Horticultural Fellowship Awards

Interim Report Form

Project title:	Maintaining and developing capability in vegetable crop pathology
Project number:	CP 113
Project leader:	Dr John Clarkson, Warwick Crop Centre, University of Warwick
Report:	Annual Report, October 2019 (Year 6)
Previous report:	Annual Report, October 2018 (Year 5)
Fellowship staff: (“Trainees”)	Dr John Clarkson & Dr Andrew Taylor
Location of project:	Warwick Crop Centre
Industry Representative:	n/a
Date project commenced:	1st November 2013
Date project completed (or expected completion date):	28th February 2021

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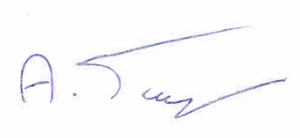
AUTHENTICATION

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.

Dr Andrew Taylor
Research Fellow
Warwick Crop Centre, University of Warwick

Signature  Date: 28/10/19

Report authorised by:

Dr John Clarkson
Reader
Warwick Crop Centre, University of Warwick

Signature  Date: 28/10/19

Progress Against Objectives

Objectives

N.B. Grey shading refers to this reporting period

Objective	Original Completion Date	Actual Completion Date	Revised Completion Date
1.1 Determine pathogenicity of a range of *Fusarium oxysporum* isolates on onion and complete DNA sequencing of a range of housekeeping genes.	31/10/15	31/10/15	
1.2 Extract DNA, prepare libraries and carry out whole genome sequencing of *F. oxysporum* f.sp. *cepae* (FOC) isolates	31/10/17	31/10/17	
1.3 Bioinformatic analyses of FOC isolate genomes and identification of potential primers for FOC diagnostics.	31/10/17	31/10/17	
1.4 Test FOC diagnostic primers *in vitro*	31/10/18	31/10/18	
1.5 Test FOC diagnostic primers using soil and bulb samples.	**31/10/18**	**31/10/18***	
1.6 Test published PCR diagnostic for *Sclerotium cepivorum*	31/10/16	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 AHDB Horticulture project FV 405 and other samples where available.	31/10/15	31/10/16	
1.10 Identify potential primers for *Itersonilia* diagnostics from existing gene sequences (or whole genome sequence).	31/10/16	31/10/16	
1.11 Test *Itersonilia* primers *in vitro*.	31/10/17	31/10/17	
1.12 Test the newly developed *Itersonilia* diagnostic test on infected parsnip seed lots and compare with the industry standard agar plate test.	31/10/17	31/10/17	
1.15 Test a range of *S. cepivorum* isolates for the presence of published pathogenicity genes	31/10/17	31/10/17	
1.16 Test the ability of sclerotia to germinate for a range of *S. cepivorum* isolates using an	31/10/17	31/10/17	

Objective	Original Completion Date	Actual Completion Date	Revised Completion Date
established assay based on diallyl disulphide (DADS)			
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	31/10/15	
2.2 Confirm identity and characterise isolates from 2.1 by gene sequencing	31/10/16	31/10/16	
2.3 Develop appropriate plant infection tests and confirm pathogenicity of isolates from 2.1	31/10/17	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	31/10/18	
3.2. Gain experience with brassica pathogens such as Turnip Mosaic Virus, *Albugo candida Hyaloperonospora brassicae* and *Xanthomonas campestris* through a work programme to be developed with Eric Holub and John Walsh.	31/10/18	31/10/18	
3.3. Gain experience of other pathogens such as *Pythium ultimum*, *Oidium. neolycopersici* through existing projects (John Clarkson)	31/10/17	31/10/17	
4.1. Synthesise Dez Barbara's unpublished work on carrot/parsnip viruses	31/10/15	31/10/15	
5.1. Attend relevant research project meetings.	**Ongoing**	**Ongoing**	
5.2. Present a poster at an industry meeting or event.	31/10/16	31/10/16	
5.3. Give a talk at an industry meeting or event.	31/10/17	31/10/17	
5.4. Work-shadowing of at least one industry collaborator.	31/10/17	31/10/17	
6.1. Contribute to writing at least one research proposal	31/10/17	31/10/17	
6.2. Initiate at least two research proposals and obtain funding for one.	31/10/18	31/10/18	
7 Test published (and unpublished) methods for extraction of DNA from larger quantities of soil.	31/10/18	31/10/18	
8 Isolate and confirm identity of the causal agent of onion pink root disease	31/10/17	31/10/17	
Added milestone (9) - Molecular characterisation of *F. oxysporum* f. sp. *narcissi* isolates. This will involve PCR amplification and sequencing of effector genes in a range of isolates.	31/10/16	31/10/16	

Objective	Original Completion Date	Actual Completion Date	Revised Completion Date
Additional milestones added in 2018			
10.1. Identify SIX genes and other effectors in FOL4 by PCR / genome analysis	**31/10/19**	**31/10/19**	
10.2. Develop and test qPCR / LAMP diagnostics for FOL4	**31/10/19**	**31/10/19**	
10.3. Develop method to produce chlamydospores of FOL4	**31/10/19**	**31/10/19**	
10.4. Test and develop lettuce differentials to confirm their utility in identifying FOL4	**31/10/19**	**31/10/19**	
10.5. Carry out preliminary resistance screening for FOL4 using Warwick lettuce diversity set	**31/10/19**	**31/10/19**	
10.6. Preliminary examination of DNA longevity in soil following FOL4 death / cell lysis	**31/10/19**	**31/10/19**	
11. Repeat dose response experiment for Narcissus bulbs with FON	**31/10/19**	**31/10/19**	
12. Discuss with Andy Richardson / Tom Will the commercial potential of FOC diagnostic assay (developed in 1.4)	**31/10/19**	**31/10/19**	
13. Give talks at relevant industry events such as the UK Brassica and Leafy salad conference	**Ongoing**	**Ongoing**	
14. Investigate the feasibility of published (and unpublished) methods for extraction of DNA from larger quantities of soil. Liaise with University of Idaho and FERA	31/10/20		
15. Develop bioinformatics skills and resources with Clubroot genomes	31/10/20		
16. Test FOC isolates which have had effector genes knocked-out for pathogenicity against onion bulbs	31/10/20		

*additional work carried out in 2019

Summary of Progress

Milestone 1.5: Test FOC diagnostic primers using soil and bulb samples. The FOC qPCR assay was tested using 39 sub-samples from a single soil sample from the high-disease pressure field at Wellesbourne and produced consistent positive results thus further validating both the assay and the optimised method for DNA extraction from soil.

Milestone 10.1 / 10.2: Identify SIX genes and other effectors in FOL4 by PCR / genome analysis and develop and test qPCR / LAMP diagnostics for FOL4. SIX genes and other putative effectors were identified in FOL1 and FOL4 by PCR and genome analysis. Differences in effector complement / sequence were observed between FOL1 and FOL4, allowing the development of a specific qPCR assay for FOL4 which appears to be highly sensitive and specific. A LAMP assay which detects both FOL1 and FOL4 was also developed and initial testing produced robust results and suggested that this assay is highly specific. These assays have been used to help track FOL4 outbreaks in the UK are being used in experiments being carried out in AHDB project FV PE 458 to quantify FOL4 in soil and lettuce roots.

Milestone 10.3: Develop method to produce chlamydospores of FOL4. Various methods of FOL chlamydospore production were tested and a method based on sterile soil culture was found to be highly effective, yielding high numbers of spores.

Milestone 10.4 / 10.5: Test and develop lettuce differentials to confirm their utility in identifying FOL4 and carry out preliminary resistance screening for FOL4 using Warwick lettuce diversity set. The Warwick lettuce diversity set was screened for resistance to FOL1 and FOL4 using previously developed inoculation procedures and lines with high levels of resistance to one or both of the races were identified. This also provided some new 'differential' lines for distinguishing FOL1 from FOL4.

Milestone 10.6: Preliminary examination of DNA longevity in soil following FOL4 death / cell lysis. It was shown that DNA from dead FOL4 spores degraded rapidly in soil and as such the presence of dead pathogen should not significantly affect the results of molecular diagnostic assays.

Milestone 11: Repeat dose response experiment for Narcissus bulbs with FON. An experiment to determine the effect of FON inoculum levels on disease development in FV POBOF 452 was repeated as a high background level of disease on the bulbs used confounded results. The repeat experiment resulted in a clear dose-response and a critical FON inoculum level for disease development was established.

Milestone 12: Discuss with Andy Richardson / Tom Will the commercial potential of FOC diagnostic assay. Following discussions with Andy Richardson, a small pilot project was started in order to test the FOC assay on commercial root and soil samples.

Milestone 13: Give talks at relevant industry events such as the UK Brassica and Leafy salad conference. Presentations were given at the UK Brassica and Leafy Salads Conference, the Hutchinsons Veg Conference and the International Allium Conference (details below).

Milestones not being reached

n/a

Do remaining milestones look realistic?

New milestones need to be developed with AHDB

Training undertaken

- Attended and gave talk (Understanding and combatting Fusarium diseases of onion and lettuce) at VeGIN meeting at Warwick Crop Centre (27th Nov 2018)
- Attended BSPP Presidential meeting at Warwick University (10th-11th Dec 2018)
- Completed 'Preparing to Teach in Higher Education' course (Dec 2018)
- Completed APP-PGR course leading to the award of an Associate Fellowship of the Higher Education Authority (Jan – Aug 2019)
- Attended and gave talk (Lettuce Fusarium wilt in the UK) at the UK Brassica and Leafy Salads Conference in Peterborough (23rd Jan 2019)
- Attended and gave talk (Research on Fusarium basal rot of onion and other vegetable diseases) at Hutchinsons Veg Conference in Peterborough (26th Feb 2019)
- Hosted guest seminar by Sascha Mooney, University of Nottingham (7th March 2019)
- Attended the Journal of Horticultural Science and Biotechnology conference in Charlecote (9th May 2019)
- Hosted visit and guest seminar by Louise Thatcher from CSIRO, Australia (17th June 2019)

- Attended and gave an invited talk (Creation and characterisation of an onion diversity set and identification of accessions with resistance to Fusarium basal rot and improved seedling vigour) at the International Allium Conference in Madison, USA (23rd – 27th July 2019)
- Visited lettuce grower to discuss Fusarium issues (26th Sept 2019)
- Attended Fusarium meeting in Utrecht and met with researchers at Wageningen (29th – 31st Oct 2019)

Expertise gained by trainees

- Improved communication skills
- Improved understanding of the lettuce industry
- Improved understanding of plant pathology
- Greater understanding of worldwide Allium research
- Expertise in Fusarium wilt of lettuce
- Greatly improved teaching skills including a formal qualification

Other achievements in the last year not originally in the objectives

- Obtained a formal teaching qualification as an Associate Fellow of the HEA
- Inoculated polytunnels with FOL4 and maintained these facilities for future projects
- A paper titled: 'Assembly and characterisation of a unique onion diversity set identifies resistance to Fusarium basal rot and improved seedling vigour' was published in Theoretical and Applied Genetics
- A paper titled: 'First report of *Fusarium oxysporum* and *Fusarium redolens* causing wilting and yellowing of wild rocket (*Diplotaxis tenuifolia*) in the UK' was published in Plant Disease.
- A paper titled: 'First report of *Fusarium oxysporum* f. sp. *lactucae* Race 4 causing lettuce wilt in England and Ireland was published in Plant Disease.
- A paper titled: 'Draft genome sequence of an onion basal rot Isolate of *Fusarium proliferatum*' was published in Microbiology Resource Announcements.
- Took on the role of Associate Editor for the Journal of Horticultural Science and Biotechnology

- Awarded 2 travel grants (Vegetable Research Trust and GCRI) totalling £2500 to attend the International Allium Conference in Madison, USA.
- Carried out commercially funded research projects on Fusarium basal rot in onion

Changes to Project

Are the current objectives still appropriate for the Fellowship?

New objectives need to be set with AHDB

GROWER SUMMARY

Headline

Molecular diagnostic assays were developed and validated for *Fusarium oxysporum* f. sp. *lactucae* (FOL), the cause of lettuce Fusarium wilt. These assays will be of great benefit for testing soil and plant material for this important recently emerged pathogen. Lettuce lines with resistance to FOL were identified, providing promise for future breeding programmes.

Background

Fusarium oxysporum and Fusarium basal rot of onion

Onion (*Allium cepa*) is an important horticultural crop which is cultivated by every agricultural nation. Soilborne diseases caused by *Fusarium oxysporum* formae speciales (isolates adapted to specific hosts, f. spp.) are major constraints to the production of many horticultural food crops worldwide including onion, leek, lettuce, tomato, brassicas, asparagus, cucurbits, peppers, coriander, spinach, basil, beans, peas, strawberry, watermelon and banana, and also affect economically important non-food crops such as carnation and narcissus (Leslie and Summerell 2006) and stocks. *F. oxysporum* was recently identified as the 5th most important plant pathogenic fungus based on its economic and scientific impact (Dean et al, 2012). *F. oxysporum* f.sp. *cepae* (FOC) is one of the most important pathogens of onion crops and infects the roots and/or basal plate at any stage of plant development (Cramer, 2000; Taylor et al, 2013). This causes a damping-off symptom on seedlings and a basal rot on more mature plants resulting in severe pre and/or post-harvest losses. In the UK, FOC is recognised mainly as being a problem at harvest and in store but in severe cases entire crops can be lost in the field. Economic losses due to FOC on onion are estimated at up to £20 million per year. FOC infection is favoured by warm temperatures and is predicted to get worse in Europe due to climate change (Cramer, 2000). It produces long-lived chlamydospores that survive in the soil for many years and hence control approaches have previously relied on the use of soil sterilisation, chemical fumigation, drenches with fungicides or seed treatments. These approaches have in some cases been unsuccessful, have undesirable environmental effects and have been banned or are threatened by legislation governing restrictions in pesticide use. In the past it has been difficult to distinguish f. spp. of *F. oxysporum* and identification has relied on pathogenicity tests. However, work from *F. oxysporum* f. sp. *lycopersici*, the f. sp. infecting tomato (Lievens et al, 2009; Ma et al, 2010) has identified a set of pathogenicity related genes which are conserved in FOC (Taylor et al,

2016). Differences in the compliment or sequences of SIX (secreted in xylem) genes between forma speciales of *F. oxysporum* can potentially be utilised to develop diagnostic assays which can be utilised to test soil and plant material for presence of the pathogen. As part of this fellowship, an assay for FOC was developed and validated (see year 5 annual report) which will allow seed, soil, roots and bulbs to be tested for the presence of FOC.

Fusarium wilt of lettuce

Fusarium wilt of lettuce, caused by *Fusarium oxysporum* f. sp. *lactucae* (FOL), is a problem in most production areas globally, causing severe economic losses in protected and field crops. Initial symptoms are stunting and yellowing, particularly on older leaves (Taylor & Clarkson 2018). A black/brown/red discoloration of the vascular tissue can be observed and infection ultimately leads to plant death. There are 4 known races of FOL, the most widespread being race 1 which affects both field and protected crops (Gilardi et al, 2017). Races 2 and 3 are only found in Japan and Taiwan. FOL was only very recently first reported in the UK in October 2017 (Taylor et al, 2019) with initial outbreaks affecting protected lettuce crops in Lancashire and Ireland. However, since then there has been local spread within these areas to other growers and also confirmed reports of FOL in Cambridgeshire (2018) and Yorkshire (2019). Genetic analysis has confirmed the causal agent as FOL race 4 (FOL4, Taylor et al, 2019). FOL4 was first reported in the Netherlands in 2013 (Gilardi et al, 2017) and so far all cases have been confined to protected crops. It is also causing severe losses for protected lettuce growers in Belgium. Currently, rapid spread of FOL4 is being prevented by hygiene measures imposed by the industry while affected growers are mitigating disease impact through use of the soil fumigant Basamid (dazomet), removal of contaminated soil or by abandoning affected growing areas. Although there is no widespread availability of resistant lettuce cultivars, these are under development. Developing a molecular test for FOL4 would mean soil, seed and other plant material could be tested for the pathogen.

Narcissus basal rot

Daffodil (Narcissus spp.) is one of the most widely cultivated bulb crops of temperate regions. The major production areas are the UK, Netherlands and USA although smaller areas are cultivated across the world (Hanks, 2002). In the UK, bulbs are particularly prone to infection by soil-borne pathogens due to the standard biennial growing system employed (Hanks, 2002). The most damaging pathogen is *Fusarium oxysporum*. f.sp. *narcissi* (FON), the cause of narcissus basal rot (Linfield, 1994). The symptoms include pale yellow leaf tips, soft bulbs, root rot and ultimately a bulb rot. Infected bulbs may not sprout and produce few or no flowers.

Controlling FON is challenging due to the production of chlamydospores as discussed above. It is not known what concentration of FON is required for infection to occur.

Do molecular techniques detect dead pathogens?

There is some debate in the literature about how long after death a pathogen can be detected by molecular techniques due to continued survival of DNA, particularly in soil. For example, research carried out on *Gaeumannomyces graminis* (a fungus causing take-all disease of cereals) suggests that fungal DNA is broken down rapidly in soil, reaching an undetectable level after 8 days (Herdina et al, 2004). However, other work has suggested that DNA can bind to soil particles or humic acids, protecting it from degradation (Alvarez et al, 1998; Crecchio et al, 1998). If molecular assays detect dead pathogen then this could affect any disease predictions based on DNA quantities in soil. Therefore, it is important to begin to understand the persistence of DNA in soil following death of the pathogen.

Summary

- A highly specific and reproducible molecular diagnostic assay (qPCR) was developed for FOC in year 5. This assay can be used to test seed, plant material and soil for FOC and is fully quantitative.
- The FOC qPCR assay was further validated by testing 39 sub-samples from a single soil sample collected from the FOC inoculated field at Wellesbourne and all samples gave similar positive results indicating that the assay and the DNA extraction method is highly reproducible.
- SIX genes and other predicted effectors were identified in FOL1 and FOL4 by PCR and genome analysis. Differences between FOL1 and FOL4 were observed, allowing the development of a specific qPCR assay for FOL4 which appears to be highly sensitive and specific.
- A rapid assay similar to PCR (loop-mediated isothermal amplification, LAMP), which detects both FOL1 and FOL4 was also developed and initial testing produced robust results and suggested that this assay is highly specific. This assay gives a diagnosis in under 1 hour and can potentially be used at grower sites.
- A method of chlamydospore production was developed for FOL4 which will allow testing of heat / disinfectant treatments against the pathogen in another AHDB project.

- The Warwick lettuce diversity set was screened for resistance to FOL1 and FOL4 and lines with high levels of resistance to one or both of the races were identified. This will allow for future breeding of resistant cultivars.
- Clear lettuce 'differential' lines for distinguishing FOL1 from FOL4 were identified, allowing definitive differentiation of races alongside molecular testing
- It was shown that DNA degrades rapidly in soil and as such dead pathogen should not significantly affect molecular diagnostic assays.
- An experiment to determine the effect of FON inoculum levels on disease development in FV POBOF 452 was repeated as a high background level of disease on the bulbs used confounded results. The repeat experiment resulted in a clear dose-response observed and the critical inoculum level for disease established. Further work will now utilise a FON-specific qPCR assay to relate DNA levels to inoculum rate and disease development. This will be an important first step in assessing the utility of the PCR tests for assessing disease risk in the field.

Financial Benefits

None to report

Action Points

None to report