragariae* and the commercially available entomopathogenic nematode *Steinernema carpocapsae* using two methods; a blood and tissue kit method which produced pure DNA and a crude heat extraction method which can produce DNA together with inhibitory components.

A Genie II (GEN-02) instrument was used to perform the DNA LAMP (Loop-mediated isothermal amplification) analysis. Initially the instrument was used to test whether the selected primers identified DNA from *A. fragariae*. Replicate samples of pooled DNA from 20 extracted *A. fragariae* and from 20 pooled *S. carpocapsae* and water controls were tested. The instrument was then used to test the sensitivity of the method i.e. whether DNA from a single *A. fragariae* could be detected. In this test, replicate samples of pooled DNA from 20 *A. fragariae* or 20 *S. carpocapsae* extracted using the blood and tissue kit method, DNA from one or four *A. fragariae* using the crude heat extraction method and water controls were tested.

Results

The initial test confirmed that the selected primers were specific for *A. fragariae* as only the pooled DNA from *A. fragariae* was amplified by the instrument (Figure 1).

The sensitivity test showed that the instrument detected DNA from *A. fragariae* from either one or four nematodes and once again did not detect DNA from *S. carpocapsae*. However, 45 minutes after the test had started, the water control also gave a positive reading, suggesting that contamination had occurred during preparing the samples for testing.

© Agriculture and Horticulture Development Board 2016. All rights reserved

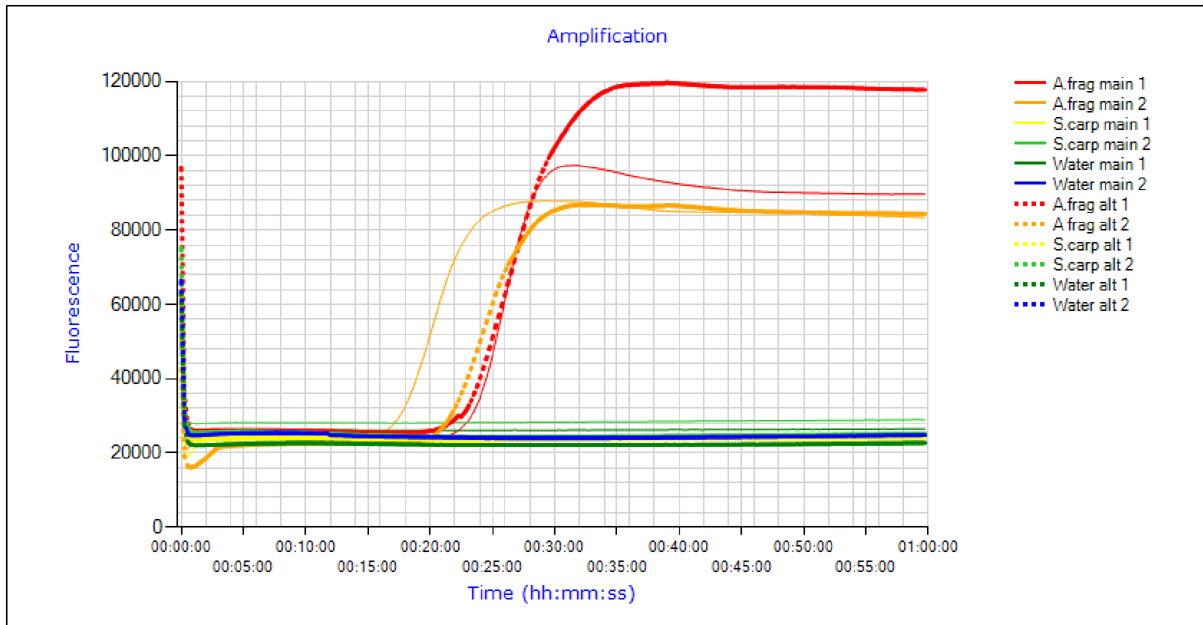


Figure 1. Amplification results from the LAMP anlaysis to confirm that the primers identified DNA from *Aphelenchoides fragariae* (increases and peaks of fluorescence in red and orange lines) but not from *Steinernema carpocapsae* (no increases in fluorescence in pale yellow and pale green lines) or from the water controls (blue and dark green lines).

Conclusions and action points

- This work showed that it is possible to detect DNA from one or more extracted *Aphelenchoides fragariae* using a rapid molecular diagnostic technique.
- Further work would be needed to design DNA primers to use the technique for detection of *A. ritzemabosi*, another commonly occurring leaf and bud nematode species.
- Further work would be needed to develop a technique for using the same instrument to detect DNA from leaf and bud nematodes more rapidly from crushed leaf material rather than from nematodes extracted in the laboratory.
- If further work was completed, the test could be made commercially available to growers
- At present growers should ask their crop protection consultant to confirm the presence of leaf and bud nematodes by sending leaves with symptoms to a laboratory offering a nematode identification service using the traditional extraction technique followed by microscopic examination.

© Agriculture and Horticulture Development Board 2016. All rights reserved

SCIENCE SECTION

Introduction

Leaf and bud nematodes, *Aphelenchoides* spp. are common, persistent and damaging pests of a range of economically important HNS plants. In the UK, both *A. ritzemabosi* and *A. fragariae* can occur. Feeding damage results in angular-shaped dark patches on the leaves, delineated by leaf veins and leaf distortion also often occurs. These damage symptoms can make infested plants unmarketable. However, growers are not always confident in recognising the damage symptoms, particularly in the early stages of infestation and on some host plants. The damage symptoms can also be confused with those of downy mildew on some host plants. There are now very few nematologists in the UK who are able to confirm *Aphelenchoides* spp. by microscopy. A novel reliable method for confirming infestation with leaf and bud nematodes will give growers the confidence to plan integrated control methods for this pest.

In AHDB Horticulture-funded project FV 415, ADAS worked with a Dutch company to validate a molecular PCR (Polymerase Chain Reaction) method for detection of stem nematode (*Ditylenchus dipsaci*) in soil in order to predict the risk of damage to onions and leeks (Ellis, 2015). The method was effective at detecting even a single stem nematode in nematode species mixes extracted from soil and offers a potential rapid and reliable commercial test as an alternative to the current UK diagnostic service which relies on microscopic examination by a restricted number of skilled nematologists.

The work in this project aimed to develop a molecular method for reliable detection of leaf and bud nematodes that could potentially be offered as a diagnostic tool for UK growers.

Materials and methods

Primer design

Target DNA in *Aphelenchoides fragariae* and *A. ritzemabosi* was identified by searching the National Center for Biotechnology Information (NCBI) database using the organism's name, 'mitochondrial' or '18sRNA' as search terms. These searches identified published sequences available for these two species.

Sequence alignments (a method of arranging DNA sequences to identify regions of similarity) were performed on the sequences found for *A. fragariae* and *A. ritzemabosi* to confirm the regions we were likely to target were different between the two species, so that any primers designed using these sequences would be species specific. A multiple sequence alignment program called 'Clustal' was used to perform sequence alignments.

© Agriculture and Horticulture Development Board 2016. All rights reserved

Significantly more published sequences were available for *A. fragariae* than for *A. ritzemabosi* and therefore this species was chosen to develop the LAMP (Loop-mediated isothermal amplification) technique for initially due to the higher confidence in its published sequences. The sequences most readily available in the database were those around the genes coding for 18S ribosomal RNA (which is a component of the small ribosomal subunit). Sequences from this region are often used as they are highly conserved among species.

The NCBI database also identified the presence of different sequences representing different isolates of *A. fragariae.* As a result, further sequence alignments were performed to make sure that the target sequences chosen would work and reflect the diversity of all the *A. fragariae* isolates.

Once the target DNA sequence was identified, Primer Explorer (https://primerexplorer.jp/e/) was used to design the primers. Primers were ordered from Sigma Aldrich. Ben Maddison in the ADAS Biotechnology group at the University of Nottingham was responsible for the primer design.

Sourcing nematodes

Live *A. fragariae* and *A. ritzimabosi* nematodes were obtained from infested leaf material. Scotland's Rural College (SRUC) provided leaves infested with leaf and bud nematodes which were from pure cultures of both *A. fragariae* and *A. ritzimabosi.*

Infested leaves were collected, cut up into small pieces (1cm x 1cm) and aerated (using a fish tank pump and air stone) in 1 litre of water for 48 hours. After 48 hours the water containing the leaves and nematodes was washed through a coarse sieve into a 45µm sieve. The nematodes were collected on the 45µm sieve and washed into a Doncaster dish (nematode counting dish). Using a mounted needle with an eyelash attached individual nematodes could be picked up and transferred.

DNA extraction

DNA was extracted from the nematodes using two methods:

1) DNA extraction using Qiagen DNeasy blood and tissue kit

DNA was initially extracted from *A. fragariae* and the commercially available entomopathogenic nematode *Steniernema carpocapsae* using the Qiagen DNeasy Blood and Tissue kit. The standardised extraction methodology provided with the kit was used. This method ensured that DNA was extracted and purified from the nematodes so that no inhibitory compounds would affect the reactions.

© Agriculture and Horticulture Development Board 2016. All rights reserved

2) Crude DNA extraction using heat

A crude DNA extraction method was developed which required heating the nematodes at 95°C for five minutes prior to running the LAMP programme. This process ruptures the nematodes causing them to release their DNA. Unlike the Qiagen method the DNA is not purified and therefore can contain inhibitory components.

To heat the nematodes, single nematodes were transferred (using an eyelash attached to a mounted needle) into an Eppendorf tube containing 2µl of nuclease free water. A microscope was used to confirm that the nematode was in the water in the tube. The live nematode in the 2µl of nuclease free water was then added to the reaction mixture using a pipette instead of 2µl of extracted DNA which is usually added. When the reactions were prepared the Genie II instrument was run at 95°C for five minutes to denature the nematodes then the LAMP programme was run.

Setting up the reactions

A Genie II (GEN-02) instrument was used to perform LAMP method. This instrument can run up to 16 reactions at a time.

Each reaction performed contained (25µl total volume)*:*

- 15 µl of Isothermal master mix (containing dye) obtained from OptiGene
- 2µl of F3 primer (at 5pmol concentration)
- 2µl of B3 primer (at 5pmol concentration)
- 2µl of BIP primer (at 20pmol concentration)
- 2µl of FIP primer (at 20pmol concentration)
- 2µl of extracted DNA/ nuclease free water containing a live nematode

Primer design

Two sets of primers were designed to test for *A. fragariae*. These were called 'A.frag Main' and 'A.frag Alt' (alternative). Each primer set identified a different region of DNA sequence around the genes coding for 18S ribosomal RNA.

Testing the primer design for detection of A. fragariae

On 21 August 2015 a LAMP was performed to test that the primers developed for *A. fragariae* detected this species. 'A.frag Main' and 'A.frag Alt' primers were tested against:

1. Pooled DNA (from four tubes each containing 20 nematodes) of *A. fragariae* extracted via the Qiagen method

© Agriculture and Horticulture Development Board 2016. All rights reserved

2. Pooled DNA (from two tubes each containing 20 nematodes) of *S. carpocapsae* extracted via the Qiagen method
3. Nuclease free water (negative control)

Two replicates of each were tested.

Testing the sensitivity of LAMP

On 16 October 2015 a LAMP was performed to test the sensitivity of the method i.e. whether it could detect a single *A. fragariae*. The ‘A.frag Main’ primers were tested against:

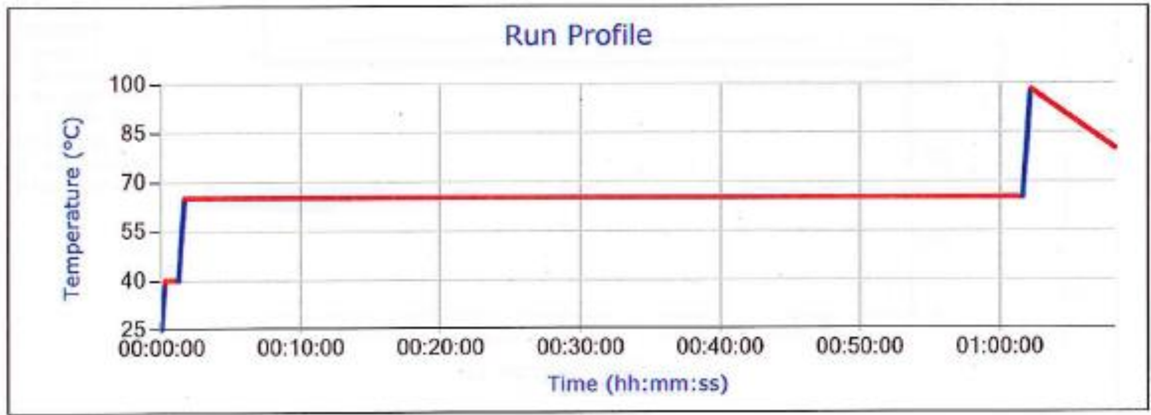
1. Nuclease free water
2. Pooled DNA (from four tubes each containing 20 nematodes) of *A. fragariae* extracted via the Qiagen method
3. DNA from one *A. fragariae* nematode extracted crudely via heating
4. DNA from one *A. fragariae* nematode extracted crudely via heating
5. DNA from four *A. fragariae* nematodes extracted crudely via heating
6. Pooled DNA (from two tubes each containing 20 nematodes) of *S. carpocapsae* extracted via the Qiagen method

Results and Discussion

LAMP profile

The profile of the isothermal amplification method used is shown in Figure 1. Initially the Isothermal period was run for 60 minutes but this was later reduced to 30 minutes as the amplification results were displayed within this time.

© Agriculture and Horticulture Development Board 2016. All rights reserved



Preheat	Yes
Temperature	40 °C
Time	1 min(s)

Isothermal	Yes
Temperature	65 °C
Time	60 min
Gradient	0 °C

Anneal	Yes
Start Temp	98 °C
End Temp	80 °C
Ramp Rate	0.05 °C/sec

Figure 1 LAMP run profile

Testing the primer design for detection of A. fragariae

Figure 2 shows the results from the LAMP. Only the pooled DNA from *A. fragariae* was amplified by the 'A.frag Main' and 'A.frag Alt' primers (i.e. the primers identified a complementary sequence) shown by the increase and peaks in fluorescence. This confirmed that the primers were specific for *A. fragariae* although a more closely related species also needs to be tested i.e. *A. ritzimabosi.*

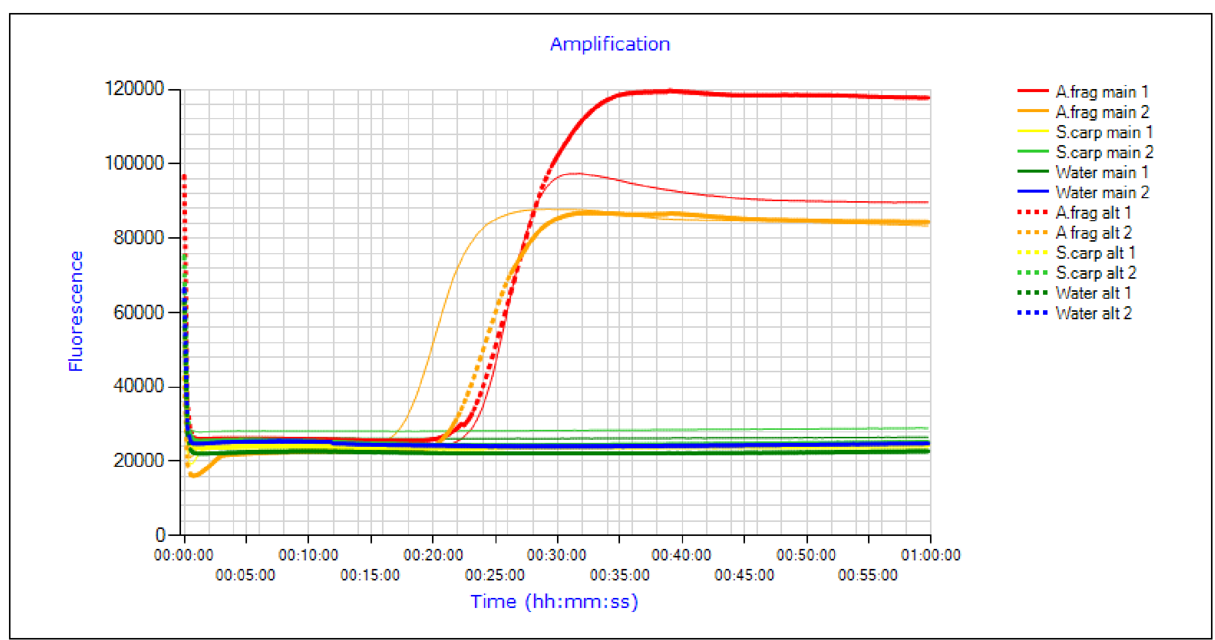


Figure 2. Amplification results from the LAMP performed to confirm the primers work on 21 August 2015.

© Agriculture and Horticulture Development Board 2016. All rights reserved

Testing the sensitivity of LAMP

Figure 3 shows the results from the LAMP. While the primers successfully amplified complementary DNA in the reactions containing one and four *A. fragariae* nematodes (i.e. the primers identified a complementary sequence), the nuclease free water also gave a positive reading suggesting that contamination had occurred during set up.

The positive amplification of the nuclease free water control did not occur until around 45 minutes into the run suggesting that only a small amount of *A. fragariae* DNA was detected. The amplification of the reaction containing four *A. fragariae* nematodes also did not occur until around 40 minutes which is likely due to the addition of inhibitory components to the reaction as a result of the crude DNA extraction method. As each individual nematode was added further inhibitory components would have been added.

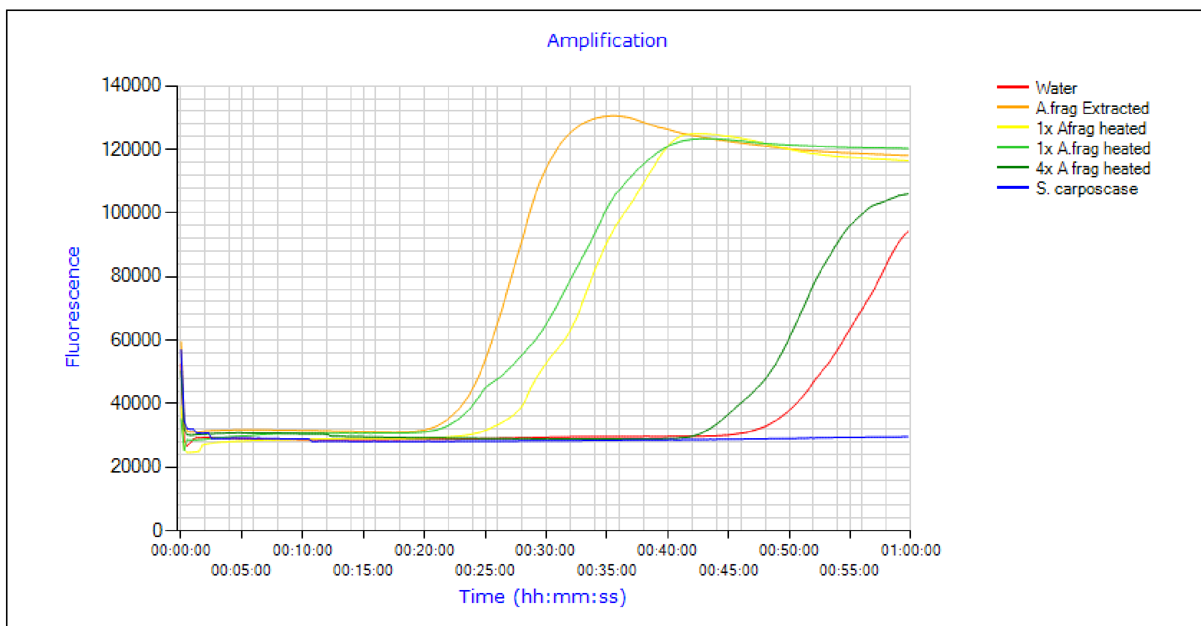


Figure 3. Amplification results from the LAMP performed to test the sensitivity of the method on 16 October 2015

Conclusions

- This work showed that it is possible to detect DNA from one or more extracted *Aphelenchoides fragariae* using a rapid molecular diagnostic technique.
- Further work would be needed to design DNA primers to use the technique for detection of *A. ritzemabosi*, another commonly occurring leaf and bud nematode species.

© Agriculture and Horticulture Development Board 2016. All rights reserved

- Further work would be needed to develop a technique for using the same instrument to detect DNA from leaf and bud nematodes more rapidly from crushed leaf material rather than from nematodes extracted in the laboratory.
- The test could be made commercially available to growers after further work as suggested above.
- At present if growers wish to confirm the presence of leaf and bud nematodes in plants they are reliant on sending leaves with symptoms to one of the few laboratories offering a nematode identification service using the traditional extraction technique followed by microscopic examination.

Acknowledgements

Thanks to:

- Idowu Rotifa from Scotland's Rural College (SRUC) for providing *A. fragariae*.
- BASF for supplying *S. carpocapsae*.

References

Ellis, S. (2015). Molecular methods for detection of stem nematode (*Ditylenchus dipsaci*) in soil and predicting risk of damage to onions and leeks. Final report to AHDB Horticulture on project FV 415.

© Agriculture and Horticulture Development Board 2016. All rights reserved