Horticultural Fellowship Awards

Interim Report Form

Project title:	Maintaining and developing capability in vegetable crop pathology
Project number:	CP113
Project leader:	Dr John Clarkson, Warwick Crop Centre, University of Warwick
Report:	Annual Report, October 2014 (Year 1)
Previous report:	n/a
Fellowship staff:	Dr John Clarkson
("Trainees")	Dr Andrew Taylor
Location of project:	Warwick Crop Centre
Date project commenced:	1st November 2013
Date project completed (or expected completion date):	30th October 2018

DISCLAIMER

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.

Andrew Taylor
HDC Fellow
Warwick Crop Centre, University of Warwick

Signature:  Date: 12/11/2014

John Clarkson
Principal Research Fellow
Warwick Crop Centre, University of Warwick

Signature:  Date: 12/11/2014

Report authorised by:
Rosemary Collier
Director, Warwick Crop Centre
Warwick Crop Centre, University of Warwick

Signature:  Date: 12/11/2014

CONTENTS

Progress Against Objectives

Objectives

1) To train the Fellow as a plant pathologist with a focus on molecular techniques and diagnostics through direct participation in research relevant to the industry.

2) To develop pathogen collections of molecularly characterised isolates with known virulence to enhance future capability for plant pathology research.

3) To train the Fellow as a plant pathologist with a broad knowledge of different pathosystems and associated plant pathology techniques.

4) To synthesise existing unpublished work/data generated by Dr Dez Barbara.

5) To give the Fellow experience of grower practice and disease problems through industry meetings, visits and contacts.

6) To mentor the Fellow in becoming an independent research leader and establish a role for him as a leading molecular plant pathologist in UK horticulture.

Objective / Milestone	**Original Completion Date**	**Actual Completion Date**	**Revised Completion Date**
1.1 Determine pathogenicity of a range of *F. oxysporum* (FOC) isolates from onion and complete DNA sequencing of a range of housekeeping genes.	31/10/15		
1.2 Extract DNA, prepare libraries and carry out whole genome sequencing of FOC isolates	31/10/17		
1.3 Bioinformatic analyses of FOC isolate genomes and identification of potential primers for FOC diagnostics	31/10/17		
1.4 Test FOC diagnostic primers in vitro.	31/10/18		
1.5 Test FOC diagnostic primers using soil and bulb samples.	31/10/18		

1.6 Test published PCR diagnostic for *Sclerotium cepivorum*	31/10/16		
1.7 Check existing *Pythium violae* specific primers using contemporary isolates / soil samples from carrot fields	31/10/14	31/10/14	
1.8 Develop qPCR for *P. violae* using WCC Roche Lightcycler	31/10/14	31/10/14	
1.9 Quantify *P. violae* in soil samples from HDC project FV405 and other samples where available.	31/10/15		
1.10 Identify potential primers for *Itersonilia* diagnostics from existing gene sequences (or whole genome sequence).	31/10/16		
1.11 Test *Itersonilia* primers in vitro.	31/10/17		
1.12 Test *Itersonilia* primers using soil and diseased parsnip roots.	31/10/17		
1.13 Extract DNA, prepare libraries and carry out whole genome sequencing of *Sclerotinia* spp. isolates including *S. subarctica*.	31/10/17		
1.14 Bioinformatic analyses of *Sclerotinia* spp. genomes.	31/10/18		
2.1 Collect new isolates of *Sclerotium cepivorum*, *Peronospora destructor* (onion downy mildew), *Botrytis squamosa* (botrytis leaf blight) and *Botrytis allii* (neck rot of onion)	31/10/15		
2.2 Confirm identity and characterise isolates from 2.1 by gene sequencing	31/10/16		
2.3 Develop appropriate plant infection tests and confirm pathogenicity of isolates from 2.1	31/10/17		

2.4 Confirm identity and characterise parsnip canker pathogen isolates as obtained through HDC project FV366 or BBSRC PhD project using gene sequencing	31/10/17		
3.1. Gain experience with lettuce pathogens such as *B. cinerea* and *B. lactucae* through a work programme to be developed with Katherine Denby and Eric Holub.	31/10/18		
3.2. Gain experience with brassica pathogens such as Turnip Mosaic Virus, A. candida *H. brassicae* and *X. campestris* through a work programme to be developed with Eric Holub and John Walsh.	31/10/18		
3.3. Gain experience of other pathogens such as *P. ultimum*, *O. neolycopersici* through existing projects (John Clarkson)	31/10/17		
4.1. Synthesise Dez Barbara's unpublished work on carrot/parsnip viruses.	31/10/15		
4.2. Analyse and publish virus sequences collected by Dez Barbara from carrots and wild umbellifers from a previous Defra project.	31/10/17		
5.1. Attend relevant research project meetings.	Ongoing		
5.2. Present a poster at an industry meeting or event.	31/10/16		
5.3. Give a talk at an industry meeting or event.	31/10/17		

5.4. Work-shadowing of at least one industry collaborator. 6.1. Contribute to writing at least one research proposal 6.2. Initiate at least two research proposals and obtain funding for one.	31/10/17 31/10/17 31/10/18		

Summary of Progress

Milestones 1.7 and 1.8: Existing published primers for detection of *P. violae* by PCR (Klemsdal et al 2008) were tested using contemporary *P. violae* isolates and soil samples from carrots. Although efficient amplification and detection of *P. violae* was achieved, these primers were subsequently found to be unsuitable for use in quantitative (q)PCR due to the formation of primer dimers. This means that detection was not possible for low concentrations of *P. violae* in the soil. In addition, the same primers were tested for their specificity to *P. violae* and found to amplify *P. sylvaticum, P. intermedium* and *P. irregulare*. A second set of published primers (Wang and White 1996) were tested and found to be suitable for qPCR. However, they were not *P. violae* specific and amplified a larger range of *Pythium* species. New primers for *P. violae* detection were therefore designed and found to be very efficient for qPCR, allowing detection of the pathogen at very low levels. However there was some weak amplification of *P. sylvaticum*, *P. intermedium* and *P. irregulare* after a high number of PCR cycles. With minor modifications to cycling conditions and/or primers, these new primers should be suitable for qPCR detection of *P. violae*.

Milestones 2.1 and 2.3: Isolates of *Peronospora destructor* were collected from four different field sites and a successful infection method for onion was developed.

Milestones not being reached

N/A

Do remaining milestones look realistic?

Yes

Training undertaken

- Attended the UK Carrot and Onion Conference (20th-21st Nov 2013) in Peterborough)
- Attended the BSPP Presidential (18th Dec 2013) in Birmingham
- Attended the 'Establishing yourself: Productivity and People' course at Warwick University. This course is designed for early career who want to progress to the research leader level (28th Feb 2014)
- Visited the 'clean lab' at the University of Warwick main site and gained experience of extracting DNA from ancient material. An infected leaf sample from 1927 was obtained from Kew and attempts are ongoing to extract the fungal DNA (20th Feb 2014)
- Visited Richard Harrison at East Malling Research from 11th – 14th March 2014. This included a 3 day bioinformatics training course (An Introduction to Bioinformatics 11th – 13th) which covered how to go from raw genome sequence data to an assembled genome and genome analysis. Many aspects of script writing were covered.
- Attended the HDC/BCGA Carrot Technical Seminar at PGRO in Peterborough (20th March 2014).
- Attended the Plant Genomics Congress in London (12-13th May 2014)
- Gained experience of using a confocal microscope to view onion roots after infection with Fusarium. Roots were stained with florescent dyes and infections structures observed with the help of Ruth Schafer (Warwick Life Sciences, September 2014).
- Attended a training session with Joana Vicente (University of Warwick) on H. brassicae- how to collect and store spores and how to infect Brassica plants (10th Sept 2014)
- Worked closely with John Clarkson to develop methods for inoculating onion plants with downy mildew (ongoing)

Expertise gained by trainees

- Isolation and maintainance of downy mildew on onion
- Developed technical skills for *P. violae* detection including a greater understanding of qPCR

- Identification of downy mildew symptoms on onion / rocket / basil / brassicas, Fusarium wilt on peas and cavity spot on carrot
- Use of confocal microscope
- Extraction of DNA from ancient material
- Knowledge of the principles of genome sequencing and assembly
- Use of genetic software such as Geneious to extract useful information from whole genome sequences

Other achievements in the last year not originally in the objectives

A paper titled 'Identification of resistance to a highly aggressive isolate of *Sclerotinia sclerotiorum* in a *Brassica napus* diversity set' based on previous work was written and accepted for publication in Plant Pathology.

Changes to Project

Are the current objectives still appropriate for the Fellowship?

Indicate any changes to the ordinal objectives that you would like to make and provide any information that you can to support this decision.

No changes at this time

If, during the course of the year, the Fellowship has undertaken research that has generated information that would be of interest to the industry please ensure that a Grower Summary and Science Section is completed for each relevant piece of work. Please provide a subtitle and number to indicate separate sections.

GROWER SUMMARY

Headline

A newly developed quantitative PCR assay for detecting *P. violae* in the field will be a valuable tool for understanding of the dynamics of the pathogen in the field.

Background

Cavity spot disease of carrots (caused predominantly by *Pythium violae*) is the most damaging disease for UK carrot growers. Infection leads to small, sunken, elliptical lesions and eventually the skin ruptures to form an open cavity (Hiltunen and White 2002). Currently, the only control option is the use of metalaxyl as a drench normally applied 6 weeks after drilling. The epidemiology and dynamics of *P. violae* is poorly understood, mainly because the pathogen is difficult to isolate from the soil. Moreover, whilst *P. violae* is present in newly formed closed lesions, once these are open and exposed to the soil, secondary infections occur which can reduce the likelihood of isolating the pathogen. A rapid and specific qPCR assay for detecting the presence of *P. violae* and the other species potentially involved in cavity spot would be a valuable tool for studies on pathogen dynamics. The aim of this part of the Fellowship was therefore to evaluate existing PCR tests and develop this approach further.

Summary

A newly developed qPCR assay for detecting *P. violae* in the field has been developed but requires some final optimisation. The assay could then be used to quantify levels of *P. violae* DNA in the soil and on carrots and enable studies on the dynamics of this pathogen. The technique could also be potentially used to assess the efficacy of control methods.

Financial Benefits

None at this time

Action Points

None at this time.

SCIENCE SECTION

During the first year of the Fellowship, research work was mainly undertaken on the detection and quantification of *Pythium violae* causing cavity spot of carrot. However, some initial work was also carried out on onion downy mildew.

Introduction

Cavity spot of carrots

Cavity spot disease of carrots (caused predominantly by *Pythium violae*) is the most damaging disease for UK carrot growers. Infection leads to small, sunken, elliptical lesions and eventually the skin ruptures to form an open cavity (Hiltunen and White 2002). Currently, the only control option is the use of metalaxyl as a drench normally applied 6 weeks after drilling. The epidemiology and dynamics of *P. violae* is poorly understood, mainly because the pathogen is difficult to isolate from the soil. Moreover, whilst *P. violae* is present in newly formed closed lesions, once these are open and exposed to the soil, secondary infections occur which can reduce the likelihood of isolating the pathogen. A rapid and specific quantitative (q)PCR assay for detecting the presence of *P. violae* and the other species potentially involved in cavity spot would be a valuable tool for studies on pathogen dynamics.

Specific primers for PCR detection of *P. violae* have been published previously (Wang and White, 1996; Klemsdal et al 2008) and those of Klemsdal et al., (2008) further developed for qPCR by Dez Barbara at Warwick. However, some technical problems with this test were outlined in subsequent work (HDC Project CP46). The research in this part of the Fellowship was aimed at evaluating the existing PCR tests and improving them further.

Onion downy mildew

Onion downy mildew (*Peronospora destructor*) is a worldwide disease that causes severe damage to onion plants in cool moist conditions (Scholten et al 2007). Infection leads to pale patches forming on the leaves which develop into purple lesions (Brewster, 2008). This is followed by sporulation, seen as a grey furry mass on the leaves, and leaf death. Once infection has occurred, further damage can only be minimised by fungicides. *P. destructor* is listed as a high research priority by the British Onion Producers Research and Development Committee. The aim of this part of the Fellowship was to collect and store isolates of *P. destructor* and develop inoculation procedures as a resource for future work.

Materials and methods

Cavity spot

Multiple techniques were used to develop a reliable qPCR assay for *P. violae*. Firstly, the published primers PviolF and PViolr (Klemsdal et al 2008) were testing using a dilution series of *P. violae* DNA (isolate 26B, isolated from Cottage field, Wellesbourne) ranging from 6.69ng/µl to 6.69fg/µl. Several primer concentrations (0.05-0.2µM and annealing temperatures (60-69°C) were tested. All samples were run in triplicate and each 10µl reaction mixture contained 2µl water, 5µl SYBR Green ® (Roche), 1µl forward primer (1/2/3µM), 1µl reverse primer (1/2/3µM) and 1µl DNA. Water was used as a negative control. The samples were then loaded in to a LightCycler® 480 Real-Time PCR system (Roche). Cycle conditions consisted of pre-incubation at 95°C for 5 minutes. Followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing for 10 seconds and extension at 72°C for 10 seconds. Following this, a melt curve was produced under the following conditions: 95°C for 5 minutes, 65°C for 1 minute and 97°C.

A second approach used a molecular beacon probe with the following structure: 5' FAM CCGAGCCGCAGTGGATGTCTAGGCTCGCACCGCTCGG BHQ 3'. A molecular beacon is a highly specific and sensitive probe which functions based on florescence resonance energy transfer (reviewed by Li et al 2008). This probe was used with the primers PviolF and PviolR. The probe was diluted to a concentration of 1µM using TE buffer and primers were diluted to the same concentration using water. The same DNA dilution series was used. This was done with both 0.1 and 0.2 µM concentrations of primer. The total reaction volume of 10µl contained 1µl PviolF (1µM), 1µl PviolR (1µM), 5µl RedTaq® ReadyMix™ , 1 or 2µl of probe (1µM), 1 or 0µl of water and 1µl DNA. Cycle conditions consisted of pre-incubation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds and extension at 72°C for 10 seconds. A melt curve was produced under the following conditions: 95°C for 5 minutes, 65°C for 1 minute and 97°C.

Another set of primers used to carry out qPCR was the *P. violae* specific primer pair ITS2 and PV1 (Wang and White 1996). The primer concentrations used were 0.1µM and 0.2µM. The previously described DNA dilution series was used. Volumes of reaction mixture components were the same as for the Pviol primers. The samples were then loaded in to the LightCycler® as previously described. Cycle conditions consisted of 40 cycles of denaturation at 95°C, annealing at 55°C and extension at 72°C. All other conditions were the same as those used for the molecular beacon qPCR.

Finally, a new set of primers were designed manually based on the ITS region (PViol NEWF and PViol NEWR) following alignments of all available *Pythium* ITS sequences using MEGA

software. Three primer concentrations were tested, 0.1µM, 0.2µM and 0.3µM. A dilution series of *P. violae* DNA was made as previously described. Each 10µl reaction mixture contained 2µl water, 5µl SYBR Green ®, 1µl NEWF (1/2/3µM), 1µl NEWR (1/2/3µM) and 1µl DNA. The samples were then loaded in to the LightCycler® as before. Cycle conditions consisted of 45 cycles of denaturation at 95°C, annealing at 67°C and extension at 72°C. All other conditions were the same as those used for the molecular beacon qPCR.

The specificity of all primer pairs was also tested using conventional PCR with pure DNA of *P. lutarium*, *P. ultimum*, *P. sylvaticum*, *P. irregulare* and *P. intermedium*. These PCRs used REDTaq® ReadyMix ™ and each reaction mix contained 10µl of REDTaq, 1µM of each primer, 1µl of DNA and 7µl of water. After initial denaturation at 94°C for 5 minutes, cycle conditions consisted of 40 cycles of 94°C denaturation for 1 minute, annealing for 1 minute, extension at 72°C for 30 seconds and final extension of 72°C for 10 minutes. PCR products were then visualised by gel electrophoresis on a 1-2% agarose gel. Initial PCR's were carried out with a limited set of *Pythium* species and these were repeated with the full range of species.

Downy mildew

Infected onions were obtained from 4 different field sites: Rugby (2 different fields), Luddington and Wellesbourne. Plants were transplanted and placed in an 'infection tent' within a cooled (20°C) glasshouse to maintain 100% humidity and induce sporulation. A humidifier was placed in the tent and set to run for 30 minutes at the start of the dark period and 30 minutes halfway through the dark period. Following one overnight period, spores were collected using two different methods. The first method was to remove the spores from the leaves using a vacuum pump and store them at -20°C. The second was simply taking leaf sections, placing in tubes and storing at -20 or -80°C. Spores taken by the vacuum method were used to re-infect healthy plants. A spore suspension was made using water and 0.01% tween then corrected to 3×10^4 spores/ml using a haemocytometer after which it was sprayed onto onion plants until run off. Leaf wetness was then maintained (using the humidifier on constant) for 24 hours to allow infection after which plants were removed from the tent and observed for symptoms after 7-14 days. Plants with possible symptoms were placed back in the tent and sporulation induced as previously described.

Results

Cavity Spot

The PViol primers (Klemsdal et al 2008) were efficient at high DNA concentrations but at lower concentrations there was a problem with primer dimers (primers binding to each other and/or themselves). This is illustrated in Figure 1 which shows *P. violae* DNA at low concentrations (green) and some DNA extracted from soil on the outside of carrot roots (red). These primers were therefore not suitable for qPCR.

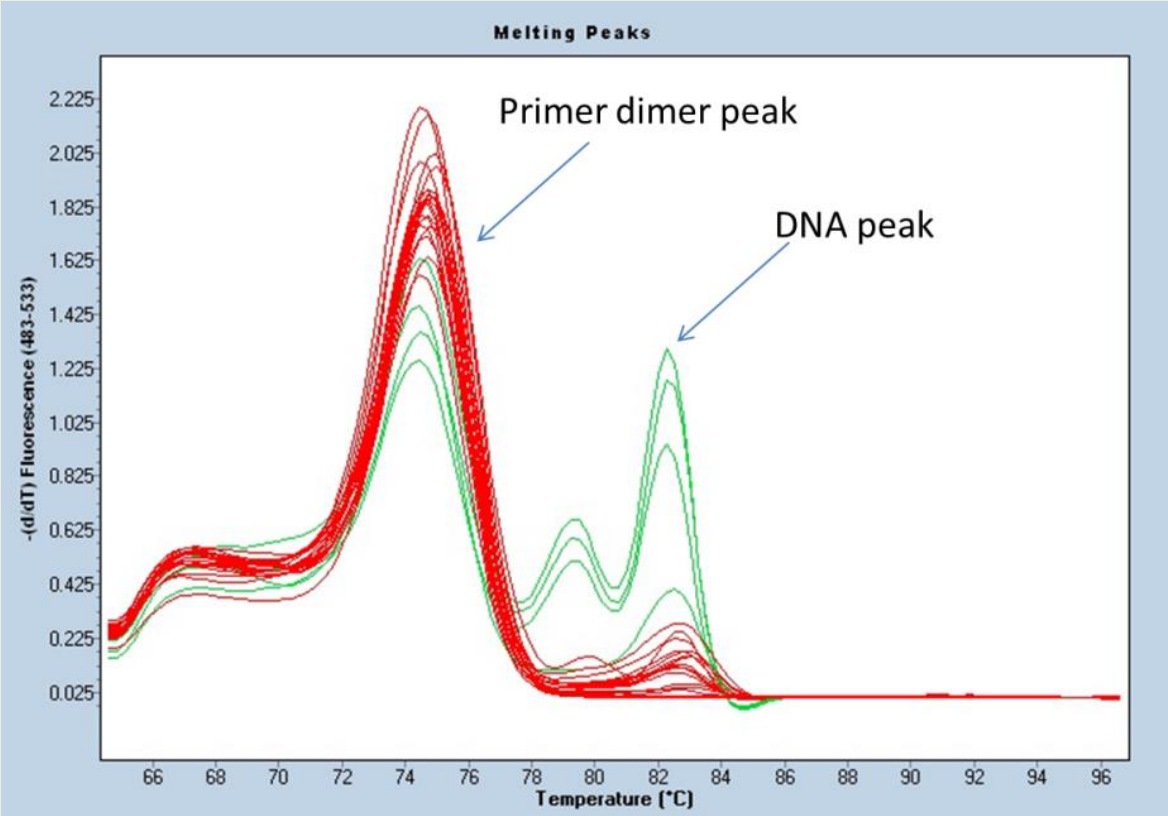
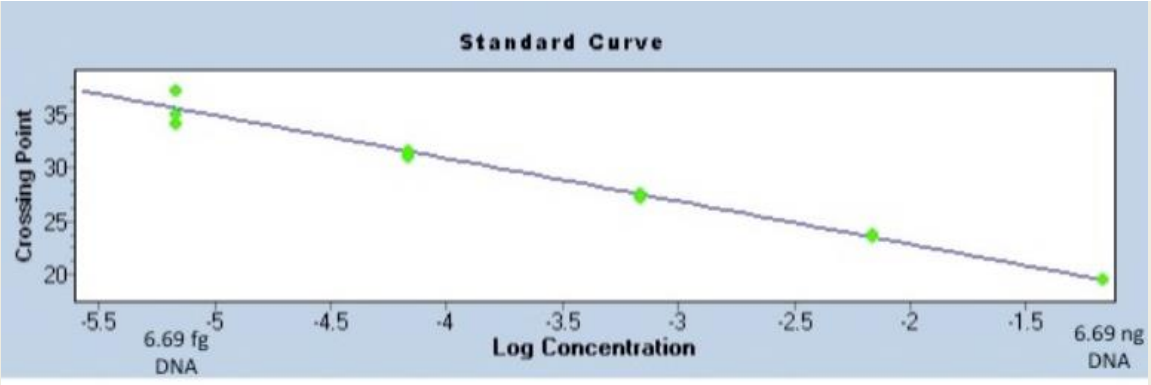


Figure 1. Melting peaks of PCR products using PViol primers.

The primers PV1 and ITS2 (Wang and White, 1996) allowed detection of *P. violae* DNA at concentrations as low as 6.69fg/µl (Figure 2a) without any problems with primer dimers (Figure 2b).

(a)



(b)

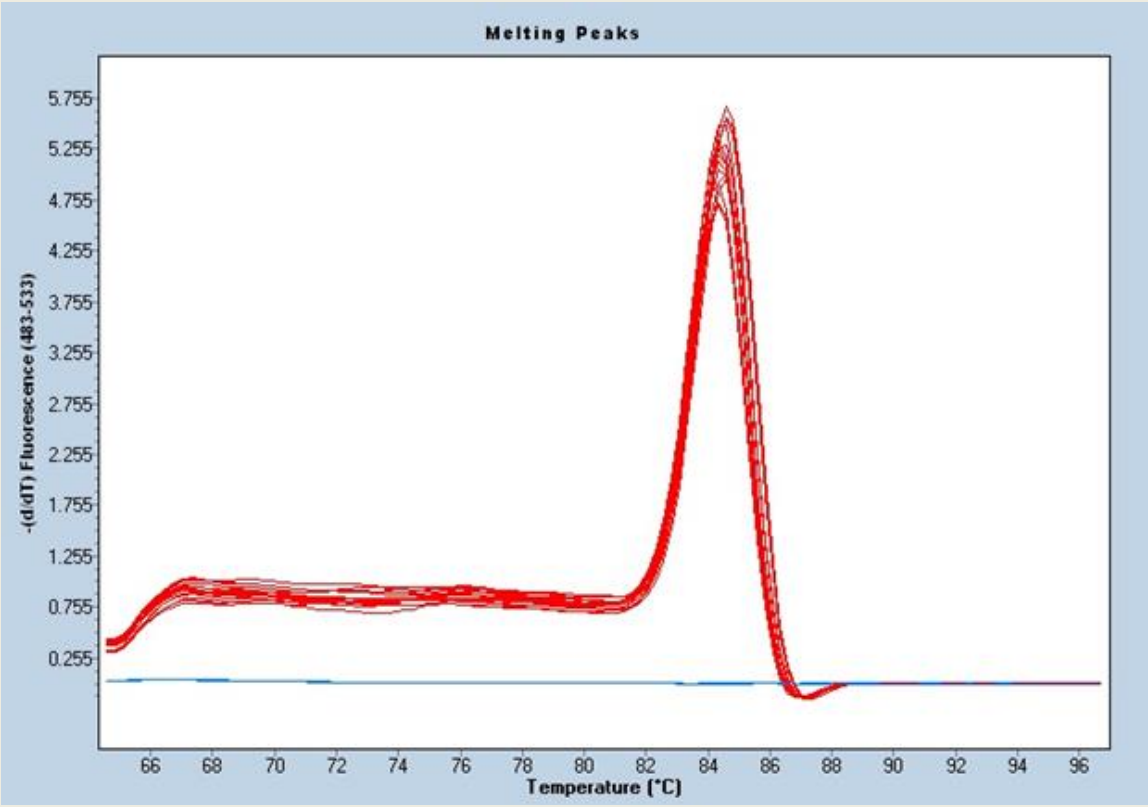
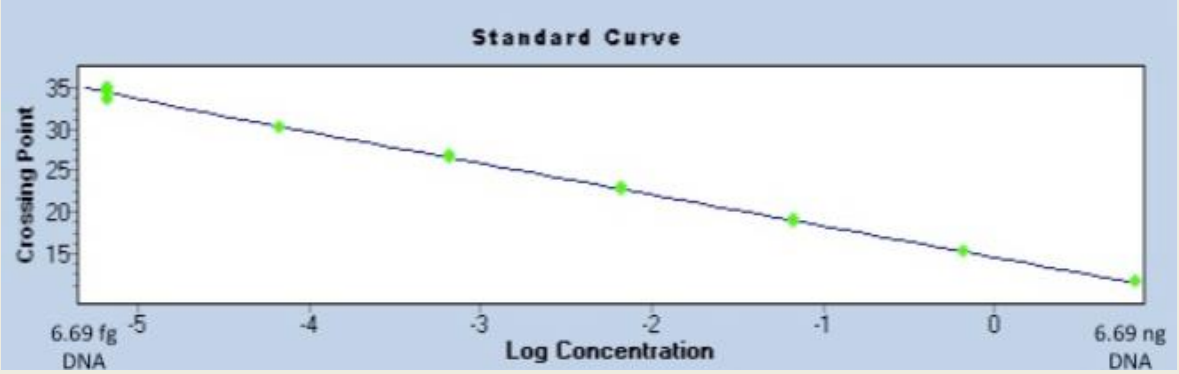


Figure 2. qPCR using PV1 and ITS2 primers showing the standard curve (a) and the melting peaks (b)

The newly designed PViolNEW primers also allowed for efficient qPCR detection of *P. violae* DNA down to a concentration of 6.69fg/µl and also did not have any issues with primer dimers (Figure 3a and b).

(a)



(b)

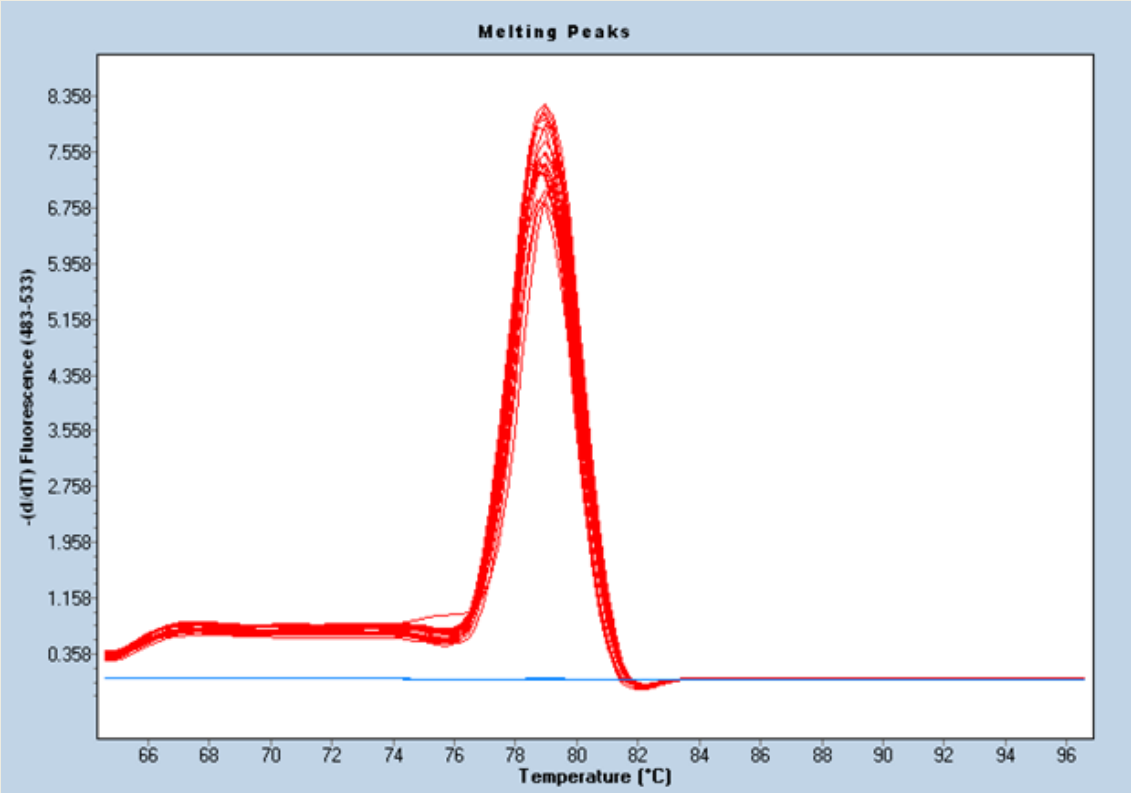


Figure 3. qPCR using PViol NEW primers showing the standard curve (a) and the melting peaks (b)

The specificity of all three primer pairs was assessed using conventional PCR. The primers PV1 and ITS2 amplified *P. ultimum* and *P. intermedium* as well as *P. violae* (Table 1). As *P. ultimum* is a common soil microbe, these primers were deemed not useful. Both PViol and PViolNEW primers amplified other *Pythium* species, but this amplification was very weak.

Table 1. Amplification of different *Pythium* species following PCR with three different primer pairs. nd=not done. The number of plus signs indicates the strength of amplification.

***Pythium* species**	**PViol (Klemsdal et al 2008)**		**PV1 / ITS2 (Wang and White 1996)**		**PViol New (this study)**	
P. violae	+++	+++	+++	nd	+++	+++
P. ultimum	-	-	++	nd	-	-
P. lutarium	-	-	-	nd	-	-
P. sulcatum	nd	-	nd	nd	nd	-
P. sylvaticum	nd	++	nd	nd	nd	+
P. intermedium	-	++	++	nd	-	+
P. irregulare	nd	++	nd	nd	nd	+

Downy mildew

P. destructor Isolates were collected and stored for future use. The inoculation method described was successful and can be used for future pathogenicity testing.

Discussion

Published primers for *P. violae* were found to be unsuitable for qPCR due to primer dimers and/or lack of specificity. New primers were developed which produced a very good standard curve and would potentially allow for detection of *P. violae* DNA in the soil at concentrations as low as 6.69 fg/µl. However, these primers weakly amplify some other *Pythium* species so cycling conditions would need to be optimised to avoid this. Another option is to modify the primers slightly by shortening them thus allowing more specific PCR amplification. In their current state, these primers are suitable for *P. violae* monitoring as the amplification of other species only occurs at very high DNA concentrations, a situation which is unlikely to occur in the soil. In the future, a whole genome sequence for *P. violae* will become available through

HDC Project FV432. This will potentially enable the design of new primers based on *P. violae* specific genes and will eliminate any doubts about primer specificity.

Conclusions

New primers were developed for qPCR detection of *P. violae* and could be useful for monitoring pathogen dynamics in the field.

A successful inoculation method was developed for *Peronospora destructor* which will allow for future characterisation of isolates and testing of efficacy of new biological and chemical products or resistant cultivars.

Knowledge and Technology Transfer

GROWER/TECHNICAL VISITS

Attended the BCGA AGM (16th Jan 2014) in Newark.

Hensborough Farm, Rugby – collected onion downy mildew (15th Aug 2014)

Vale Fresco (28th October 2014) in Hampton Lucy, Warks - observed downy mildew in rocket in a field situation. Samples were collected for a KTP project

Strawsons and Freshgro in Nottinghamshire (29th October 2014) - spoke with both companies about carrot production and particularly cavity spot.

CONFERENCES ATTENDED

UK Carrot and Onion Conference (20th-21st Nov 2013) in Peterborough)

BSPP Presidential (18th Dec 2013) in Birmingham

BCGA AGM (16th January 2014) in Newark.

HDC/BCGA Carrot Technical Seminar at PGRO in Peterborough (20th March 2014).

Plant Genomics Congress in London (12-13th May 2014)

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Hiltunen, L.H. and White, J.G. (2002) Cavity spot of carrot (*Daucus carota*). Annals of Applied Biology 141:201-223.

Li, Y, Zhou, X and Ye, D (2008) Molecular beacons: An optimal multifunctional biological probe. Biochemical and Biophysical Research Communications 373:457-461.

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Wang, P.-H. and White, J. (1996). Development of a species-specific primer for *Pythium violae*. MONOGRAPHS-BRITISH CROP PROTECTION COUNCIL: 205-210.