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 2018 (Year 5)
Previous report:	Annual Report, October 2017 (Year 4)
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):	31/12/19

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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: 29/10/18

Report authorised by:

Dr John Clarkson
Reader
Warwick Crop Centre, University of Warwick

Signature  Date: 29/10/18

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Progress Against 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**		
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	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 established assay based on diallyl disulphide (DADS)	31/10/17	31/10/17	
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	

Objective	Original Completion Date	Actual Completion Date	Revised Completion Date
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	
ADDITIONAL MILESTONE- 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	

Summary of Progress

Milestone 1.4/1.5: *Fusarium oxysporum* f. sp. *cepae* (FOC) diagnostic primers were developed and shown to be highly specific to FOC. Primers were tested against soil, root and bulb samples and shown to be very effective at detecting the pathogen.

Milestone 3.1/3.2: Where possible, experience of a range of pathogens has been gained throughout the fellowship. This includes experience of working with *Fusarium* on a range of crops (e.g. onion, leek, asparagus, lettuce, rocket, stocks, celery, fenugreek, coriander, pea, narcissus); white rot on onion (*Sclerotium cepivorum*); cavity spot of carrot (*Pythium* species); downy mildews (e.g. onion downy mildew, *Peronospora destructor*); *Botrytis* spp.; onion pink root (*Setophoma terrestris*); *Aphanomyces* and *Didymella* (part pf pea footrot complex); *Stemphylium* on onion / narcissus; *Sclerotinia* on a range of crops; *Itersonilia* on parsnip and Brassica clubroot (*Plasmodiophora brassicae*).

Milestone 6.2: During the fellowship, I have been involved in numerous research proposals. In 2017, I led a successful proposal to carry out a review on lettuce Fusarium wilt (AHDB project CP17/18-1006). I also carried out preliminary work and contributed to a BBSRC proposal titled: Development of resistance to Fusarium basal rot in onion and functional analysis of *Fusarium oxysporum* effectors. Unfortunately, this was unsuccessful. Recently, I was involved in writing a proposal titled 'Lettuce: biology and management of Fusarium wilt caused by *Fusarium oxysporum* f.sp. *lactucae* race 4' which was submitted to the AHDB and successfully funded with a start date of December 2018. In addition, I was involved in writing a proposal to test chemical / biological control agents against *Fusarium oxysporum* f.sp. *lactucae* race 4 as part of the AHDB SCEPTREplus project which was successful (SP25).

Milestone 7: A range of methods for extraction of DNA from soil were tested, including large sample methods. An optimised method (based on a commercial kit) was developed and found to be both effective and repeatable.

Milestones not being reached

None

Do remaining milestones look realistic?

Due to changes in the end date of the fellowship, additional milestones will be added and the remaining milestones reviewed. This will be discussed and agreed with AHDB.

Training undertaken

- Visited an asparagus field and met with Phil Langley (G's). Trained in identifying and sampling Fusarium crown rot of asparagus (3rd Oct 2017)
- Hosted a visitor from Bayer USA – various meetings and exchange of materials (Oct 2017)
- Attended UK carrot and onion conference in Nottingham (14th Nov 2017)
- Attended seminar by Matt Back (Harper Adams University) on biofumigation (30th Nov 2017)
- Attended and gave talk (Update on diagnostics for lettuce Fusarium wilt and discussion on sampling and monitoring) at Lettuce Fusarium workshop in Skelmersdale (14th Dec 2017)
- Visited Enza Zaden in the Netherlands to discuss lettuce Fusarium wilt (8th Jan 2018)
- Skype meeting with Rijk Zwaan to discuss lettuce Fusarium wilt (24th Jan 2018)
- Attended and gave talk (*Fusarium oxysporum*: understanding and combatting a highly aggressive, global plant pathogen) at Crop Science workshop, Warwick University (1st Feb 2018)
- Attended Warwick University School of Life Sciences industry day (28th Feb 2018)
- Skype meeting with Pietro Spanu (Imperial College) to discuss Fusarium on stocks (5th March 2018)
- Attended and gave talk (Update on lettuce Fusarium wilt: minimising impact on the UK lettuce industry) at Outdoor and Protected Leafy Salad Technical Day at Stockbridge House (20th March 2018)
- Hosted guest seminar by Steven Penfield (John Innes) on seasonal sensing by plants (24th May 2018)
- Visited rocket grower to discuss Fusarium problems (4th June 2018)
- Attended Crop Science workshop, Warwick University (7th June 2018)
- Hosted guest seminar by Malcolm Hawkesford (Rothamsted) on scanalyzers and drones (5th July 2018)
- Hosted guest seminar by Max Newbert (Syngenta) on RNAi (19th July 2018)

- Attended and gave talk (Understanding the genetic control of pathogenicity and resistance to *Fusarium oxysporum* in onion) at the 10th Australasian Soilborne Diseases Symposium in Adelaide (4th – 7th Sept 2018)
- Attended a personal tutor training session (Small Group Teaching) organised by Vivid Communication (27th Sept 2018)
- Attended and gave talk (Lettuce Fusarium wilt: potential management options) at Growing media developments in vegetable propagation in Doddington, Cambs (9th Oct 2018)
- Attended and gave talk (Lettuce Fusarium wilt: potential management options) at BLSA Protected R & D Committee meeting in Warwick (16th Oct 2018)
- Hosted a guest seminar on Fusarium diseases by Kim Hammond-Kosack, Rothamsted (18th Oct 2018)

Expertise gained by trainees

- Improved communication skills
- Improved understanding of the lettuce industry
- Improved knowledge of the rocket industry
- Improved knowledge of the carrot and onion industries
- Greater understanding of global plant pathology and soilborne diseases
- Ability to identify and isolate the cause of Fusarium crown rot of asparagus
- Expertise in Fusarium wilt of lettuce
- Expertise in Fusarium wilt of stocks
- Improved teaching skills

Other achievements in the last year not originally in the objectives

- A paper entitled 'Inoculum potential of *Sclerotinia sclerotiorum* sclerotia depends on isolate and host plant' was published (Plant Pathology, 67: 1286-1295).
- A paper entitled 'Shifts in diversification rates and host jump frequencies shaped the diversity of host range among *Sclerotiniaceae* fungal plant pathogens' was published (Molecular Ecology, 27: 1309-1323).
- A paper entitled 'Characterisation of pathogen-specific regions and novel effector candidates in *Fusarium oxysporum* f. sp. *cepae'* was published (Scientific Reports, 8: article number 13530).

- Continued in my role as an editor for Exchanges: the interdisciplinary research journal (http://exchanges.warwick.ac.uk/index.php/exchanges).
- Maintained the quarantine field areas at Wellesbourne. Involved in trials in the *Fusarium* area, being run by commercial companies.
- Designed and carried out an experiment to monitor SIX gene expression *in planta* following infection of Narcissus scales with FON. This produced excellent data which is being written into a publication (continued from 2017 annual report).
- Increased involvement in teaching including taking on a year 1 undergraduate tutorial group.
- Awarded 2 travel funds (BSPP and VRT) totalling £2000 to attend the 10th Australasian Soilborne Diseases Symposium in Adelaide.
- Developed assays to infect lettuce plants with *Fusarium oxysporum* and carried out glasshouse assays to determine the race of new isolates. A new disease report has been submitted.
- Developed glasshouse assays to infect rocket plants with *Fusarium*.

Changes to Project

Are the current objectives still appropriate for the Fellowship?

Due to changes in the end date of the fellowship, additional milestones will be added and the remaining milestones reviewed. The following future milestones are suggested:

<u>Preliminary work to support AHDB FV/PE 458 Lettuce: biology and management of fusarium wilt and further research</u>

- Identify SIX genes and other effectors in FOL4 by PCR / genome analysis
- Develop and test qPCR / LAMP diagnostics for FOL4
- Develop method to produce chlamydospores of FOL4
- Test and develop lettuce differentials to confirm their utility in identifying FOL4
- Repeat dose response experiment for Narcissus bulbs with FON
- Carry out preliminary resistance screening for FOL4 using Warwick lettuce diversity set

Developing future projects

Soilborne pathogen diagnostics and quantification

- Investigate the feasibility of published (and unpublished) methods for extraction of DNA from larger quantities of soil. Liaise with University of Idaho and FERA.
- Begin to examine the longevity of DNA in the soil following death / cell lysis.
- Discuss with Andy Richardson / Tom Will potential of FOC diagnostic – further testing on field soil and bulbs for storage?

Resources and information for new projects

- Develop bioinformatics skills and resources with Clubroot genomes.

Impact

- Give talks at relevant industry events such as the UK Brassica and Leafy salad conference.

Other

- Test FOC isolates which have had effector genes knocked-out for pathogenicity against onion bulbs.

GROWER SUMMARY

Headline

A molecular diagnostic assay for FOC was shown to be specific and reproducible for detecting the pathogen in soil and plant samples. An optimised method for extracting DNA from soil has been developed.

Background

Onion diseases

Fusarium basal rot

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 £10-11 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 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.

DNA extraction from soil

Extracting DNA from soil can be challenging as samples may be very diverse and contain many different PCR inhibitors (Schrader et al, 2012). Such contaminants can inhibit PCR, even when present at low concentrations. Consequently, commercial kits have been developed which remove inhibitors from soil and can be used to extract high quality DNA. However, these kits rely on very small starting samples, usually 0.25-0.5g. This makes field soil sampling challenging, particularly when pathogens are involved as many pathogens are notoriously patchy in their occurrence in the field. Therefore, larger scale extraction protocols have been developed to overcome this sampling issue, allowing for extraction from up to 500g of soil (e.g. Woodhall et al. 2012).

Summary

- A highly specific and reproducible molecular diagnostic assay (qPCR) was developed for FOC. This assay can be used to test seed, plant material and soil for FOC and is fully quantitative.
- The assay was tested against a panel of 62 fungi and shown to be highly specific
- The assay was tested on soil, root and bulb samples and shown to produce consistent amplification of the target
- Field monitoring showed a peak in FOC DNA levels in August, and the pathogen was detectable in a commercial field 12 months after an infected onion crop
- A range of methods for DNA extraction from soil were tested and an optimised method, based on a commercial kit, was developed
- Large scale extraction methods produced a good yield of DNA but PCR results were disappointing, likely due to inefficient removal of inhibitors.
- This optimised method produced consistent results when tested for detection of FOC using the FOC-specific primers.

Financial Benefits

None to report

Action Points

None to report

SCIENCE SECTION

Introduction

Onion diseases

Fusarium basal rot

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 £10-11 million per year and FOC infection is favoured by warm temperatures and is predicted to get worse in Europe due to climate change (Cramer, 2000). FOC 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.

FOC is one of more than 120 *Fusarium oxysporum formae speciales* (isolates adapted to specific hosts) (Michielse & Rep, 2009) which 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). *F. oxysporum* was recently identified as the 5th most important plant pathogenic fungus based on its economic and scientific impact (Dean et al, 2012).

The genetically heterogeneous nature and lack of reliable morphological characters in this *F. oxysporum* complex means that distinguishing between different pathogenic f. spp. and also and between pathogenic and non-pathogenic isolates is difficult and can only be done using laborious and time consuming pathogenicity tests on different hosts. The factors which determine the host specificity and pathogenicity of different *F. oxysporum* f. spp. are poorly understood although recent studies have identified the role of secreted effector proteins (SIX genes) and mobile pathogenicity chromosomes in *F. oxysporum* f. sp. *lycopersici*, the f. sp. infecting tomato (Lievens et al, 2009; Ma et al, 2010). The genetic basis for pathogenicity

appears to be partially conserved in FOC with 7 of the 14 known SIX genes identified and predicted to have an important role in pathogenicity (Taylor et al, 2016). Differences in the compliment and sequences of SIX genes between *formae speciales* of *F. oxysporum* including FOC holds promise for the development of specific diagnostic assays for each f.sp. which could be utilised to test soil and plant material for presence of these important pathogens.

Extracting DNA from soil

Extracting DNA from soil can be challenging as samples may be very diverse and contain many different PCR inhibitors such as humic and fulminic acids (Schrader et al, 2012). Such contaminants can inhibit PCR, even when present at low concentrations. Consequently, commercial kits have been developed which remove inhibitors from soil and can be used to extract high quality DNA. However, these kits rely on very small starting samples, usually 0.25-0.5g. This makes field soil sampling challenging, particularly when pathogens are involved as many pathogens are notoriously patch in their occurrence in the field. Therefore, larger scale extraction protocols have been developed to overcome this sampling issue, allowing for extraction from up to 500g of soil (e.g. Woodhall et al. 2012). Some examples of methods available to extract DNA from soil are listed in Table 1.

Table 1: Methods to extract DNA from soil. *PREDICTA B is offered as a diagnostics service by The South Australian Research and Development Institute (SARDI) and the method is not publicly available.

Method	**Publication / manufacturer**	**Maximum sample size (g)**
PowerSoil	Qiagen	0.25
FastDNA™ SPIN Kit for soil	MP Biomedicals	0.5
Exgene™ Soil SV	GeneAll Biotechnology	0.5
Woodall method	Woodhall et al. 2012	500
Devi method	Devi et al. 2015	1
PREDICTA B method*	http://pir.sa.gov.au/research/services/	500

Materials and methods

Milestone 1.4 Test FOC diagnostic primers *in vitro* / 1.5 Test FOC diagnostic primers using soil and bulb samples

Development of specific primers based on the SIX5 gene was reported in the 2017 annual report. For full specificity testing, primers were tested against a panel of 62 different *F. oxysporum* f.spp., *Fusarium* species, other common soilborne oomycetes / fungi as well as selected onion pathogens (Table 2) using quantitative PCR (qPCR). A dilution series of FOC isolate FUS2 ranging from 100 ng/µl -1 pg/µl was prepared and all DNA samples from non-target organisms were diluted to 10ng/µl. All qPCRs were carried out in 10µl reactions containing primers (0.5 µM), 5 µl SensiFAST™ SYBR® No-ROX Kit mastermix and 1 µl of DNA. Conditions were as follows: 1 cycle of 95°C for 3 mins followed by 45 cycles of 95°C for 5s, 60°C for 10s and 72°C for 10s. All samples were run in triplicate and a melt curve analysis carried out.

In order to test the sensitivity of this assay, a modified dilution series of FOC (isolate FUS2) DNA was prepared ranging from 10ng/µl – 0.5pg/µl. qPCR was carried out as described above and the efficiency of the assay analysed using Cp values.

Table 2: Fungal isolates used for testing the specificity of a newly developed qPCR assay for FOC.

	Genus	Species	f. sp	Isolated from	Country	Code
1	Fusarium	oxysporum	cepae	onion	UK	FUS2
2	Fusarium	oxysporum	conglutinans (Race 2)	Brassica/Arabidopsis	USA	NRRL 54008
3	Fusarium	oxysporum	cubense	banana	UK	E421A-3
4	Fusarium	oxysporum	dianthi	dianthus	UK	BX13/113
5	Fusarium	oxysporum	freesia	freesia	Netherlands	NRRL26990
6	Fusarium	oxysporum	gladioli	gladioli	Netherlands	NRRL 26993
7	Fusarium	oxysporum	lini	flax	UK	FOLIN
8	Fusarium	oxysporum	lycopersici	tomato	UK	FOL R1
9	Fusarium	oxysporum	lycopersici	tomato	UK	FOL R2
10	Fusarium	oxysporum	lycopersici	tomato	USA	FOL R3 (MN25)-NRRL 54003
11	Fusarium	oxysporum	mathioli	stocks	UK	Stocks 4 (10A-4)
12	Fusarium	oxysporum	melonis	melon	Mexico	NRRL 26406
13	Fusarium	oxysporum	narcissi	daffodil	UK	FON63
14	Fusarium	oxysporum	phaseoli	bean	USA	ATCC90245
15	Fusarium	oxysporum	pisi race 1	pea	UK	FOP R1
16	Fusarium	oxysporum	pisi race 2	pea	UK	FOP R2
17	Fusarium	oxysporum	pisi race 5	pea	UK	FOP R5
18	Fusarium	oxysporum	radicis-lycopersici	Lycopersicum	USA	NRRL 26381

19	Fusarium	oxysporum	statice	statice	UK	30A-9
20	Fusarium	oxysporum	vasinfectum	cotton	China	NRRL 25433
21	Fusarium	oxysporum		rocket	UK	FR3
22	Fusarium	oxysporum		leek	UK	L2-1
23	Fusarium	oxysporum		onion	UK	Fo47
24	Fusarium	avenaceum		leek	UK	L5
25	Fusarium	begoniae		begonia	UK	775
26	Fusarium	cerealis		?	UK	831
27	Fusarium	coeruleum		potato	UK	F88
28	Fusarium	culmorum		wheat	UK	Fc/01/W001
29	Fusarium	equiseti		rocket	UK	NL1
30	Fusarium	flocciferum		asparagus	UK	AT4
31	Fusarium	graminerum		onion	UK	WR21
32	Fusarium	lactis		pepper	UK	P9
33	Fusarium	langsethiae		wheat	UK	34f.l.003.2
34	Fusarium	poae		wheat	UK	Fp/01/W/001
35	Fusarium	proliferatum		onion	UK	A40
36	Fusarium	pseudocircinatum		banana	UK	
37	Fusarium	redolens		onion	UK	NL96
38	Fusarium	sacchari		banana	UK	18RFB 2015
39	Fusarium	sambucinum		potato	UK	F37
40	Fusarium	solani		pea	UK	PG14
41	Fusarium	torulsoum		?	UK	102
42	Fusarium	tricinctum		Brassica	UK	CO
43	Alternaria	infectoria		onion	UK	AT2
44	Botrytis	cinerea		onion seed	UK	WRAR-4
45	Botrytis	aclada		onion	UK	9736
46	Botrytis	allii		onion	UK	9745
47	Cylindrocarpon	destructans		parsnip	UK	CD10
48	Itersonilia	perplexans		parsnip	UK	IP10
49	Microdochium	majus		wheat	UK	MM/X/W/003
50	Microdochium	nivale		wheat	UK	MN/X/W/003
51	Mycocentrospora	acerina		parsnip	UK	Ma5
52	Phoma	sp.		?	UK	
53	Pythium	ultimum		carrot	UK	3b/P174
54	Pythium	violae		carrot	UK	2C/P2d
55	Rhizoctonia	solani		?	UK	R5(A92-1)
56	Sclerotinia	sclerotiorum		lettuce	UK	L6
57	Sclerotium	cepivorum		onion	UK	RUG1-1
58	Setophoma	terrestris		onion	UK	PQF4
59	Stemphylium	sp.		onion	UK	SQ3
60	Trichoderma	sp.		onion	UK	WRLG11
61	Phytophthora	cactorum		Strawberry	UK	P414
62	Verticillium	albo atrum		potato	UK	PD693

To test the assay on soil and plant samples, soil samples were taken from a quarantine field at Wellesbounre that was previously inoculated with FOC (Fusarium QF) and DNA extracted using the optimised Soil SV method described for milestone 7. In addition, onion root samples were taken from the same field (*A. fistulosum* plants not showing obvious symptoms of infection). DNA was extracted using the Soil SV method. Conventional PCR was used to test the assay. PCRs were carried out in 20µl reactions containing primers (0.5 µM), 5µl of RedTaq (Sigma) and 1µl of DNA with the following conditions: 1 cycle of 94°C for 2 mins followed by 35 cycles of 94°C for 45s, 60°C for 30s and 72°C for 30s followed by 1 cycle of 72°C for 5 mins. PCR products (10µl) were run on a 1.2% agarose gel.

To further validate the assay, and as a disease monitoring exercise, soil samples were taken from the Fusarium QF approximately every month from 22.04.17 to 11.05.18. Three beds were selected and each was sampled in a W formation (5 points) on every sampling occasion. The 5 samples from each bed were pooled before processing. DNA was extracted from 0.5g using the Soil SV method and qPCR set up as described above. In addition, soil samples were taken from a commercial field following a cereal crop on 20/09/17. In the previous year, an onion crop was grown and a high level of basal rot was observed. Samples were processed as described.

To further validate the assay, onions from 2 commercial stores with variable symptoms of basal rot were received from Vegetable Consultancy Services (VCS). These were cut longitudinally and half of the basal plate excised, flash frozen in liquid N and freeze dried. Samples were homogenised using a pestle and mortar and DNA was extracted from 20mg using a DNeasy Plant mini kit (Qiagen), following the manufacturer's guidelines with the exception of an additional centrifugation step after cell lysis (13,000 rpm for 5 mins). qPCR was carried out as described above.

The other half of the basal plate was excised, cut into 5 sections, surface sterilised in 70% ethanol for 30s, rinsed twice in sterile water and plated onto PDA containing 20 µg/ml chlorotetracycline. Plates were incubated at room temperature (approx. 20°C) for 3 days and scored for the presence / absence of *Fusarium*. Results from the platings was compared to data from qPCR using a Pearson correlation (Excel).

Milestone 7: Test published (and unpublished) methods for extraction of DNA from larger quantities of soil.

Woodhall method for DNA extraction from 500g of soil

A large scale DNA extraction method was tested based on a published method (Woodhall et al 2012). Initially, two solutions were prepared: 1M Sodium phosphate buffer- solution A: 138g of NaH_2PO_4-H_2O in 1 litre dH_2O; solution B: 142g of Na_2HPO_4 in 1 litre of dH_2O. 423ml of solution A was mixed with 577ml solution B, adjusted to pH 8, autoclaved and stored at room temperature. For spiking, a soil sample (1-2kg) was taken from an area with no recent history of onion production (Cottage Field, Wellesbourne), air dried for 72 hours, mixed well and sieved through a 2mm sieve before mixing again. A 100g sample was placed in a 500ml Nalgene bottle with 10 beads (stainless steel, 1 inch diameter) and spiked with 5 million FOC spores (isolate FUS2, 192µl of a spore suspension at 2.6 x 10^7 cfu/ml). 200ml of grinding buffer (120mM sodium phosphate buffer (120mls), 2% CTAB (20g), 1.5M sodium chloride (500 ml of 3M), pH7-8, made up to 1 litre and autoclaved) and 9ml of antifoam B (Sigma) were added. Extracts were mixed vigorously by manual shaking for 5 min before transferring 50ml to a fresh tube and centrifuging at 4100 rpm for 5 mins. 20ml of supernatant was transferred to a fresh tube containing 2ml of 5M potassium (or ammonium) acetate and the mixture vortexed before Incubating on ice for 10 mins the centrifuging at 4100 rpm for 20 mins. The supernatant was transferred to a fresh tube containing 15ml isopropanol and 800µl of 100% silicon dioxide suspension (100g of silica made up to 1 litre with dH_2O, homogenise and settle for 36 hours, pour off water, repeat (overnight) and add dH2O to ~40mls to give appearance of thick paint. HCl (~200µl conc. HCl) was added to adjust to pH2 and autoclave) and placed on a flatbed shaker for 15 mins at 120rpm, mixing halfway through. Tubes were then centrifuged at 4100 rpm for 20 mins, the supernatant discarded and 2mls of Buffer AP1 (DNeasy plant mini kit, Qiagen), 5µl of RNase A and a scoop of polyvinylpolypyrrolidone (PVPP) added to the silica. This was mixed well before incubating the tubes at 65°C for 11 mins, inverting by hand every 2 mins. Tubes were then centrifuged at 13,000 rpm for 5 mins and 1000µl processed using a DNeasy kit (from step 9 onwards). DNA was eluted in 200µl of buffer AE (100 + 100). DNA quality was checked using a nanodrop and by running 5µl on a 1.2% agarose gel. PCR was then carried out on neat DNA and DNA diluted 1 in 10 and 1 in 100 in TE buffer using the FOC-specific primers and previously described conditions. PCR was subsequently set up using 1-5µl of DNA.

This method was subsequently tested using soil from the Fusarium QF, an area infected with a high level of FOC. The same method was followed with the exception that processing 1000

µl and 1400 µl through the DNeasy kit was compared. DNA was extracted from the same samples using a commercial kit (PowerSoil, 0.25g). PCR was carried out as previously described.

Due to weak PCR amplification, an extra clean-up step was attempted using a Zymo PCR inhibitor removal kit, following the manufacturer's guidelines.

Testing a pre-concentration method and variable starting quantities

Using soil taken from the Fusarium QF, a pre-concentration step was tested. 5 or 10 g of soil was placed in a 50ml tube and 40ml of 1 x PBS added. This was soaked for 1 hour, vortexing every 5-10 mins. The sample was then allowed to settle for 3 or 6 mins before centrifuging at 4100rpm for 20 mins at 8-10°C. The pellet was resuspended in 1.4 ml of PBS and transferred to a 2ml tube before centrifuging at 13,000 rpm for 5 mins. The supernatant was removed and the contents of the bead tube (PowerSoil kit) was added before extracting DNA following the PowerSoil protocol with the addition of an extra homogenisation step in a Fastprep machine (5.5m/s for 30 secs). As a second method, a range of starting quantities from 0.25 – 0.65g were tested (dried soil from the same sample) using the PowerSoil kit. PCR was carried out as previously described.

Devi method for economical DNA extraction from 1g of soil

DNA extraction was attempted from 1g of Fusarium QF soil using a published method (Devi et al., 2015). PCR was carried out as previously described.

Testing a new soil DNA extraction kit – Soil SV

A new soil DNA extraction kit became available (Soil SV, GeneAll) which offers the advantage of being more rapid than other protocols. This kit was tested and compared to the PowerSoil kit and also FastDNA™ for soil kit (MP Biomedicals). In order to fully homogenise samples, soil from the Fusarium QF field (approx. 200g) was mixed, sieved through a 4mm sieve, air dried for 72 hours, mixed, sieved through a 2mm sieve, hit with a wooden mallet and given a final mix. The following homogenisation methods were tested: 2 x Fastprep (5.5m/s for 20s); vortexing on maximum speed for 10 mins; Tissuelyser (Qiagen) 30Hz for 1 min, rotated and repeated. Methods were also tested on root samples taken from onion plants growing in the FOC quarantine field.

Optimising the Soil SV method

A number of modifications were tested (using soil from the Fusarium QF). These included an additional Fastprep step, extending the elution step to 5 mins and pre-heating the extraction buffer to 60°C. In addition, a pre-step method was tested (as used by Prof. Gary Bending, University of Warwick) whereby 5g of soil was added to 40ml of sterile water (in duplicate), the tubes inverted ten times, vortexed for 20s then centrifuged at 4000 rpm for 10 mins. The water was then poured away and the sample weighed before adding 1ml of water per gram of soil and combining the contents of the 2 tubes. The sample was then vortexed for 20s before transferring 1000 µl to a 2ml tube and centrifuging at 12,000 rpm for 10 mins. The supernatant was discarded then the contents of a Soil SV bead tube added and DNA extracted following the standard protocol. The success of modifications was tested using FOC primers in conventional PCR as previously described.

Results

Milestone 1.4: Test FOC diagnostic primers *in vitro* / 1.5 Test FOC diagnostic primers using soil and bulb samples

As there was only a single copy of SIX5 in FOC and this gene has only previously been identified in FOL (all races), this provided a good target for designing FOC diagnostic primers. Using a dilution series of genomic DNA, it was found that efficient amplification could be achieved with a minimum detection level of 0.5pg of DNA (Fig. 1). The efficiency of the assay was 100% with a slope of -3.309. An R^2 value of 0.99 showed that the assay is very accurate.

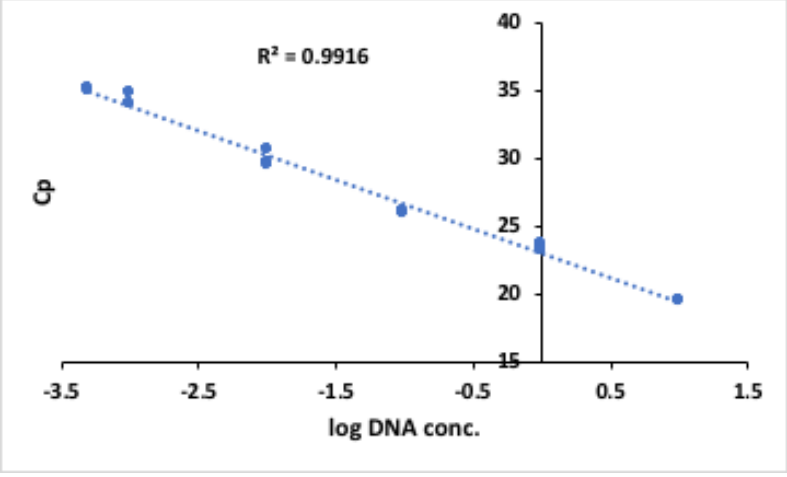


Fig. 1: Testing the FOC assay using a DNA dilution series from 10ng/µl – 0.5 pg/µl.

When tested against the panel of fungi / oomycetes, the primers were found to be highly specific to FOC with no amplification of DNA from any of the other fungi / oomycetes tested with the exception of an *F. oxysporum* isolate from leek which has been subsequently shown to be pathogenic on onion and hence designated as FOC (Fig. 2).

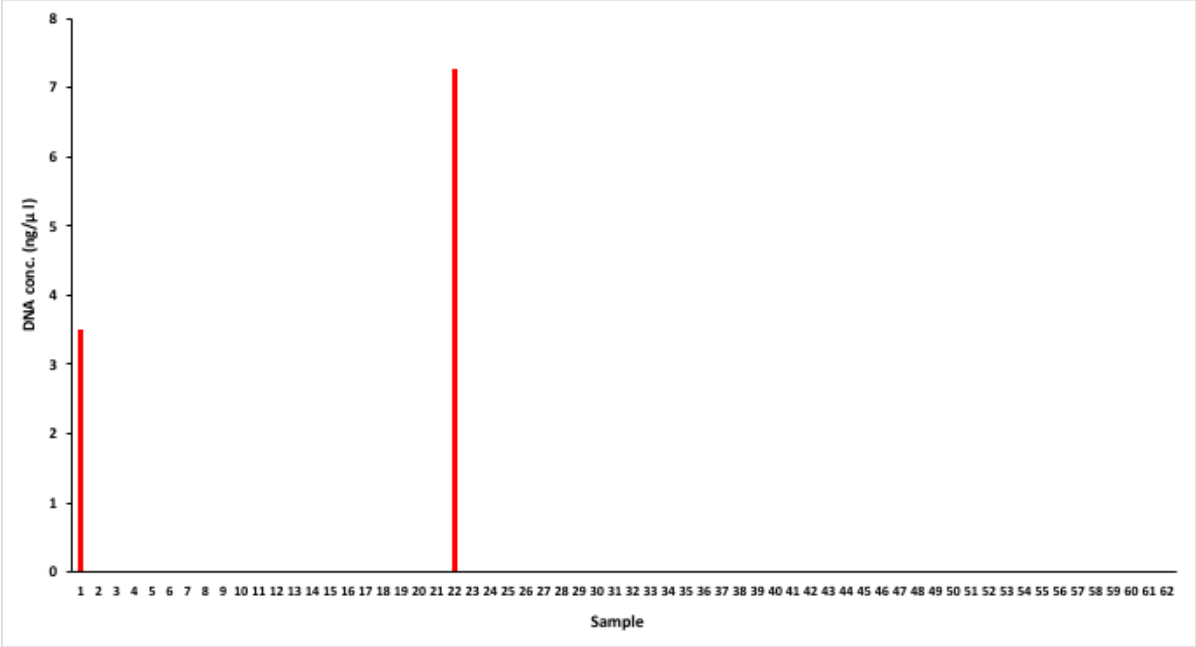


Fig. 2: Amplification of PCR products from a panel of 62 DNA samples following qPCR with FOC primers. Samples are listed in Table 2. Samples 1 and 22 are FOC isolates.

When tested on soil and root samples from the Fusarium QF, good amplification was observed in all samples (Fig. 3), illustrating the consistency of the assay. As expected, brighter bands were observed for root samples than soil samples.

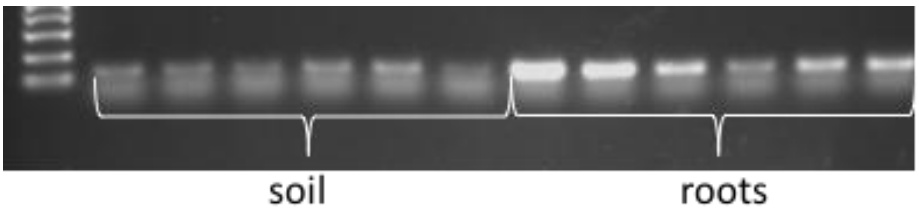


Fig. 3: Amplification of FOC from soil and root samples taken from the Fusarium QF using FOC-specific primers.

The assay was used to monitor FOC levels in the Fusarium QF at Wellesbourne. The three beds monitored produced consistent results with a peak in FOC DNA levels in August, corresponding to the warmest time of year and likely peak time for infection. FOC DNA was

detectable in every sample tested (Fig. 4). In addition, the 4 samples taken from a commercial field 12 months after a FOC infected onion crop, were all positive for FOC using the assay (Table 3). DNA concentrations in these samples ranged from 4 – 26 pg/µl thus showing that the FOC assay can be used effectively in a commercial field.

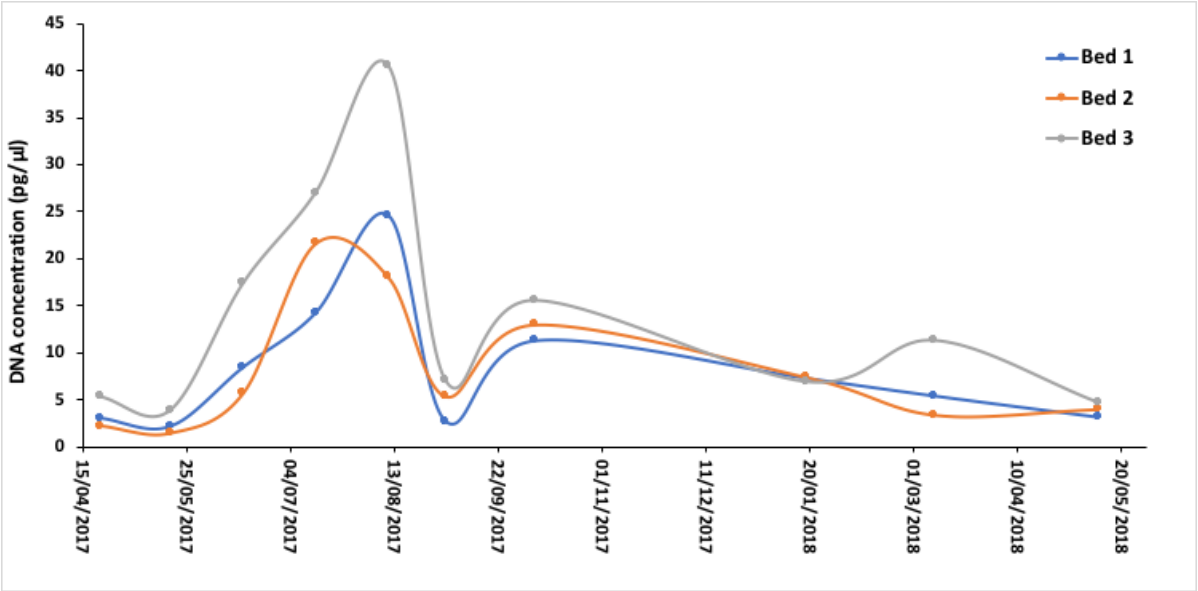


Fig. 4: Concentration of FOC DNA in field soil samples over a 12-month period (Fusarium QF).

Table 3: Concentration of FOC DNA in 4 samples taken from a commercial field, 12 months after an infection onion crop.

Sample	DNA conc. (pg/µl)
claypit1	25.8
claypit2	15.8
claypit3	5.4
claypit4	4.2

The FOC assay was also tested against commercial bulb samples. All clearly infected samples (Fig. 5) had 100% growth from basal plate sections on PDA and produced high FOC DNA concentrations (Table 4). All healthy samples had no growth on PDA and very low / undetectable DNA values. Samples with 'mild infection' showed variable results for both plating and qPCR, possibly reflecting the subjective nature of describing the symptoms. Samples which were selected as unknown or 'corky' led to no Fusarium growth on PDA and very low / undetectable DNA levels leading to the conclusion that a corky appearance is not

a symptom of FOC infection. The correlation analysis showed a strong correlation between DNA concentration (log) and growth on PDA ($r = 0.86$, $P<0.001$, Fig. 6). It should be noted that plating on PDA almost always results in an all or nothing result.

Fig. 5: The range of basal rot symptoms observed on onion bulbs from VCS used to test a qPCR assay for FOC.

Table 4: Testing the FOC qPCR assay on samples of commercial onion bulbs from 2 stores. The percentage growth on PDA was calculated based on 5 sections of basal plate from the same onion bulb as the DNA was extracted from.

Site	sample details	DNA conc (pg/ul)	% Fusarium growth on PDA
1	healthy	1.0	0
1	healthy	below detection	0
1	healthy	below detection	0
1	healthy	below detection	0
1	infected	21133.3	100
1	infected	9420.0	100
1	unknown/corky	2.6	0
1	unknown/corky	below detection	0
1	unknown/corky	below detection	0
1	unknown/corky	below detection	0
1	unknown/corky	below detection	0
2	healthy	below detection	0
2	healthy	1.9	0
2	healthy	1.6	0
2	healthy	1.5	0
2	healthy	below detection	0
2	infected	2043.3	100
2	infected	3350.0	100
2	infected	4.5	100

2	infected	5630.0	100
2	infected	505.7	100
2	mild infection	below detection	0
2	mild infection	1803.3	100
2	mild infection	3.0	100
2	mild infection	below detection	20
2	unknown/corky	below detection	0
2	unknown/corky	2.4	0
2	unknown/corky	below detection	0
2	unknown/corky	below detection	0
2	unknown/corky	below detection	0

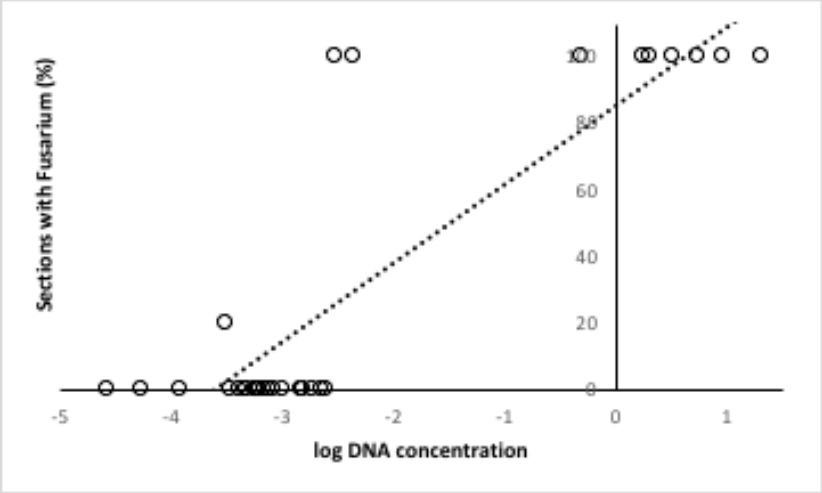


Fig. 6: Correlation between *Fusarium* growth from basal plate sections on PDA and DNA levels detected following qPCR.

Milestones 3.1 and 3.2 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 / 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.

The work for this milestone was tailored around the projects that have been active at Warwick during this fellowship. This has mainly revolved around work on *Fusarium* on a range of crops. Predominantly as part of AHDB project POBOF 452, experience of working with Fusarium on onion, leek, asparagus, lettuce, rocket, stocks, celery, fenugreek, coriander, pea, narcissus) was gained (see final report for details). This mainly included isolating the pathogen, molecular identification and in some cases pathogenicity testing. As reported in previous annual reports, experience was also gained of working with white rot on onion (*Sclerotium cepivorum*); cavity spot of carrot (*Pythium* species); downy mildews (e.g. onion downy mildew, *Peronospora destructor*); *Botrytis* spp.; onion pink root (*Setophoma terrestris*); *Stemphylium* on onion / narcissus and *Itersonilia* on parsnip. As part of an Innovate UK project (in collaboration with Nottingham University and PGRO) I have also gained experience of working with *Aphanomyces* and *Didymella* (part pf pea footrot complex). Through various projects throughout the duration of the fellowship, I have gained experience of working with *Sclerotinia* on a range of crops including brassica, carrot, lettuce, bean, peas and potatoes and now have a number of publications on *Sclerotinia*. In the past 12 months I have recently been able to observe and identify the symptoms of clubroot infection (caused by *Plasmodiophora brassicae*) on brassica and rocket. Additional details on specific work objectives can be found in previous annual reports (years 1-4).

Milestone 7: Test published (and unpublished) methods for extraction of DNA from larger quantities of soil.

<u>Woodhall method for DNA extraction from 500g of soil</u>

Both samples (replicates) extracted from soil spiked with FOC produced intact DNA (Fig. 7) at concentrations of 27 and 21 ng/µl. The 260/280 values were 1.62 and 1.46 where 1.8 indicates a pure DNA sample. However, when PCR was carried out, only weak bands were observed, especially considering the high level of spiking with FOC (Fig. 7). Using a single microlitre seems to be the optimum for PCR.

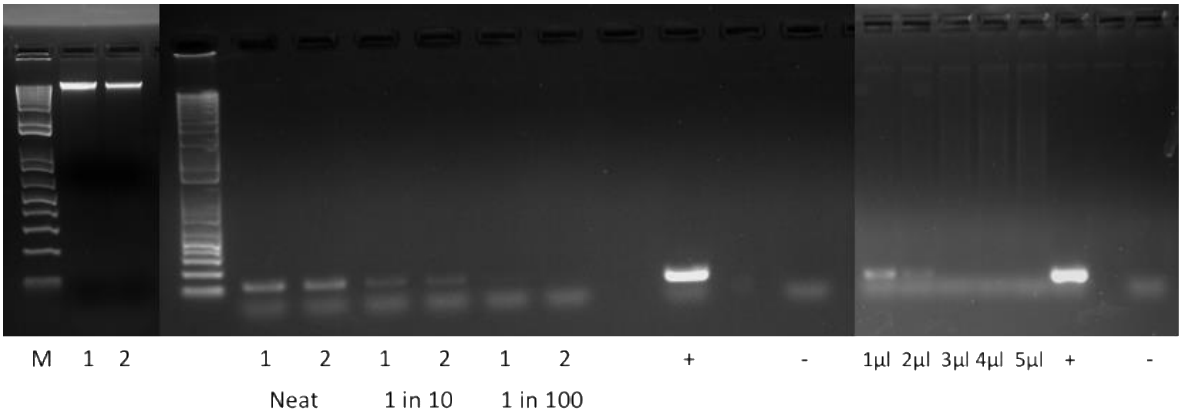


Fig. 7: Testing the Woodhall large scale DNA extraction method using spiked soil. 5µl of DNA samples 1 and 2 was loaded on a gel (left); PCR was carried out using FOC primers with DNA at various dilutions (middle and right).

The protocol was repeated using soil from the Fusarium QF. Processing 1400 µl of extract rather than 1000 µl resulted in an improved DNA yield (66ng/µl compared to 43ng/µl). Both samples had a good purity with 260/280 values of 1.72 and 1.73. Samples were compared to extraction using a standard soil DNA kit (PowerSoil) using PCR. The PowerSoil kit produced brighter bands than the Woodhall method despite only starting with 0.25g compared to 100g (Fig. 8). It was also consistent between the two replicate samples.

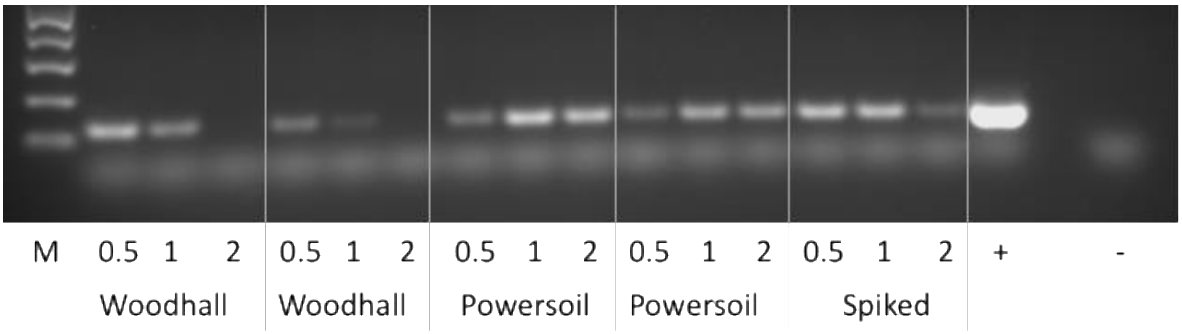


Fig. 8: Comparing the Woodhall large scale DNA extraction method with a commercial kit (PowerSoil) using samples taken from the Fusarium QF. PCR was carried out using FOC primers and 0.5 – 2 µl of DNA.

Due to weaker than expected amplification with samples from the Woodhall method, an extra clean-up step was added. Amplification was then compared to DNA extracted by the PowerSoil method, with or without a pre-concentration step. Even with the addition of a clean-

up step, no amplification of FOC DNA was observed during qPCR for any sample extracted by the Woodhall method (Table 5). As such, the Woodhall method was not explored any further. The addition of a pre-concentration step led to a slightly later CP value (32) than following the standard method (31).

Table 5: Comparison of the Woodhall large scale DNA extraction method with a commercial kit (PowerSoil) using qPCR. Soil samples that were not spiked were taken from the Fusarium QF.

Method	details	CP value
PowerSoil	pre-concentration	31.7
PowerSoil	pre-concentration	32.1
PowerSoil		30.8
PowerSoil		31.3
Woodhall	spiked soil	no amplification
Woodhall	spiked soil	no amplification
Woodhall		no amplification
Woodhall		no amplification
Woodhall	spiked soil, Zymo clean-up	no amplification
Woodhall	spiked soil, Zymo clean-up	no amplification
Woodhall	Zymo clean-up	no amplification
Woodhall	Zymo clean-up	no amplification

Devi method for economical DNA extraction from 1g of soil

All three replicate samples (extracted from the same bulk soil batch taken from the Fusarium QF) extracted by this method produced a good DNA yield (43 – 63 ng/ µl) with high purity (260/280 values from 1.72-1.79). However, no PCR amplification was observed when using either 1 or 2 µl of DNA per reaction (Fig. 9)

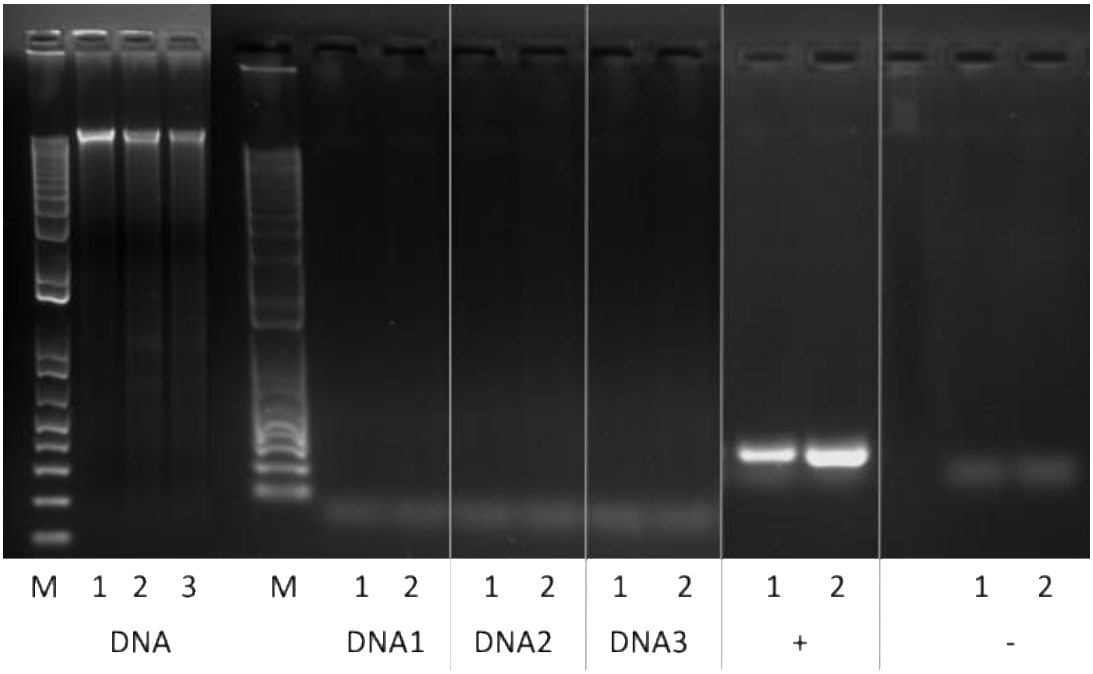
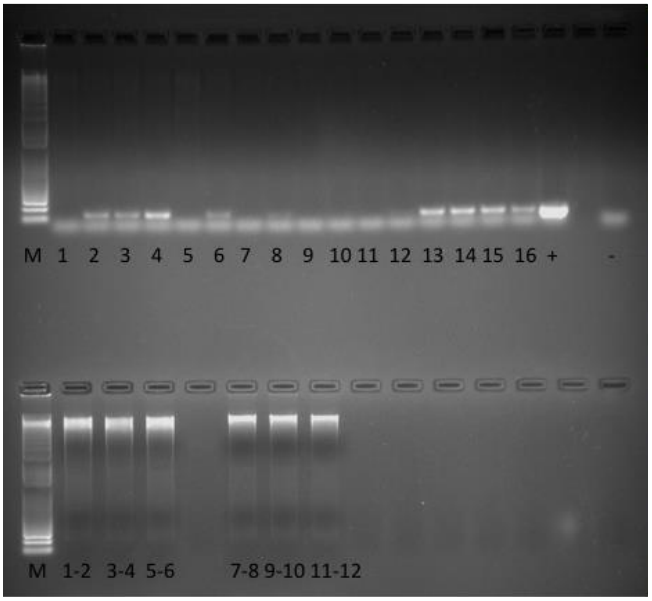


Fig. 9: Testing a published DNA extraction method (Devi et al 2015) on soil samples from the Fusarium QF using FOC primers. 3µl of DNA samples 1-3 was loaded on a gel (left); PCR was carried out using FOC primers with either 1 or 2 µl of DNA.

Testing a new soil DNA extraction kit – Soil SV

The soil SV method has the advantage that it is much more rapid than any of the other methods tested. The DNA yields (soil from Fusarium QF) were found to be 50 – 64 ng/µl with 260/280 values of 1.82-1.85 showing very pure DNA. This compares favourably with the PowerSoil method which generally yields DNA at around 10ng/µl (the two samples used here were 7.8 and 11.6ng/µl). When compared with the PowerSoil method, amplification was similar although it was noted that 0.5 µl of DNA was optimal with the soil SV kit compared to 1 µl with the PowerSoil kit (Fig. 10). Homogenisation by vortexing was ineffective.



Sample	Kit	Homogenisation	DNA added
1	Soil SV	Fastprep	1µl
2	Soil SV	Fastprep	0.5µl
3	Soil SV	Fastprep	1µl
4	Soil SV	Fastprep	0.5µl
5	Soil SV	Fastprep	1µl
6	Soil SV	Fastprep	0.5µl
7	Soil SV	Vortex	1µl
8	Soil SV	Vortex	0.5µl
9	Soil SV	Vortex	1µl
10	Soil SV	Vortex	0.5µl
11	Soil SV	Vortex	1µl
12	Soil SV	Vortex	0.5µl
13	Powersoil	Fastprep	1µl
14	Powersoil	Fastprep	0.5µl
15	Powersoil	Fastprep	1µl
16	Powersoil	Fastprep	0.5µl

Fig. 10: Comparing the Soil SV kit with the PowerSoil kit using the FOC assay and either 0.5 or 1 µl of DNA as a template. 5 µl of DNA was also run on a 1.2% gel (bottom). All soil samples were taken from the Fusarium QF.

When the Soil SV kit was compared with the FastDNA™ method, similar amplification was observed (Fig. 11).

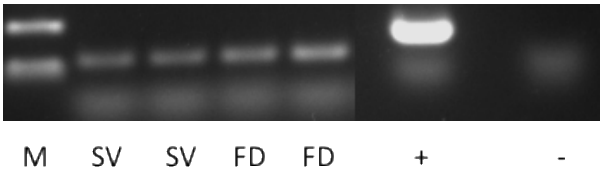


Fig. 11: Comparing the Soil SV (SV) kit with the FastDNA™ (FD) kit using the FOC assay. All soil samples were taken from the Fusarium QF.

Optimising the Soil SV method

The addition of a pre-mixing step (adding water to a larger soil sample and processing through various steps) was directly compared with the standard protocol of sieving, mixing and drying soil. Using soil samples from the Fusarium QF, DNA yields were higher with the pre-mixing (44-63 ng/ µl compared to 22-32 ng/ µl). However, amplification was stronger in the samples extracted using the standard method (Fig. 12). In addition, the results were more consistent for the standard method, illustrating the utility of this method for pathogen monitoring.

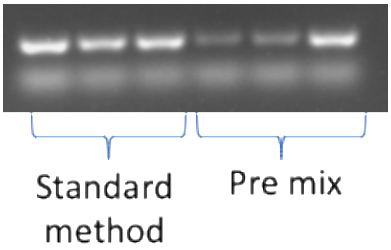


Fig. 12: Effect of the addition of a pre-mixing step to the Soil SV protocol on PCR results using FOC primers with soil samples taken from the Fusarium QF.

The Soil SV method was further optimised by testing the addition of differing volumes of sterile water to soil samples (from Fusarium QF) in the extraction tube. It was found that adding 200 µl produced the best amplification of FOC (Fig. 13). Without the addition of water, amplification was very poor (data not shown)

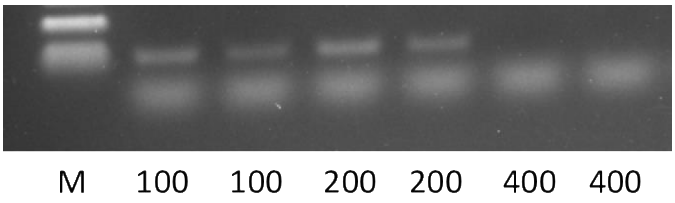


Fig. 13: Effect of adding 100- 400 µl of sterile water to the soil sample (from Fusarium QF) before extracting DNA using a Soil SV kit and carrying out PCR using FOC primers.

It was previously noted that the DNA should be diluted for optimal amplification. Therefore, a range of dilutions were tested. It was found that a 1 in 6 dilution was optimal (Fig. 14).

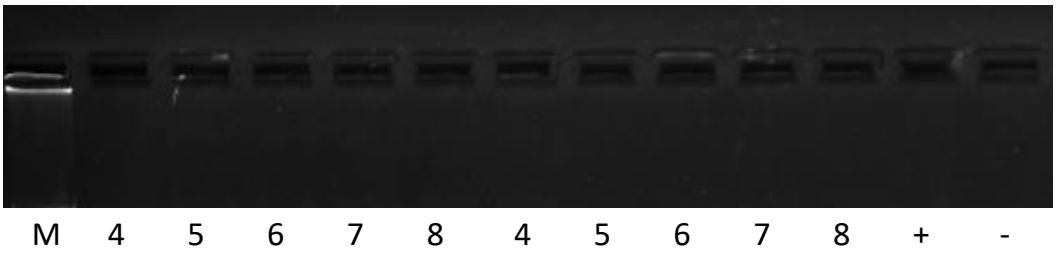


Fig. 14: Testing dilutions of DNA extracted from the Fusarium QF by the soil SV method using the FOC PCR. Dilutions ranged from 1 in 4 to 1 in 8.

Additional optimisations included adding an extra Fastprep step and extending the elution time to 5 mins. Increased DNA yields were observed with the modifications (Table 6). In addition, combining an extra Fastprep step with an extended elution step produced better

amplification on conventional PCR (Fig. 15). Preheating either the extraction buffer or elution buffer had no effect on the amplification (data not shown).

Table 6: DNA yields after modifying the Soil SV method. All soil samples were taken from the Fusarium QF.

Method	Method number	Yield (ng/µl)
Standard	1	19.7
Standard	1	23.7
3 x Fastprep	2	38.5
3 x Fastprep	2	33.3
Standard + 5 min elution	3	33.2
Standard + 5 min elution	3	27.2
3 x Fastprep + 5 min elution	4	35.0
3 x Fastprep + 5 min elution	4	35.9

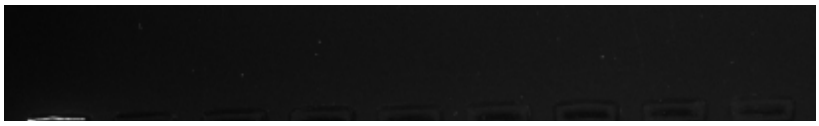


Fig. 15: Comparing modifications of the Soil SV method. method 1 = standard; method 2 = 3 x Fastprep; method 3 = standard + 5 min elution; method 4 = 3 x Fastprep + 5 min elution. PCR was carried out using FOC primers. Soil samples were taken from the Fusarium QF.

Samples extracted from the Fusarium QF by this method have been tested using qPCR with cq values of 30-35 cycles observed. This is comparable with the PowerSoil method (Table 5). DNA was extracted from soil samples taken at several time-points over a 12 month period and all qPCR results were positive (Fig. 4).

Discussion

Milestone 1.4: Test FOC diagnostic primers *in vitro* / 1.5 Test FOC diagnostic primers using soil and bulb samples

A high quality, long-read assembly of the FOC isolate FUS2 genome has been greatly beneficial for diagnostic assay development. PCR primers were designed based on the SIX5 single copy pathogenicity gene which is present in all FOC isolates and absent in all non-pathogenic *F. oxysporum* (Taylor et al. 2016). Furthermore, with the exception of FOL, no other *F. oxysporum* f.sp. or any other fungal species has been reported to contain homologues of SIX5. Specificity testing confirmed that the PCR assay only amplifies DNA from FOC. In addition, the assay can accurately detect FOC DNA at concentrations as low as 0.5pg/µl, something which is essential of use on soil samples. Testing on soil, root and bulb samples showed that the assay is both accurate and reproducible and could be of great benefit for the onion industry for testing FOC DNA levels in a field as well as testing onion bulbs prior to storage. The assay was able to detect FOC in a commercial field, 12 months after an infected onion crop showing its potential use in the future. Further development is planned with a more comprehensive set of commercial bulb samples. A FOC-specific assay based on the SIX3 gene has been previously reported (Sazaki et al 2015). However, the FOC genome analysis revealed two copies of SIX3 which may mean that some isolates may only carry a single copy, complicating any quantitative analyses. In addition, when we tested this SIX3 based assay, amplification was inefficient, possibly suggesting some secondary structure in the product (data not shown).

Milestone 7: Test published (and unpublished) methods for extraction of DNA from larger quantities of soil.

Various methods were tested for extraction of DNA from large quantities of soil (e.g. Woodhall et al 2012). Whilst these methods all produced good DNA yields, PCR results were disappointing and did not compare well with kit-based methods. This is likely due to the inefficient removal of inhibitors such as humic acid as even very small amounts of such substances can inhibit PCR (Schrader et al 2012). After many rounds of testing and optimisation, it was found that the Soil SV kit produces consistent results. The chosen method avoids the need to extract from a large volume of soil by sampling a larger volume before drying, sieving to a very small size and homogenising thoroughly. The addition of an extra Fastprep step in the extraction and an extended elution step also produced improved PCR results. The added advantage of the Soil SV method is that it is very rapid compared to all the other methods tested and as such DNA can be extracted from up to 30 samples in as little

as 2 hours. In addition, we have done preliminary testing on a range of soil types and this method was effective for all samples tested. The question will always remain about the 'patchy' nature of a pathogen in the field, and sampling strategies must be rigorous in order to capture the true nature of a pathogen in the field.

Conclusions

- A high quality FOC genome sequence has provided crucial genomic information for this pathogen and allowed the design of a specific diagnostic assay based on a pathogenicity gene (SIX5).
- This assay was shown to be highly reproducible and is fully quantitative.
- The assay was tested against a panel of 62 fungi / oomycetes and shown to be highly specific
- The assay was tested on soil, root and bulb samples and shown to produce consistent amplification
- Field monitoring showed a peak in FOC DNA levels in August, and the pathogen was detectable in a commercial field 12 months after an infected onion crop
- A range of methods for DNA extraction from soil were tested and an optimised method, based on a commercial kit, was developed
- Large scale extraction methods produced a good yield of DNA but PCR results were disappointing, likely due to inefficient removal of inhibitors.
- This optimised method produced consistent results when tested for detection of FOC using the FOC-specific primers.

Knowledge and Technology Transfer

- Visited an asparagus field and met with Phil Langley. Exchanged knowledge on Fusarium crown rot of asparagus (3rd Oct 2017)
- Hosted a visitor from Bayer USA – various meetings and exchange of materials (Oct 2017)
- Attended UK carrot and onion conference in Nottingham (14th Nov 2017)
- Attended seminar by Matt Back (Harper Adams University) on biofumigation (30th Nov)

- Attended and gave talk (Update on diagnostics for lettuce Fusarium wilt and discussion on sampling and monitoring) at Lettuce Fusarium workshop in Skelmersdale (14th Dec 2017)
- Visited Enza Zaden in the Netherlands to discuss lettuce Fusarium wilt (8th Jan 2018)
- Skype meeting with Rijk Zwaan to discuss lettuce Fusarium wilt (24th Jan 2018)
- Attended and gave talk (*Fusarium oxysporum*: understanding and combatting a highly aggressive, global plant pathogen) at Crop Science workshop, Warwick University (1st Feb 2018)
- Attended Warwick University School of Life Sciences industry day (28th Feb 2018)
- Skype meeting with Pietro Spanu to discuss Fusarium on stocks (5th March 2018)
- Attended and gave talk (Update on lettuce Fusarium wilt: minimising impact on the UK lettuce industry) at Outdoor and Protected Leafy Salad Technical Day at Stockbridge House (20th March 2018)
- Hosted guest seminar by Steven Penfield (John Innes) on seasonal sensing by plants (24th May 2018)
- Visited rocket grower to discuss Fusarium problems (4th June 2018)
- Attended Crop Science workshop, Warwick University (7th June 2018)
- Hosted guest seminar by Malcolm Hawkesford (Rothamsted) on scanalyzers and drones (5th July 2018)
- Hosted guest seminar by Max Newbert (Syngenta) on RNAi (19th July 2018)
- Attended and gave talk (Understanding the genetic control of pathogenicity and resistance to *Fusarium oxysporum* in onion) at the 10th Australasian Soilborne Diseases Symposium in Adelaide (4th – 7th Sept 2018)
- Attended and gave talk (Lettuce Fusarium wilt: potential management options) at Growing media developments in vegetable propagation in Doddington, Cambs (9th Oct 2018)
- Attended and gave talk (Lettuce Fusarium wilt: potential management options) at BLSA Protected R & D Committee meeting in Warwick (16th Oct 2018)
- Hosted a guest seminar on Fusarium diseases by Kim Hammond-Kosack, Rothamsted (18th Oct 2018)

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