Horticultural Fellowship Awards

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 2015 (Year 2)
Previous report:	Annual Report, October 2014 (Year 1)
Fellowship staff: ("Trainees")	Dr John Clarkson & Dr Andrew Taylor
Location of project:	Warwick Crop Centre
Industry Representative:	
Date project commenced:	1st November 2013
Date project completed (or expected completion date):	30th October 2018

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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.

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Signature: **Date: 29/10/15**

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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4) To synthesise existing unpublished work/data generated by Dr Dez Barbara.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	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		
1.3 Bioinformatic analyses of FOC isolate genomes and identification of potential primers for FOC diagnostics (end Yr 4).	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 *S. cepivorum*	31/10/16		
1.7 Check existing *P. 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		31/10/16
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	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	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.	31/10/17		
6.1. Contribute to writing at least one research proposal	31/10/17		
6.2. Initiate at least two research proposals and obtain funding for one.	31/10/18		

* All pathogens except *Botrytis squamosa* were isolated. See explanation below.

Summary of Progress

1.1: The pathogenicity of 33 *F. oxysporum* isolates (32 from onion) was tested using replicated assays on both seedlings and bulbs. All isolates were characterised through sequencing of housekeeping genes and putative effector genes and eighteen isolates were found to be pathogenic in all tests. Although an incomplete relationship between housekeeping gene sequence and virulence of isolates was observed, there was a very strong relationship between the presence of a putative effector gene and the ability of isolates to cause disease.

2.1: New isolates of *Sclerotium cepivorum* (causing white rot) *Peronospora destructor* (causing downy mildew) and *Botrytis allii* / *aclada* (causing neck rot) were collected from onions across the UK. Samples with symptoms of *Botrytis squamosa* (leaf blight) infection were collected from seven locations but the pathogen did not grow in culture, possibly due to in-field fungicide sprays.

2.2: Molecular characterisation of new pathogen isolates from 2.1 has begun and an efficient DNA extraction protocol identified and tested.

3.1/3.3: Disease areas were developed in the Wellesbourne Quarantine Field for *Fusarium* and white rot on onion and *Sclerotinia* on lettuce using artificial inoculation.

4.1: Carrot virus sequences were collated, aligned and phylogenetic trees constructed for parsnip yellow fleck virus (PYFV), anthriscus yellows virus (AYV) and carrot redleaf virus (CRLV).

5.4: Work-shadowing was carried out with Hobson Farming and PGRO.

Milestones not being reached

1.9: Quantification of *P. violae* in soil samples has not been completed due to some technical issues. Although the qPCR assay we currently have works very well, it is not yet 100% specific for *P. violae*. However, we have recently sequenced the *P. violae* genome which will allow us to improve the assay and thus this milestone will be completed in the next year.

Do remaining milestones look realistic?

Yes

Training undertaken

- Work-shadowing with Rodger Hobson (Hobson Farming) (14-15 Jan 2015)
- Attended and presented a poster at the new technologies for crop improvement workshop in Antalya, Turkey (22-27 Feb 2015).
- Attended a seminar on 'Grantsmanship' by Mark Pallen from the Warwick University Medical School. This provided excellent tips for grant writing and success (23 March 2015)
- Attended the BCGA technical seminar at PGRO (26 March 2015)
- Presented a seminar in the Warwick Crop Centre seminar series (16 April 2015)
- Attended a seminar by Karl Ritz 'New views of the underworld: visualising the structure and functioning of soil communities' (14 May 2015)
- Attended and gave an oral presentation at The International Symposium on Edible Alliaceae (ISEA) 2015 in Nidge, Turkey (21-25 May 2015)
- Attended a qPCR seminar at Warwick Life Sciences (21 July 2015)
- Visited 24 pea fields near Hull which were sampled for foot rot (*Fusarium* spp.) and aided PhD student with isolations (22-23 July 2015). Trained on identifying foot rot by staff from PGRO.
- Attended an AHDB Biofumigation meeting at Harper Adams University (9 Sept 2015)
- Attended and presented a poster at AHDB Studentship Conference (16-17 Sept 2015)
- Attended and manned Warwick Crop centre stand Elsoms Open Day (14 Oct 2015)
- Ongoing training with John Clarkson on isolating and handling different pathogens

Expertise gained by trainees

- A greater understanding of cavity spot and it's commercial impact
- A greater knowledge of new technologies (genomic) used in crop improvement
- A greater understanding of the grant writing process
- Improved presentation and communication skills
- Improved knowledge of qPCR and optimisation
- The ability to recognise pea foot rot in the field and isolate the causal pathogen
- The ability to recognise a range of onion pathogens in the field and isolate them
- Improved knowledge of biofumigation
- The ability to recognise *Fusarium* infection on rocket and isolate the pathogen (gained by visiting a grower at Fosse Way Farms)

- Inoculating on a field scale- oversaw the inoculation of fields with *Sclerotinia sclerotiorum* and *Fusarium oxysporum*.

Other achievements in the last year not originally in the objectives

- A paper entitled: 'Growth and nutritional responses to arbuscular mycorrhizal fungi are dependent on onion genotype and fungal species' was accepted and published in Biology and Fertility of Soil.
- A paper entitled: 'Identification of pathogenicity-related genes in *Fusarium oxysporum* f. sp. *cepae*' was accepted for publication in Molecular Plant Pathology (pending revisions)
- Awarded a fully-funded place (following a successful application) at the new technologies for crop improvement workshop in Antalya, Turkey (22nd-27th Feb 2015)
- Successfully developed disease areas in the Wellesbourne Quarantine Field for *Fusarium* and white rot on onion and *Sclerotinia* on lettuce. These will be valuable resources for future projects and/or for commercial trials. See Science section results for further details.

Changes to Project

Are the current objectives still appropriate for the Fellowship?

Based on the results obtained by Lauren Chappell (BBSRC Case Studentship) it appears that *Itersonilia* is only a minor pathogen of parsnip and *Mycocentrospora* / *Cyclindrocarpon* are more prevalent in cankers. Therefore, it seems appropriate to change objectives 1.10-1.12 to develop tools for these pathogens as opposed to *Itersonilia*. A small modification to Objective 2.1 is also suggested; due to problems isolating *B. squamosa* and the fact that is only listed as medium priority on the BOPA research priorities, it is proposed that no further isolations are attempted. However, some isolates have been located in historic collections and these could be characterised using PCR. All other objectives are still appropriate. A new objective is also suggested; molecular characterisation of *F. oxysporum* f. sp. *narcissi* isolates. This will involve PCR amplification and sequencing of effector genes in a range of isolates (work to be carried out in year 3 to be completed by 31/10/16).

GROWER SUMMARY

Headline

The pathogenicity of a set of *Fusarium oxysporum* isolates from onion was determined. A strong correlation between the presence of an effector gene and the ability of isolates to cause disease on seedlings or bulbs was observed. This gene (and others) may therefore form the basis of a future diagnostic test for *Fusarium oxysporum* f.sp. *cepae* (FOC) infecting onion.

Background

Onion diseases

Onion is a crucially important crop both in the UK and worldwide. Losses due to fungal pathogens can be devastating and annual losses due to Fusarium basal rot alone are estimated at £10-11 million. The pathogen causes symptoms at every stage of crop development and can occur in stores even when seemingly symptomless bulbs are harvested. Therefore, understanding the pathogen and developing diagnostic tools would be hugely beneficial for future control and monitoring of FOC.

Carrot diseases

Two major virus complexes can affect carrot / parsnip; parsnip yellow fleck virus (PYFV) / anthriscus yellows virus (AYV) and carrot redleaf virus (CRLV) / carrot mottle virus (CMoV) / carrot redleaf associated RNA (CLRaRNA). The incidence of these viruses on wild hosts was previously being investigated by Dez Barbara as they potentially provide a means of survival of viruses between crops and hence may be important sources of inoculum or pathogen vectors for crop plants. The data from these studies however had not been collated and summarised.

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 applied 6 weeks after drilling but losses due to the disease can still be high. In order to understand the dynamics of the pathogen and improve management, a specific and quantifiable diagnostic is required.

Summary

Onion diseases

A set of 33 *F. oxysporum* isolates from UK onions were characterised through pathogenicity testing on onion seedlings and bulbs. These assays clearly distinguished pathogenic and non-pathogenic isolates, which are common soil inhabitants. DNA was extracted from all isolates and a set of genes analysed / sequenced. A partial correlation between the sequence of housekeeping genes ('standard' genes that are always expressed and present in all isolates) and pathogenicity on onion bulbs and seedlings was observed. However, there was a very strong correlation between the presence / absence of an effector gene (*SIX7*) and pathogenicity. Furthermore, the sequence of this gene is unique to FOC allowing it to be distinguished from other forms of *F. oxysporum* pathogenic on different crops and hence has the potential as the basis for a future diagnostic test. Such a test would allow field / stored onions and sets to be assessed for levels of FOC and potential risk of disease. Other effector genes in FOC are also being investigated in a related BBSRC HAPI project.

Plants with symptoms of onion white rot and downy mildew were sampled and the pathogens isolated. DNA was extracted and molecular characterisation is in progress. Isolates associated with *Botrytis* neck rot (*B. allii* and *B. aclada*) are also being characterised. These new fungal isolates are now in long-term storage and will provide a resource for future resistance screening or other work.

Carrot diseases

Wild umbellifer hosts were tested for the presence of carrot / parsnip virus RNA and positive samples were sequenced. The analysis of CRLV sequences showed that the vast majority of genotypes from carrot were quite different from those from the wild hosts. However, some samples from carrots did have the same genotype as those from wild hosts suggesting that transmission to carrots from one of many potential wild hosts could potentially pose a future problem. Overall, wild hosts do not represent a significant source of CRLV inoculum for carrots and hence control approaches should focus on the crop. In contrast, many of the PYFV genotypes found in carrot were also found in cow parsley and hogweed suggesting that wild umbellifers present a source of inoculum. Control measures for PYFV should take into account wild hosts but further work is needed to investigate the changes in virus frequency in the most important wild umbellifers over time. Finally, AYV genotypes were found in all wild hosts but not carrot. This supports previous findings that carrot is not a host for AYV and it may not be a limiting factor as a 'helper' virus enabling PYFV infection in carrots.

A quantitative test (quantitative PCR) has been developed in order to assess levels of *P. violae* DNA in the soil throughout the growing season.

Financial Benefits

None to report at this time.

Action Points

None to report at this time.

SCIENCE SECTION

Introduction

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). *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 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. Although *F. oxysporum* is an important pathogen, non-pathogenic isolates also commonly occur in the soil as saprophytes while some have been identified as biocontrol agents and endophytes. 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 and mobile pathogenicity chromosomes in *F. oxysporum* f. sp*. lycopersici*, the f. sp. infecting tomato (Lievens *et al* 2009; Ma *et al* 2010). If the genetic basis for pathogenicity could be similarly identified in FOC, then this

information could be utilised to provide molecular tools for distinguishing it from other f. spp. and non-pathogenic isolates hence potentially allowing detection in soil, onion seeds and sets as part of developing a risk assessment strategy for onion growers.

White rot

Onion white rot, caused by the fungus *Sclerotium cepivorum,* is one of the most important diseases of Alliums, causing major losses for onion and garlic growers worldwide (Woodall *et al* 2012). Initially, the root system becomes infected and as the infection progresses through the stem or bulb, leaf yellowing, stunting and plant death occurs (Crowe, 2008). The pathogen produces large numbers of sclerotia which can survive in the soil for up to 20 years, even without a plant host (Woodall *et al* 2012). These sclerotia germinate in response to volatile compounds released by a new *Allium* crop, leading to new infection (Entwistle, 1990).

Botrytis leaf blight / Botrytis neck rot

Botrytis species cause a range of diseases on onions including leaf blight and neck rot. Leaf blight is caused predominantly by *B. squamosa* leading to lesions which are whitish in color, 1 to 5 mm in length and surrounded by a white halo (Tremblay *et al* 2003). Conidia are the main source of inoculum and are produced at night. Neck rot can be caused by a number of species, the main characterised species being *B. allii*, *B. aclada* and *B. byssoidea* (Chilvers and du Toit 2006). Symptoms of neck rot include soft, watery decay, mycelial growth in the bulb and sclerotia on the outer scales (Khan *et al* 2013). Infection may be latent in the field and occur after several months of storage. The pathogen can enter the bulb through several routes including through flowers which can lead to seed transmission (Maude and Presly 1977).

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 turn into purple lesions (Brewster, 2008). This is followed by sporulation, seen as a grey furry mass on the leaves, and leaf senescence. Once infection has occurred, damage can only be minimised by fungicides. It is listed as a high research priority by the British Onion Producers Research and Development Committee.

Carrot diseases

Cavity spot of carrots

Cavity spot 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 applied 6 weeks after drilling which is not always effective. The biology of *P. violae* is poorly understood and it is difficult to isolate conventionally from soil. Moreover, whilst the pathogen is present in newly formed (and still closed lesions), once these are open and exposed to the soil secondary infections occur and the pathogen is difficult to isolate from mature lesions. A rapid and specific qPCR assay for *P. violae* and the other species potentially involved in cavity spot would be a valuable tool for further study of the disease.

Carrot / parsnip viruses

The role of wild plant hosts in the epidemiology and impact of plant diseases has largely been ignored, despite the fact that they often harbour the same pathogens and are often intimately associated with agricultural ecosystems. Wild hosts can provide a means of survival between crops and hence be important sources of inoculum or pathogen vectors for crop plants. In addition, they may harbour sources of pathogen variation which can affect the dynamics of plant disease epidemics. Two virus complexes affecting carrot / parsnip, parsnip yellow fleck virus (PYFV) / anthriscus yellows virus (AYV) and carrot redleaf virus (CRLV) / carrot mottle virus (CMoV) / carrot redleaf associated RNA (CLRaRNA), were previously being investigated by Dez Barbara (DEFRA project IF0188). The first complex is sporadic in carrot and parsnip but can be very important in some years. The second complex causes carrot motley dwarf disease every year but its importance varies according to the timing of infection. Both virus complexes involve dependent viruses. Although PYFV and AYV are encapsidated separately, PYFV is only transmitted when AYV is present as a helper virus. As AYV cannot infect carrots, all infection of carrots by PYFV arises as primary infections from a doubly infected wild host and cannot be subsequently spread from carrot to carrot. There is little information on the host range of either PYFV or AYV in wild plants. In the CRLV / CMoV complex, the latter virus is dependent on CRLV for transmission but in this case transencapsidation is involved (i.e. both viruses are contained within a single capsid coded for by CRLV). The occurrence, host range and genetic variation of the different virus biotypes, was investigated primarily in wild umbellifers.

Materials and Methods

Objective 1.1: Determine pathogenicity of a range of *F. oxysporum* isolates from onion and complete DNA sequencing of a range of housekeeping genes.

A set of 31 *F. oxysporum* isolates (Table 1) from UK onions and the non-pathogenic isolate Fo47 were assessed for their pathogenicity on onion seedlings using a previously published method (Taylor *et al* 2013). Briefly, isolates were grown on PDA for 10 days at 20°C, a spore suspension prepared in sterile distilled water (SDW) and adjusted to 1 x 10^6 spores ml^{-1}. Seeds were soaked in 5 ml of spore suspension for 1 hour before sowing in Levingtons F2 + S compost (Scotts) in modular trays which were placed in a temperature controlled glasshouse (25°C day, 18°C night, 16h photoperiod). Full details can be found in Taylor *et al* 2015.

The same 32 isolates were also assessed for pathogenicity on healthy, stored onion bulbs (cv. Napoleon) supplied by the Allium and Brassica Centre (Kirton, UK) as described by Taylor *et al* 2015. This method involves cutting off the basal plate, inoculating with agar plugs and scoring the area of bulb infected after 9 weeks. Three independent replicates (four onion bulbs per replicate) were set up for each *F. oxysporum* isolate and significant differences between isolate disease area data analysed using REML in GenStat.

Molecular characterisation of all 32 *F. oxysporum* isolates (as well as 21 other f. spp. of *F. oxysporum* and *Fusarium* species, Table 1) was carried out by sequencing three housekeeping genes, *Translation Elongation factor 1-alpha* (*EF-1α*), *RNA polymerase II second largest subunit* (*RPB2*) and *β-Tubulin* (*TUB2*) as described by Taylor *et al* 2015. The isolates were also tested for the presence of a known effector gene, Secreted in Xylem 7 (*SIX7*) as described by Taylor *et al* 2015. Sequences were aligned and phylogenetic trees constructed as described.

Table 1: *Fusarium* isolates used for pathogenicity testing and / or molecular characterisation in this study (taken from Taylor *et al* 2015).

Fusarium species	**Isolate code**	**Location**	**Origin**	**Source[1]**	**Year isolated**
Isolates used for pathogenicity testing and/or molecular characterisation					
F. oxysporum	A13	Bedfordshire, UK, site 1	onion bulb	V. Vagany, WCC	2009
F. oxysporum	A23	Bedfordshire, UK, site 2	onion bulb	V. Vagany, WCC	2009
F. oxysporum	A28	Bedfordshire, UK, site 2	onion bulb	V. Vagany, WCC	2009
F. oxysporum	A35	Bedfordshire, UK, site 3	onion bulb	V. Vagany, WCC	2009
F. oxysporum	F1	Bedfordshire, UK, site 4	onion bulb	V. Vagany, WCC	2010
F. oxysporum	195	Suffolk, UK, site 1	onion bulb	C. Handy, WCC	2012
F. oxysporum	224	Suffolk, UK, site 1	onion bulb	C. Handy, WCC	2012
F. oxysporum	244	Suffolk, UK, site 1	onion bulb	C. Handy, WCC	2012
F. oxysporum	A21	Suffolk, UK, site 2	onion bulb	V. Vagany, WCC	2009
F. oxysporum	R3	Suffolk, UK, site 3	onion bulb	V. Vagany, WCC	2009
F. oxysporum	M1	Suffolk, UK, site 4	onion bulb	V. Vagany, WCC	2010
F. oxysporum	M9	Suffolk, UK, site 4	onion bulb	V. Vagany, WCC	2010
F. oxysporum	G12	Suffolk, UK, site 5	onion bulb	V. Vagany, WCC	2009
F. oxysporum	K3b	Suffolk, UK, site 6	onion bulb	V. Vagany, WCC	2009
F. oxysporum	S1B	Essex, UK, site 1	onion bulb	V. Vagany, WCC	2009
F. oxysporum	A14	Essex, UK, site 2	onion bulb	V. Vagany, WCC	2009
F. oxysporum	A19	Essex, UK, site 2	onion bulb	V. Vagany, WCC	2009
F. oxysporum	NL70/7	Essex, UK, site 3	onion bulb	V. Vagany, WCC	2010
F. oxysporum	A1_2	Warwickshire, UK	onion bulb	V. Vagany, WCC	2008

F. oxysporum f. sp. *cepae*	FUS2	Lincolnshire, UK	onion bulb	R. Noble, East Malling Research	unknown
F. oxysporum	55	Lincolnshire, UK, site 1	onion bulb	C. Handy, WCC	2012
F. oxysporum	84	Lincolnshire, UK, site 1	onion bulb	C. Handy, WCC	2012
F. oxysporum	125	Lincolnshire, UK, site 1	onion bulb	C. Handy, WCC	2012
F. oxysporum	RO2	Lincolnshire, UK, site 2	onion bulb	V. Vagany, WCC	2010
F. oxysporum	FUS1	Nottinghamshire, UK	onion bulb	R. Noble, East Malling Research	unknown
F. oxysporum	FUS3	Nottinghamshire, UK	onion bulb	R. Noble, East Malling Research	unknown
F. oxysporum	PG	Cambridgeshire, UK	onion bulb	T. O'Neill, ADAS	unknown
F. oxysporum	CB3	UK	onion set	C. Handy, WCC	2012
F. oxysporum	HB17	UK	onion set	C. Handy, WCC	2012
F. oxysporum	HB6	UK	onion set	C. Handy, WCC	2012
F. oxysporum	JB4	UK	onion set	C. Handy, WCC	2012
F. oxysporum	NRRL 54002 (FO47)	France	Soil	ARS collection	unknown
F. oxysporum	HAZ	USA	onion bulb	H. van den Biggelaar, Hazera seeds	unknown
F. oxysporum	L2-1	UK, site 1	Leek	A. Taylor, WCC	2011
F. oxysporum	L9-1	UK, site 2	Leek	A. Taylor, WCC	2011
F. oxysporum	ATCC90245	Colorado, USA	pinto bean	ATCC collection	1990
F. oxysporum f. sp. *pisi* race 1	FOP1	UK	Pea	C. Linfield, WCC	unknown
F. oxysporum f. sp. *pisi* race 2	FOP2	UK	Pea	C. Linfield, WCC	unknown
F. oxysporum f. sp. *pisi* race 5	FOP5	UK	Pea	C. Linfield, WCC	unknown
F. oxysporum f. sp. *pisi*	NRRL36311	Netherlands	Pea	ARS collection	unknown
F. oxysporum f. sp. *lini*	FOLIN	UK	Linseed	C. Linfield, WCC	2010
F. oxysporum f. sp. *dianthi*	R207	UK	Carnation	C. Linfield, WCC	unknown
F. oxysporum f. sp. *narcissi*	FOXN7	UK	Daffodil	C. Handy, WCC	2013
F. oxysporum f. sp. *narcissi*	FOXN139	UK	Daffodil	C. Handy, WCC	2013
F. oxysporum f. sp. *freesia*	NRRL26990	Netherlands	Freesia	ARS collection	unknown

F. oxysporum f. sp. *freesia*	NRRL26988	Netherlands	Freesia	ARS collection	unknown
F. oxysporum f. sp. *cubense*	E421A3	UK	Banana	C. Nellist, WCC	unknown
F. avanaceum	L5	UK, site 1	Leek	A. Taylor, WCC	2011
F. proliferatum	A8	Beds, UK, site 3	onion bulb	V. Vagany, WCC	2009
F. proliferatum	A40	Beds, UK, site 3	onion bulb	V. Vagany, WCC	2009
F. proliferatum	SP1-2	Spain	onion bulb	V. Vagany, WCC	2010
F. redolens	NL96	Essex, UK, site 3	onion bulb	V. Vagany, WCC	2010
F. oxysporum f. sp. *lycopersici* race 3	NRRL54003 (MN25)	USA	Tomato	ARS Collection	unknown
Genome sequenced isolates used for comparison in molecular characterisation					
F. oxysporum f. sp. *pisi*	NRRL37622 (HDV247)	unknown	Pea	ARS Collection	unknown
F. oxysporum	NRRL32931 (FOSC 3-a)	USA	Human	ARS Collection	unknown
F. oxysporum f. sp. *conglutinans*	NRRL54008 (PHW808)	USA	Brassica	ARS Collection	unknown
F. oxysporum f. sp. *raphani*	NRRL54005 (PHW815)	France	Radish	ARS Collection	unknown
F. oxysporum f. sp. *radicis-lycopersici*	NRRL26381 (CL57)	USA	Tomato	ARS Collection	unknown
F. oxysporum f. sp. *cubense*	NRRL54006 (II5)	Indonesia	Banana	ARS Collection	unknown
F. oxysporum f. sp. *melonis*	NRRL26406	USA	Melon	ARS Collection	unknown
F. oxysporum f. sp. *vasinfectum*	NRRL25433	China	Cotton	ARS Collection	unknown
F. oxysporum f. sp. *lycopersici* race 2	NRRL34936 (FOL4287)	USA?	Tomato	ARS Collection	unknown
F. oxysporum	Fo5176	Australia	*Brassica oleracea*	ARS Collection	unknown

[1] WCC=Warwick Crop Centre, University of Warwick; ARS=Agricultural Research Service culture collection, USA; ATCC=ATCC culture collection

Objectives 1.8/1.9: Develop qPCR for *P. violae* using WCC Roche Lightcycler / Quantify *P. violae* in soil samples from HDC project FV405 and other samples where available

This work was carried out to support the studentship of Kathryn Hales (AHDB Studentship FV 432). An alternative qPCR approach for *P. violae* detection was tested due to lack of specificity with a previous assay (see year 1 report). A Taqman® probe (designed to be *P. violae* specific) was obtained from Mogens Nicolaisen (Aarhus University) and following initial optimisation, the probe was tested against a range of *Pythium* species. Reactions were set up in triplicate, as described in Table 2 using a qPCRBIO Probe Mix Lo-ROX (PCR Biosystems). The samples were loaded into a 384-well plate and reactions run on the LightCycler 480 Real-Time PCR system (Roche). Cycling conditions consisted of initial denaturation at 95ºC for 1 minute followed by 40 cycles of denaturation at 95ºC for 5 seconds and annealing and extension at 60ºC for 30 seconds, with a final 30 seconds of cooling at 40ºC. Additional primers were designed for standard qPCR and have been tested for specificity (see FV 432 year 1 report). These primers are based on the gene *OCM1*, a flagella-related gene.

Table 2: Taqman probe based qPCR reactions

Reagent	**Volume per reaction (µl)**
Probe mix (2x)	7.5
Forward primer (10µM)	0.6
Reverse Primer (10µM)	0.6
Probe (5µM)	0.8
DNA	1.0
Water	4.5

Objective 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)

Onion plant samples with symptoms of downy mildew (Fig. 1a), *Botrytis* leaf blight (Fig. 1b) and white rot (Fig. 1c) were collected from locations in Warwickshire, Cambridgshire, Norfolk and Lincolnshire (Table 4). For downy mildew, leaf sections were frozen at -80°C for storage. For white rot, a small amount of mycelium was removed from the bulb using a sterile scalpel and placed on PDA containing chlorotetracycline. This was subcultured after 2-3 days growth at 20°C, grown for a further 3 days and transferred to PDA slopes for storage at 5°C. For *Botrytis* leaf blight, isolations were attempted from leaves (7 locations) using three published

methods (Chilvers and du Toit 2006; Misawa and Takeuchi 2015; Carisse and Tremblay 2007). These methods involved incubating leaf sections on filter paper, tissue or PDA. All methods were attempted with and without surface sterilisation and were repeated. DNA from *Botrytis* species associated with neck rot was obtained from Dr. Steve Roberts. Live isolates will be obtained and cultured in the near future. In addition, isolations were made from infected bulbs from Wellesbourne and Loosegate, Lincolnshire. 5mm sections of infected tissue were cut out of the bulbs, surface sterilised in 70% ethanol for 30 secs, rinsed twice in SDW and placed on PDA containing chlorotetracycline. This was subcultured after 3 days growth, grown for a further 3 days and transferred to PDA slopes for storage.

Objective 2.2: Confirm identity and characterise isolates from 2.1 by gene sequencing

For Onion downy mildew, spore suspensions were prepared by adding a small volume of SDW to leaf sections and shaking manually. 2ml was then transferred to a screw-cap tube and centrifuged for 5 mins at 13,000 rpm. The supernatant was removed and DNA extracted using a method from Stephen Rehner (USDA-ARS, Beltsville, USA). 0.5g each of 0.1 mm zirconia ceramic beads and acid washed 2.5-3.5 mm glass beads were added to the tubes. Tubes were placed in a FastPrep machine (MpBio) for 20 sec at 4.5m/s. This step was repeated twice then 300µl of filter sterilised extraction buffer (2.1g sodiummetasilicate, 0.5g citric acid, 2.64ml 2-butoxy ethanol, 13.5ml 1M Tris-HCL pH 7.0, 194ml SDW) added. Tubes were shaken and incubated at 100°C for 10 mins before centrifuging at full speed for 10 mins. After this, 175µl of the supernatant was removed and used 1 in 10 diluted (in TE pH 8) for PCR.

For white rot, liquid cultures were set up by placing 3 agar plugs (5mm) in a 50ml tube containing 25ml of sterile PDB. These were incubated at 20°C for 7 days, rinsed in SDW and freeze-dried. DNA was extracted from freeze-dried mycelium using the method described for downy mildew

Figure 1: Symptoms of downy mildew (a), *Botrytis* leaf blight (b) and white rot (c).

Objectives 3.1/3.3: Gain experience with lettuce pathogens such as *B. cinerea* and *B. lactucae* / Gain experience of other pathogens such as *P. ultimum*, *O. neolycopersici* through existing projects

Additional work was done under this objective to develop disease areas in the Wellesbourne Quarantine Field for *Fusarium* and white rot on onion and Sclerotinia on lettuce using artificial inoculation

Creating an artificially infested FOC area

In April 2014, an area of the quarantine field at Warwick Crop Centre (0.175ha) was cultivated in preparation for inoculation. A shipment of approximately 2.5 tonnes of onions with basal rot symptoms was received from a failed store (courtesy of VCS Agronomy). The bulbs were spread out evenly across the field and isolations carried out (according to Taylor *et al* 2013) to confirm the presence of FOC. Molecular identification was carried out using PCR with primers for *EF-1α* (Taylor et al 2015). The field was then rolled to break open the bulbs.

Additional inoculum was prepared in flasks (isolate FUS2), using the method in Taylor *et al* 2013. This inoculum was quantified and mixed into 110kg of F2 + S compost (Levingtons) using a cement mixer and spread evenly across the field to give an estimated field dose of 6 x 10^3 cfu per ml. Rotting onion bulbs and the inoculum mixture were then lightly chopped and incorporated to a depth of approx. 10cm and the area irrigated regularly to encourage pathogen growth on onion material. Onion seed (a 50:50 mix of *Allium fistulosum* cv. Performer and *Allium cepa* cv. White Lisbon) was then drilled in the field in July and irrigation maintained for establishment. Disease was monitored and isolations made from symptomatic plants.

Reviving an artificially inoculated white rot area

Another area of the quarantine field at Warwick Crop Centre (0.25ha) had been inoculated with *Sclerotium cepivorum* in previous research over 10 years ago. To test whether this area was still infested, it was cultivated and prepared for drilling in May 2015 and onions sown (*Allium fistulosum* cv. Performer and *Allium cepa* cv. White Lisbon) in June. The area was irrigated and monitored for symptoms of white rot with suspected infected plants taken for isolations as described for Objective 2.1.

Creating an artificially infested Sclerotinia area

In May 2015, a third area of the quarantine field at Warwick Crop Centre (0.05 ha) was cultivated in preparation for lettuce planting. In June 2015, lettuce seed (cv. Senna) was sown in modular trays in a glasshouse and three weeks later, 6348 lettuces were transplanted into the field. After 7 weeks, the plants were inoculated as follows: sclerotia of *Sclerotinia sclerotiorum* (isolate L6) were bisected and placed on PDA. After 3 days these were subcultured onto fresh PDA and grown for a further 4 days. Lettuces were wounded and inoculated with 5 x 1cm plugs per plant (placed at several places in the heart). 806 out of the 6348 lettuces (12.7%) were inoculated in an even pattern across the field. Plants were irrigated after inoculation and when needed thereafter. The area was then monitored for disease symptoms.

Objective 4.1: Synthesise Dez Barbara's unpublished work on carrot/parsnip viruses

Virus sequences had been found in different wild umbellifer species collected from different areas of England and Scotland from 2010-2012 in Dez Barbara's work. .In this research, RNA had been extracted from each plant type and the virus sequences generated using the primers and RT-PCR conditions described by Vercruysse *et al* 2000. In the current project

the sequences were all collated and phylogenetic trees constructed using MEGA version 5.1 for parsnip yellow fleck virus (PYFV), anthriscus yellows virus (AYV) and carrot redleaf virus (CRLV) following alignment and editing. The trees were constructed using Maximum likelihood and the nucleotide substitution model which gave the best fit and the complete deletion option for missing data/gaps. Bootstrap values were calculated from 1000 replicates. Where possible, a sequence from a closely related virus was used to root the phylogeny. Any publically available sequences of the viruses examined were also included. Following a meeting with Adrian Fox (FERA) to discuss the results, the sequences and RNA samples were transferred to him with the aim of generating further data on detection of other carrot viruses for a joint publication.

Results

Objective 1.1: Determine pathogenicity of a range of *F. oxysporum* isolates from onion and complete DNA sequencing of a range of housekeeping genes.

In the onion seedling tests, significant differences were observed in the pathogenicity of the 32 *F. oxysporum* isolates for the two experiments using Napoleon and HZS onion cultivars (Fig. 2, $P<0.001$). The pathogenicity of each *F. oxysporum* isolate was highly correlated between the two cultivars ($r=0.97$, $P<0.001$). For both onion cultivars, 18 of the 32 isolates were significantly pathogenic. The remaining 14 isolates (including Fo47) were non-pathogenic, having little or no effect on seedling survival (Fig. 2). Two isolates (A1_2 and 55) caused significant seedling mortality on cv. Napoleon, but not on HZS. Over all the isolates, Napoleon was more susceptible to *F. oxysporum* than HZS. The most pathogenic *F. oxysporum* isolates on Napoleon and HZS were S1b (11.7% seedling survival) and isolate 125 (31.5% seedling survival, Fig. 2).

In the bulb test (cv. Napoleon), significant differences were observed in disease levels amongst the 32 *F. oxysporum* isolates ($P<0.001$; Fig. 3). Twenty-one pathogenic isolates resulted in 8.7-58.6% bulb area affected compared to the control (0%) while eleven non-pathogenic isolates (including Fo47) had no significant effect (0-5.0% bulb area affected). Of the 21 pathogenic isolates, A1_2 (8.7%) and HB6 (19.1%) showed low levels of pathogenicity while isolate 55 showed an intermediate level of pathogenicity (29.1%), which was significantly greater than A1_2 and HB6, but significantly smaller than the 18 highly pathogenic isolates. Highly significant correlations were observed between *F. oxysporum* isolate pathogenicity in the bulb test and the seedling tests with cv. Napoleon ($r=-0.91$, $P=0.001$) and cv. HZS ($r=-0.88$, $P=0.001$).

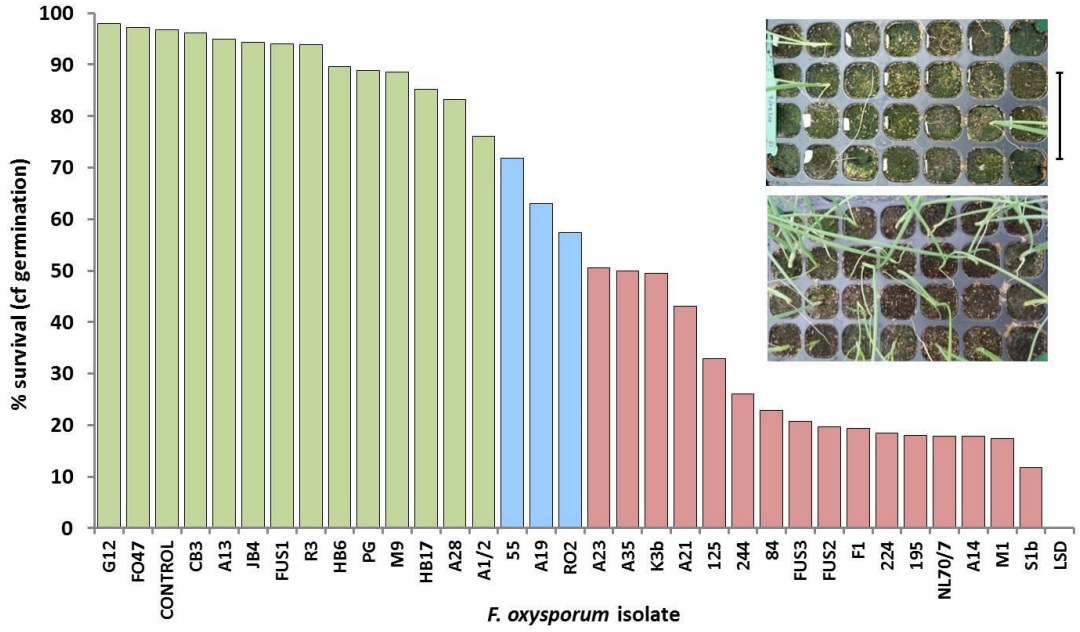


Figure 2: Pathogenicity of a set of *F. oxysporum* isolates on onion seedlings (after Taylor *et al* 2015). Green = non-pathogenic, red=pathogenic, blue=intermediate.

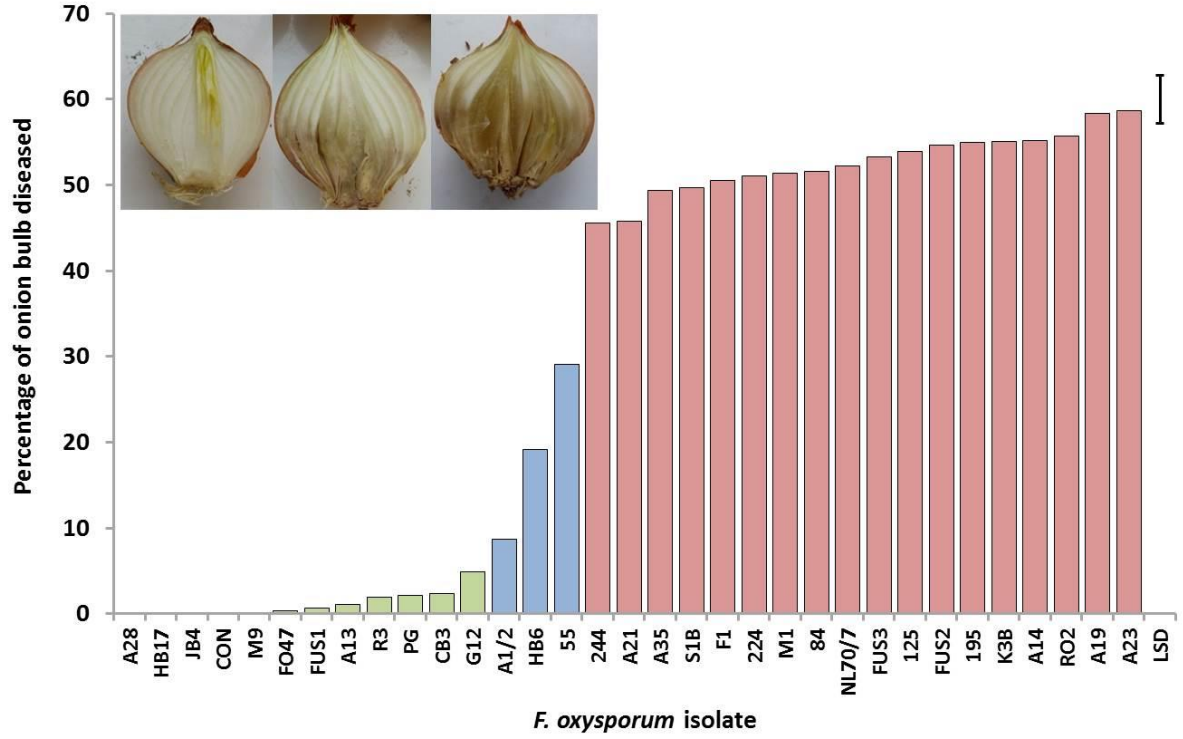


Figure 3: Pathogenicity of a set of *F. oxysporum* isolates on onion bulbs (after Taylor *et al* 2015) Green = non-pathogenic, red=pathogenic, blue=intermediate.

A concatenated phylogenetic tree based on EF-1α, RPB2 and TUB2 sequences resulted in the majority of the *F. oxysporum* isolates being separated into six clades (Fig. 4). Clade 1 contained all the isolates that showed some level of pathogenicity towards onion in either the seedling or bulb tests (or both) with the exception of A1_2. Clade 1 also included isolate L2-1 from infected leeks, which has also been shown to be pathogenic on onion bulbs. However, clade 1 also included the non-pathogenic isolate HB17 (from onion sets) and isolates of other f. spp. The non-pathogenic isolates were scattered throughout the tree. The other f. spp. were seen throughout the tree, with f. sp. cubense forming its own distinct clade. The other *Fusarium* species (*F. proliferatum*, *F. redolens* and *F. avenaceum*) formed distinct outgroups.

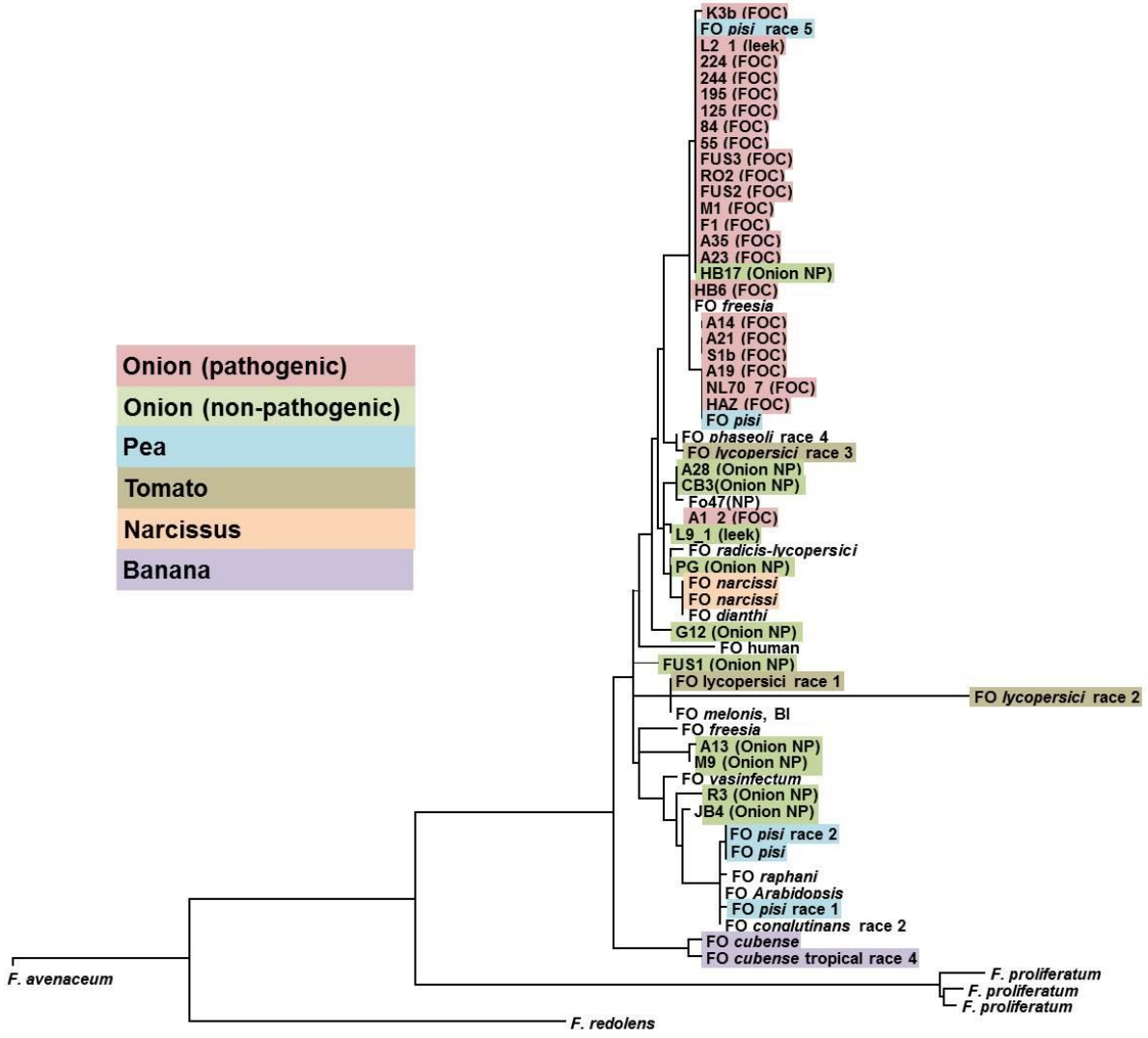


Figure 4: Maximum likelihood tree of *Fusarium* isolates from onion and other hosts based on a concatenated alignment of EF-1α, RPB2 and TUB2 genes. The tree is rooted through L5

(*F. avenaceum*) and this branch has been collapsed due to its distance from *F. oxysporum*. (After Taylor *et al* 2015).

There was a very strong correlation between the presence of the SIX7 gene and pathogenicity on onion (Table 3). All isolates that were pathogenic in all assays contained SIX7. In addition, an isolate from leek which has been shown to be pathogenic on onion also contained SIX7. All SIX7 sequences for the onion isolates were identical and were distinct from other *F oxysporum* f.spp. (Fig. 5).

Table 3: Correlation between presence of an effector gene (SIX7) and pathogenicity of *F. oxysporum* isolates against onion (Adapted from Taylor *et al* 2015).

***Fusarium* species**	**Host**	**Isolate code**	**Pathogenicity***	**Presence of SIX7**
F. oxysporum (FOC)	Onion	A23	B/S1/S2	+
F. oxysporum (FOC)	Onion	A19	B/S1/S2	+
F. oxysporum (FOC)	Onion	RO2	B/S1/S2	+
F. oxysporum (FOC)	Onion	A14	B/S1/S2	+
F. oxysporum (FOC)	Onion	K3B	B/S1/S2	+
F. oxysporum (FOC)	Onion	195	B/S1/S2	+
F. oxysporum (FOC)	Onion	FUS2	B/S1/S2	+
F. oxysporum (FOC)	Onion	125	B/S1/S2	+
F. oxysporum (FOC)	Onion	FUS3	B/S1/S2	+
F. oxysporum (FOC)	Onion	NL70/7	B/S1/S2	+
F. oxysporum (FOC)	Onion	84	B/S1/S2	+
F. oxysporum (FOC)	Onion	M1	B/S1/S2	+
F. oxysporum (FOC)	Onion	224	B/S1/S2	+
F. oxysporum (FOC)	Onion	F1	B/S1/S2	+
F. oxysporum (FOC)	Onion	S1B	B/S1/S2	+
F. oxysporum (FOC)	Onion	A35	B/S1/S2	+
F. oxysporum (FOC)	Onion	A21	B/S1/S2	+
F. oxysporum (FOC)	Onion	244	B/S1/S2	+
F. oxysporum (FOC)	Onion	55	B/S1	-
F. oxysporum (FOC)	Onion	HB6	B	-
F. oxysporum (FOC)	Onion	A1_2	B/S1	-
F. oxysporum	Onion	G12	-	-
F. oxysporum	Onion	CB3	-	-
F. oxysporum	Onion	PG	-	-
F. oxysporum	Onion	R3	-	-
F. oxysporum	Onion	A13	-	-
F. oxysporum	Onion	FUS1	-	-
F. oxysporum	Onion	M9	-	-
F. oxysporum	Onion	JB4	-	-
F. oxysporum	Onion	HB17	-	-
F. oxysporum	Onion	A28	-	-
F. oxysporum	-	FO47	-	-

F. oxysporum	Onion	HAZ	(+)	+
F. oxysporum	Leek	L2-1	(+)	+
F. oxysporum	Leek	L9-1	(-)	-
F. proliferatum	Onion	A8	(+)	-
F. proliferatum	Onion	A40	(+)	-
F. proliferatum	Onion	SP1-2	(+)	-
F. avenaceum	Leek	L5	(+)	-
F. redolens	Onion	NL96	(-)	-

*B=bulb assay, cv. Napoleon, S1=seedling assay, cv. Napoleon, S2=seedling assay, cv. HZS

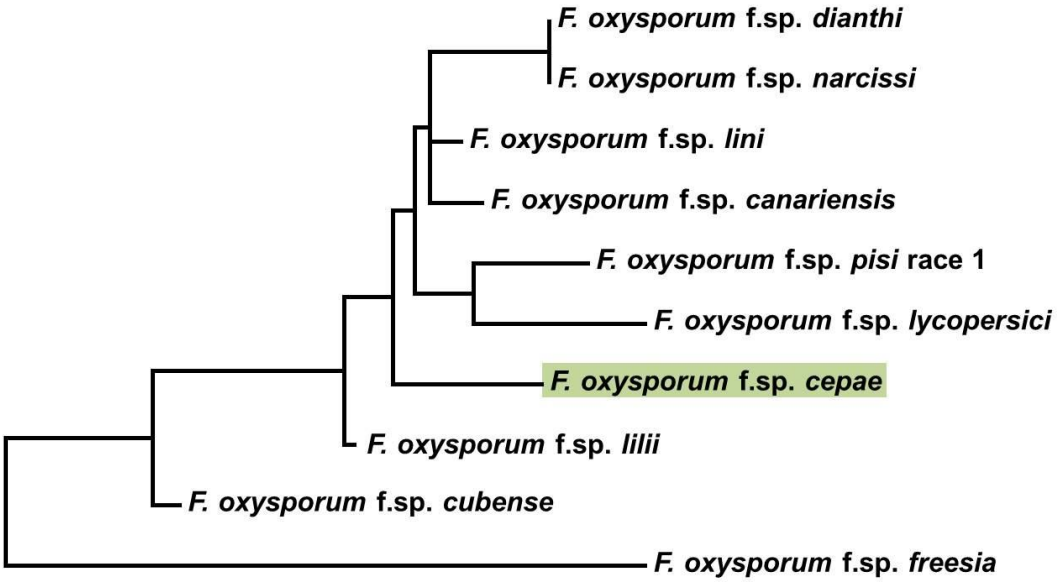


Figure 5: Maximum likelihood trees of *Fusarium* isolates from onion and other hosts based on SIX7 gene sequences (After Taylor *et al* 2015).

Objectives 1.8/1.9: Develop qPCR for *P. violae* using WCC Roche Lightcycler/ Quantify *P. violae* in soil samples from HDC project FV405 and other samples where available

The Taqman probe did not specifically bind to *P. violae* DNA and PCR products were also observed for *P. intermedium*, *P. irregulare*, *P. sylvaticum* and *P. sulcatum* (Fig. 6). However, these products occurred after a larger number of PCR cycles than for *P. violae*. No products were observed for *P. ultimum* or *P. lutarium*. Additional primers were designed and tested for standard qPCR using a published *OCM1* gene associated with (Robideau *et al* 2014) and showed a much higher level of specificity (see FV432 year 1 report). The only other *Pythium* species that was amplified with these primers was *P. intermedium* where amplification was very weak. Hence, as a practical tool, this could be suitable for monitoring *P. violae* dynamics. It was decided that using the qPCR to quantify *P. violae* in soil samples (Objective 1.9) should

be delayed until year 3 to allow extra time for assay optimisation. Soil samples are currently being tested for *P. violae* using standard PCR in project FV405 and will be retained to allow direct comparison with an optimised method.

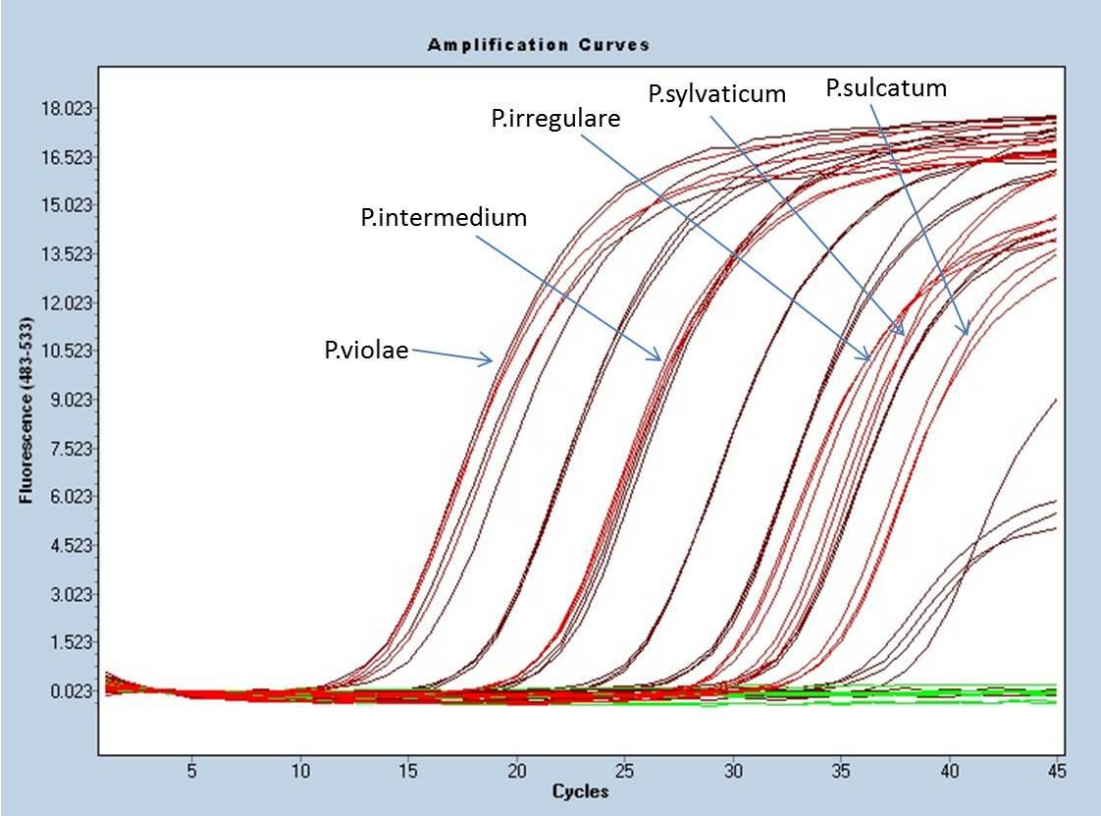


Figure 6: Specificity test for Taqman probe designed to specifically amplify *P. violae*. The brown lines are from a *P. violae* dilution series (10ng/µl to 10 fg/µl).

Objective 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)

Putative isolates of *P. destructor*, *S. cepivorum* and *Botrytis* species were collected from diseased onions form different UK locations (Table 4). DNA was successfully extracted from downy mildew spores (data not shown) and isolates of *S. cepivorum* and *Botrytis* were placed into long-term storage for DNA extraction and molecular identification in year 3.

Table 4: Collection locations and dates for onion pathogens isolated 2014-5.

Location	Date collected	Onion cultivar	Pathogen isolated	Isolate name (s)
Rugby, Warks	15/08/2014	Centro	Downy mildew	RU1
Rugby, Warks	15/08/2014	Hysky	Downy mildew	RU2
Cubbington, Warks	26/08/14	Salad onions	Downy mildew	CU1
Wellesbourne, Warks	29/08/14	Various	Downy mildew	WE1
Wellesbourne, Warks	29/08/14	Jaune des Cevennes	Downy mildew	WE2
Thetford, Norfolk	20/05/15	Toughball	Downy mildew	Ratpit
Thetford, Norfolk	20/05/15	Toughball	Downy mildew	LH
Loosegate, Lincs	11/06/15	Tornado	White rot	LG1-LG15
Aswick Grange, Lincs	10/06/15	Unknown	White rot	AR1-AR17
Littleport, Cambs site 1	12/08/15	Red Baron	White rot	DN1-DN6
Littleport, Cambs site 2	12/08/15	Coded	White rot	GS1-GS6
Rugby, Warks field 1	20/08/15	Sturon sets	White rot	RUG1-1, RUG1-2
Rugby, Warks, field 2	20/08/15	Sturon sets	White rot	RUG2-1 to RUG2-4
Methwold, Norfolk	25/08/15	Centro	White rot	JD1 and JD2
Wellesbourne, Warks	25/08/15	White Lisbon and Performer	White rot	WR1-WR6
Wellesbourne, Warks	27/05/15	Unknown (red onions)	Botrytis neck rot	B7
Loosegate, Lincs	11/06/15	Tornado	Botrytis neck rot	LGB

Objectives 3.1/3.3: Gain experience with lettuce pathogens such as *B. cinerea* and *B. lactucae* / Gain experience of other pathogens such as *P. ultimum*, *O. neolycopersici* through existing projects

Development of diseased areas in quarantine field

Fusarium: The onion bulbs which were incorporated into the basal rot field were shown to be infected with FOC through visual identification, culturing and sequencing of *EF-1α* PCR products. Eight *Fusarium* isolates were obtained from diseased onion plants grown in the

inoculated area, six of which were identified as *F. oxysporum* and had an identical *EF-1α* sequence to the isolate used for inoculation (FUS2). The remaining 2 isolates were identified as *F. solani*. Early observations of the onions that were drilled in this field suggests that some symptoms are appearing (Fig. 7)

White rot: Very clear disease symptoms were observed on onions grown in the white rot area indicating that the inoculum is still viable (Fig. 8). Isolations were made from affected plants for storage as listed in Table 4.

Sclerotinia: The inoculation of lettuces with *S. sclerotiorum* was successful with severe plant symptoms observed (Fig. 9) and large numbers of sclerotia produced in the diseased area.

Figure 7: Initial symptoms of *Fusarium* basal rot in an inoculated field

Figure 8: Symptoms of white rot (*S. cepivorum*) in a previously inoculated field

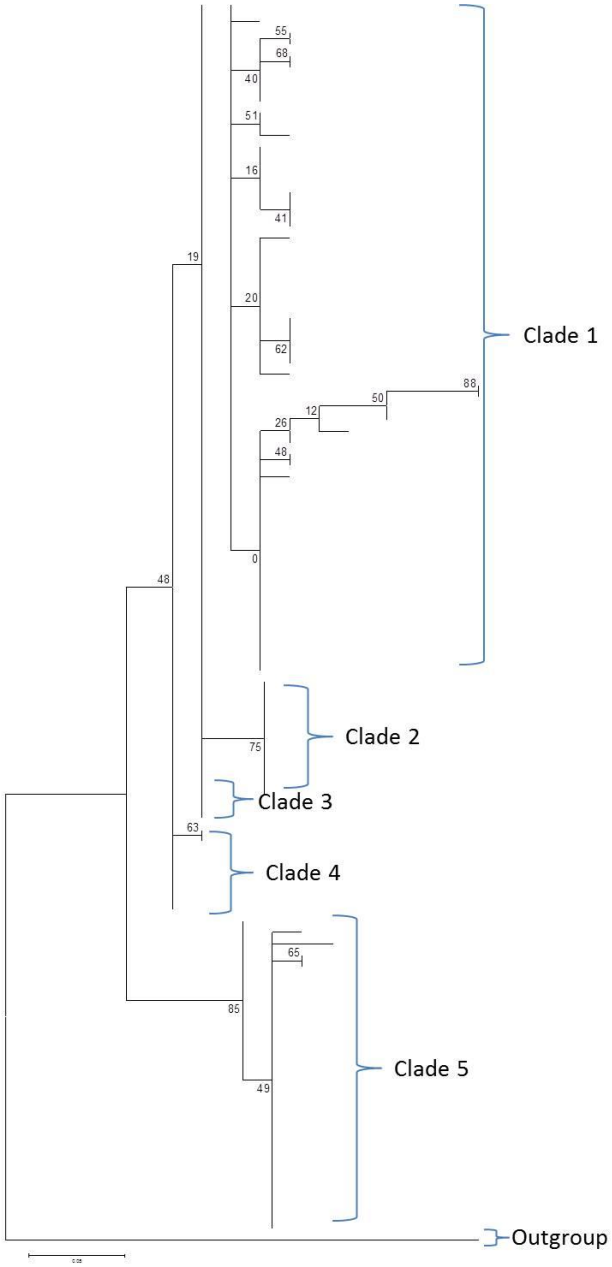
Figure 9: Symptoms of *Sclerotinia* on lettuce.

Objective 4.1 Synthesise Dez Barbara's unpublished work on carrot/parsnip viruses

Five main groups of carrot virus genotypes were identified for CRLV (Fig. 10). Clades 1 and 2 contained genotypes from many different wild hosts and locations but only very few from carrot. Clades 3 and 4 only contained genotypes from cow parsley. Clade 5 was found to be specific to carrot and wild carrot and was a clearly defined clade which is a separate lineage to Clades 1-4 with high bootstrap support. This clade also contained the sequence obtained from the CRLV strain which has been genome-sequenced.

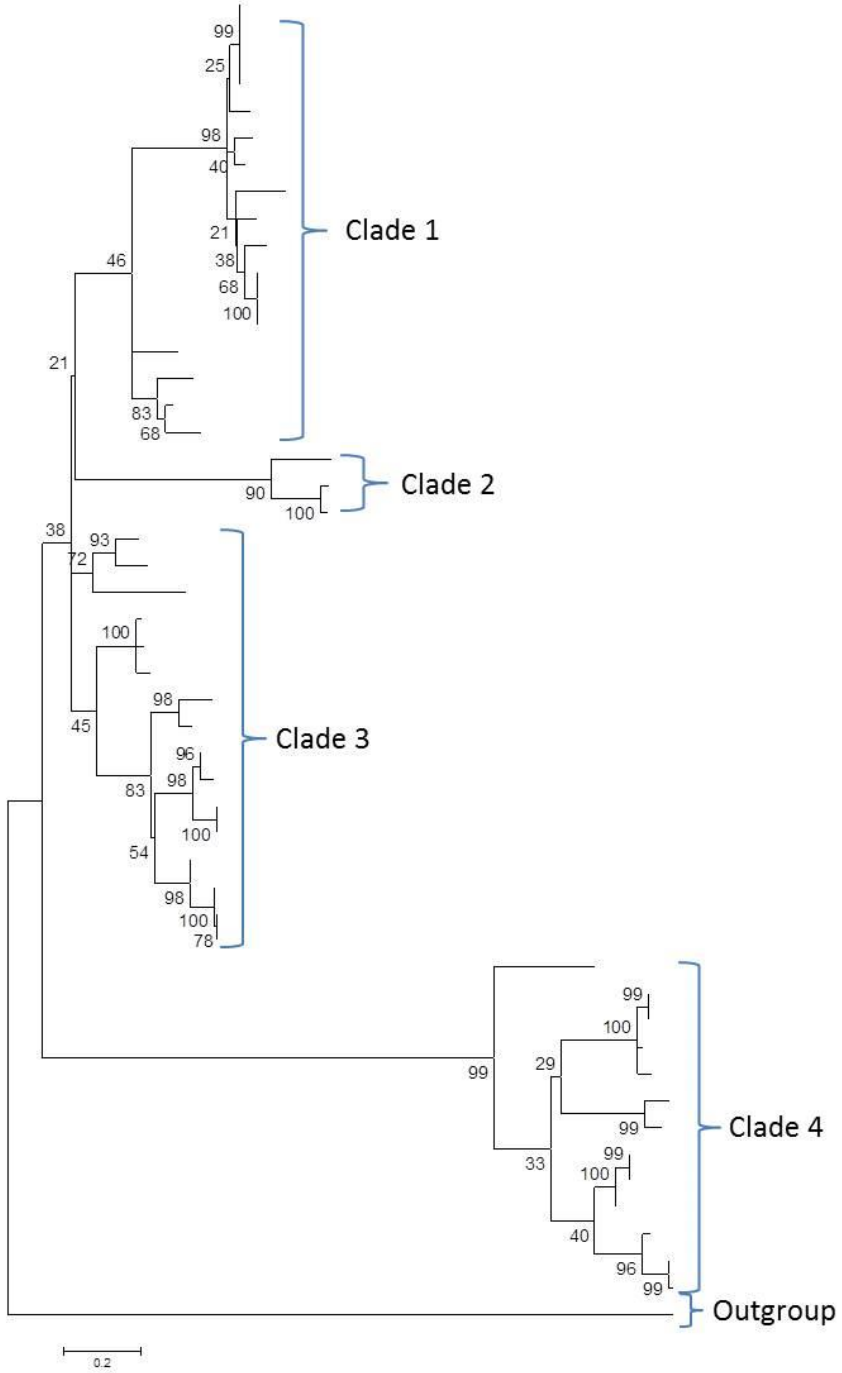
Four main groups of carrot virus genotypes were identified for PYFV (Fig. 11). Clade 1 was found exclusively in cow parsley and hogweed (but showed no geographical specificity) while Clades 2 and 3 contained genotypes from cow parsley, hogweed and carrot. Clade 2 may be specific to South West England. Clade 4 contained genotypes from hogweed, cow parsley, parsnip and celery and is more distant from the other clades. No carrot genotypes were found in this clade. This clade also contained the isolate that has been genome-sequenced.

Seven different groups of carrot virus genotypes were identified for AYV (Fig. 12). Clade 1 contained strains from many hosts and locations. Clades 2,3,5,6 and 7 only contained strains from cow parsley and are potentially location specific. However, these clades often only contained a single sequence. Clade 4 seems to show a specific sequence type that is only found in the East.



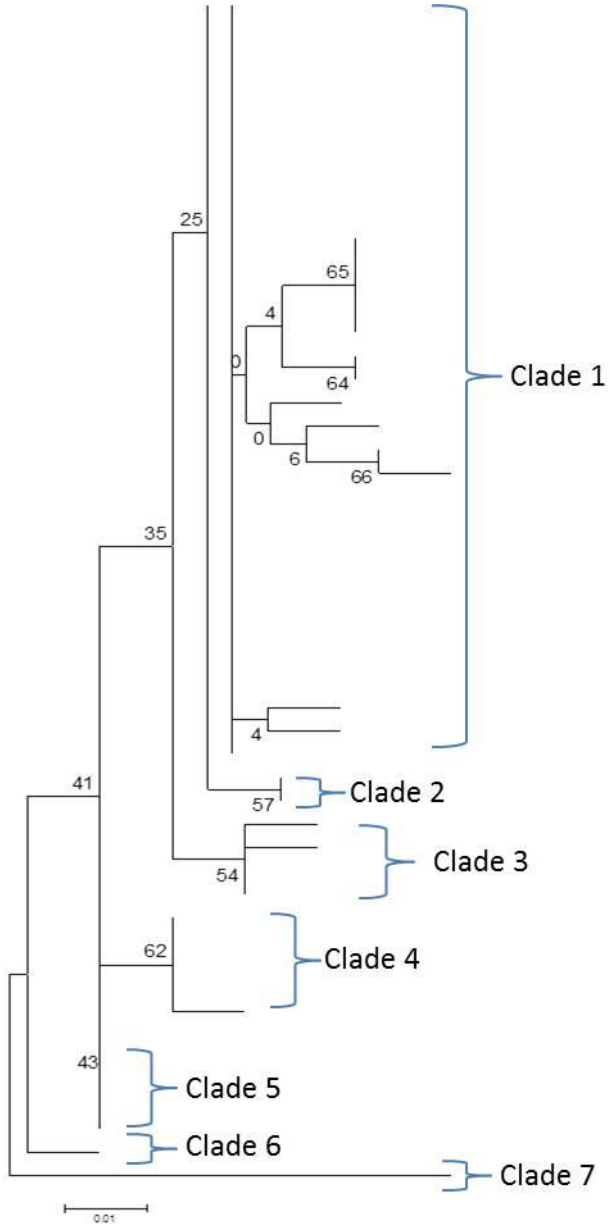
Clade	Host	Origin
1	Cow parsley, hogweed, pignut, sweet ciceley, wild parsnip, wild carrot, carrot, hemlock, rough chervil	Warks, Cumbria, Cambs, Norfolk, Yorks, Hereford, Peak District
2	Ground elder, hogweed, cow parsley	Cumbria, Warks, Cambs
3	Cow parsley	Yorks, Warks
4	Cow parsley	Cambs, Warks
5	Carrot, wild carrot	Warks, Notts, genome sequenced isolate (from wild carrot)

Figure 10: Phylogenetic tree showing evolutionary relationships among different strains of CRLV. Numbers represent bootstrap values from 1000 replicates. This tree is rooted using a sequence extracted from cereal yellow dwarf virus full genome sequence.



Clade	Host	Origin
1	Cow parsley, hogweed	Warks, Cumbria, Cambs
2	Cow parsley, hogweed, carrot	Cambs, Kent
3	Cow parsley, hogweed, carrot	Norfolk, Warks, Cumbria
4	Hogweed, cow parsley, parsnip, celery	Warks and genome sequenced isolate (from parsnip)

Figure 11: Phylogenetic tree showing evolutionary relationships among different strains of PYFV. Numbers represent bootstrap values from 1000 replicates. This tree is rooted using a sequence extracted from lettuce mottle virus full genome sequence.



Clade	Host	Origin
1	Cow parsley, pignut, hogweed, ground elder, sweet cicely, alexanders, hemlock	Cambs, Warks, Cumbria, Norfolk, Lincs, Yorks
2	Cow parsley	Warks, Norfolk
3	Cow parsley	Cumbria, Norfolk
4	Cow parsley, hogweed	Cambs, Norfolk, Lincs
5	Cow parsley	Norfolk
6	Cow parsley	Cambs
7	Cow parsley	Yorks

Figure 12: Phylogenetic tree showing evolutionary relationships among different strains of AYV. Numbers represent bootstrap values from 1000 replicates.

Discussion

Objective 1.1: Determine pathogenicity of a range of F. oxysporum isolates from onion and complete DNA sequencing of a range of housekeeping genes.

A range of pathogenicity was observed amongst the *F. oxysporum* isolates from onion and the results of the pathogenicity tests was consistent for each isolate. There was also a strong correlation between isolate pathogenicity results for the two onion cultivars used in the seedling assays, supporting the suggestion from previous work that there is no cultivar x isolate interaction (Taylor *et al* 2013). It is important to test the pathogenicity of isolates as the isolation of non-pathogenic isolates is common and they are likely to be either endophytes or saprophytes which are secondary colonisers of infected roots or bulbs. A range of housekeeping gene profiles was observed for the isolates, showing that there is genetic variation within the collection. All pathogenic isolates (with one exception) were placed in the same clade of a phylogenetic tree. However, other f. spp. were also placed in this clade showing that whilst this sequence type is indicative of pathogenic FOC isolates, other it does not distinguish FOC from other f.spp.. However, the presence / absence of an effector gene (*SIX7*) showed a very strong correlation with pathogenicity and as the sequence is unique to FOC, this is potentially useful for developing a diagnostic test in the future. Presence and sequence variation in SIX genes has been used to distinguish f. spp. and races in other studies and is a rapidly developing area (reviewed by de Sain and Rep, 2015). We now also have the whole genome sequences for six *F. oxysporum* isolates from onion and are currently investigating further potential pathogenicity markers in a related BBSRC HAPI project. One such marker is SIX3, and this gene has recently been used to create some putative FOC specific primers (Sazaki *et al* 2015). However, these primers need more rigorous testing against a range of fungi which will be carried out in year 3.

Objectives 1.8/1.9: Develop qPCR for *P. violae* using WCC Roche Lightcycler/ Quantify *P. violae* in soil samples from HDC project FV405 and other samples where available.

The problem of lack of specificity still persists for the *P. violae* qPCR assay but it should be noted that it was tested against highly concentrated DNA of each *Pythium* species, which is not representative of the field situation. Therefore, an optimised assay based on the *OCM1* gene may still be suitable for examining *P. violae* dynamics in the field. In addition, we have recently sequenced the *P. violae* genome which will potentially reveal further gene targets for primer / probe design and we expect to have a highly specific assay developed in the near future.

Objective 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)

Contemporary isolates of white rot, downy mildew and *Botrytis* were collected and molecular characterisation will take place in year 3 in order to confirm identify and identify any different groups of each pathogen. This collection will be a useful resource for future work.

Objective 4.1 Synthesise Dez Barbara's unpublished work on carrot/parsnip viruses

The analysis of CRLV sequences showed that the vast majority of genotypes from carrot were quite different from those from the wild umbellifers (Clade 5, Fig. 11). It is possible that these CRLV genotypes evolved from wild carrot. However, the few genotypes obtained from carrot in Clade 1 (closely related to strains in clades 2-4) suggests transmission to carrots from one of many potential wild hosts and could potentially pose a future problem. This is in agreement with previous work (Morgan, 2004) which examined CRLV sequences from cow parsley and carrot and found distinct clades with only two exceptions where sequences from carrot grouped with those from cow parsley. Overall, wild hosts do not represent a major source of CRLV inoculum for carrots and hence control approaches should focus on the crop. Further work needs to establish how far CRLV can spread and if there are any consistent regional variations in incidence. Given this knowledge then one control approach would be to have a break from carrots over an entire region to eliminate the virus. In contrast, many of the PYFV genotypes found in carrot were also found in cow parsley and hogweed suggesting that in this case, wild umbellifers present a source of inoculum. This is in agreement with a previous, smaller scale study (Morgan, 2004). The group of genotypes infecting parsnip/celery was different from those infecting carrot also supporting previous findings (Morgan, 2004) that there are different 'parsnip' and 'carrot' genotypes for PYFV. Control measures for PYFV should therefore take into account wild hosts but further work needs to investigate the changes in virus frequency in the most important wild umbellifers such as cow parsley and hogweed over time to try and then predict in which years PYFV may be be important, at what stage aphids then entre the crop and hence when vector control is most important. Finally AYV genotypes were found in all wild hosts but not carrot. This supports previous findings that carrot is not a host for AYV (Morgan, 2004) and its presence in a wide range of hosts and locations suggests that it may not be a limiting factor as a 'helper' virus enabling PYFV infection in carrots.

Conclusions

- A range of pathogenicity was observed across *F. oxysporum* isolates from onion and a strong association between the presence of an effector gene (SIX7) and pathogenicity was observed.
- Contemporary isolates of downy mildew, white rot and *Botrytis* pathogens were collected as a resource for future research.
- Diseased areas were created in the quarantine field for white rot and Fusarium diseases of onion and Sclerotinia disease of lettuce. These will provide an excellent resource for future research or commercial work testing of control methods.
- Wild hosts do not represent a source of CRLV inoculum but wild umbellifers may present a source of inoculum for PYFV.

Knowledge and Technology Transfer

Grower / Technical visits

- Visited Hobson Farming (6th Nov 2014), a carrot grower in York. Spoke about carrot production and cavity spot with Roger Hobson.
- Work-shadowing with Rodger Hobson (Hobson Farming)- 14th-15th Jan 2015
- Attended the BCGA technical seminar at PGRO (26th March)
- Visited Abrey Farms in Norfolk (20th May) and sampled 2 fields for downy mildew.
- Visited a site in Lincs (near Loosegate, 11th June) to collect samples of onions infected with downy mildew and white rot. Spoke to Robert Aldershaw and Andy Richardson.
- Visited 24 pea fields near Hull which were sampled for foot rot (Fusarium spp.) and aided PhD student with isolations (22-23rd July). Trained on identifying foot rot by staff from PGRO and discussed future work.
- Collected samples infected with downy mildew, white rot and leaf blight from Cambs and Norfolk (12th and 25th Aug) – visited G's and met with James Howell (VCS Agronomy)
- Visited Hensborough Farm in Rugby (20th Aug) and collected samples with white rot and downy mildew
- Visited a rocket grower (Fosse Way Farms, 20th Aug) with suspected *Fusarium* problems. Discussed the problem and our work on Fusarium. Symptoms were observed and plants brought back to the lab for isolation.
- Attended and presented a poster at Elsoms Open Day (14th Oct)

Conferences / seminars

- Attended the new technologies for crop improvement workshop in Antalya, Turkey (22nd-27th Feb). Presented a poster.
- Presented a seminar in the Warwick Crop Centre seminar series (16th April)
- Gave a talk to visitors from Australia (Rohan Kimber is from SARDI-Adelaide and is a bean/legume pathologist mainly, and Kurt Limbeck is a Plant Pathologist from the NSW Department of Primary Industries, Wagga Wagga Agricultural Institute)19th May
- Attended and gave an oral presentation at The International Symposium on Edible Alliaceae (ISEA) 2015 in Nidge, Turkey (21-25th May)
- Attended and presented a poster at AHDB Studentship Conference (16-17th Sept)

Publications

Taylor, A., Vagany, V, Jackson, A.C., Harrison, R.J., Rainoni, A., Clarkson, J.P. (2015) Identification of pathogenicity-related genes in *Fusarium oxysporum* f. sp. *cepae*. Molecular Plant Pathology, accepted pending revisions.

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