Project title:	Understanding the ecology and epidemiology of *Pythium violae* to enable disease management in carrot crops.
Project number:	FV 432
Project leader:	Dr John Clarkson, Warwick Crop Centre, University of Warwick, CV35 9EF
Report:	Annual report, September 2016
Previous report:	Annual report, September 2015
Key staff:	Kathryn Hales, Warwick Crop Centre, University of Warwick, CV35 9EF
Location of project:	Warwick Crop Centre, University of Warwick, School of Life Sciences, Wellesbourne, Warwick, CV35 9EF
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Date project commenced:	1 October 2014
Date project completed (or expected completion date):	30 September 2018

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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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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GROWER SUMMARY

Headline

- *P. violae* is the most common species associated with cavity spot of carrot.
- Methods have been developed to improve sampling and detection of *P. violae* from soil.
- Inoculation of carrot seedlings and mature plants has resulted in disease development but with some variability.

Background

Cavity spot disease of carrot

Cavity spot is the most important disease problem for carrot growers and regularly results in losses of £3-5 million per season (Martin, 2013). The disease was first recognised in the UK in 1960 and has been reported widely across the globe Hiltunen and White (2002). Typical symptoms on carrot are dark, sunken elliptical lesions that result in an unmarketable crop (Fig. 1).

Figure 1. Symptoms of cavity spot.

In the 1980's the fungicide metalaxyl was found to reduce the severity of cavity spot (Lyshol *et al*., 1984), and the discovery that the oomycete *Pythium* was the causal agent (Groom and Perry, 1985). A range of *Pythium* species have since been associated with the disease in different parts of the world including *P. violae*, *P. sulcatum*, *P. ultimum* and *P. irregulare* (Hiltunen and White, 2002). In the UK, *P. violae* is now thought to be the most significant cause of cavity spot (White, 1986, Groom and Perry, 1985), although *P. sulcatum* is also known to be associated with the disease (White, 1988, Lyons and White, 1992). Although *P. violae* is reported to be the major *Pythium* species causing cavity spot in the UK, it is still unclear whether the proportion of different *Pythium* species causing disease varies between different fields or carrot growing areas. The symptoms of cavity spot can also vary significantly, from small clean and dry looking shallow lesions to large dark lesions (Fig. 1). It is unclear however,

whether this variation is caused by environmental factors or is related to the species or isolate of *Pythium* causing the infection.

Control of cavity spot

In the absence of resistant carrot cultivars, the fungicide metalaxyl has been the primary means of managing cavity spot. Since the first report of this fungicide's utility in combating disease (Lyshol *et al*., 1984), control has largely improved (Hiltunen and White, 2002), but recently, results have been variable and defining the most appropriate time of application is proving challenging (Gladders, 2014). Some of this variability in control may be due to the enhanced degradation of the active molecule by microbes in the soil (Davison and McKay, 1999). New fungicide treatments have been tested recently (Gladders, 2014) but results were disappointing and demonstrating efficacy was hampered by lack of high enough disease levels in many of the trials. The dependency on metalaxyl as the single fungicide for control of cavity spot is concerning as its long-term sustainability is questionable.

Pythium violae

As indicated above*, P*. *violae* is thought to be the principal plant pathogen associated with cavity spot in the UK and is in the class Oomycota, making it distinct from 'true fungi'. The genus *Pythium* contains a large number of species, most of which are plant pathogens (Hendrix and Campbell, 1973). *P. violae* can infect many plant species including wheat, alfalfa and cucumber, although it does not cause disease in all of these hosts (Schrandt *et al*., 1994). It may also utilise a variety of weed hosts (Barbara, 2010, Kretzschmar, 2010). The ability of *P. violae* to exploit a wide range of hosts may explain why long rotations between carrot crops may sometimes be ineffective as a management strategy.

P. violae epidemiology

Detection and isolation of *P. violae* both from the soil and from carrots can be difficult as it has a very heterogeneous distribution in soil, and secondary infections can also occur on carrots (Hiltunen and White, 2002). Representative sampling is challenging as only 0.25 g of soil is routinely used for DNA extraction and detection limits are unclear. Previous work studying *P. violae* dynamics by Barbara and Martin (2007) used a PCR assay developed by Klemsdal (2008) to monitor five *Pythium* species in field sites but no predicative information was obtained that would be useful to growers. A DEFRA funded project (Anon., 2009), which followed the dynamics of *P. violae* using a semi-quantitative PCR, suggested that *P. violae* was usually undetectable in soil pre-planting, but increased from low levels in April in newly sown carrot crops, to reach a peak in late August/September as the plants matured, before

disappearing from the soil at an unpredictable and variable rate. It is unlikely though that *P. violae* does not survive in the soil as it produces oospores, and hence the failure to detect the pathogen pre-planting and post-harvest may be due to issues with sampling or the sensitivity of the PCR test. The production of oospores by the pathogen allows survival in soil for many years and also provides the primary inoculum for infection (Stanghellini and Burr, 1973, Hall et al., 1980). However, further investigation of the early infection events of carrots is needed, as information regarding oospore germination, infection routes and the effect of inoculum concentration on disease development, is sparse. The effect of environmental factors on disease development in the field has also been studied, with rainfall (soil moisture) and temperature (Barbara, 2010, Martin, 2013) being identified as particularly important. However quantifying these effects has been challenging, mainly due to the variability in results between different years and locations.

Artificial inoculation

Cavity spot research continues to be hampered by a lack of effective and reproducible methods to induce cavity spot symptoms in pot-grown carrots or in the field. The lack of knowledge concerning the inoculum levels required to induce disease and the ability to accurately quantify the pathogen in soil has also hindered progress. A number of methods have been investigated in an attempt to artificially inoculate carrots, but with only limited success (Suffert and Montfort, 2007, Kretzschmar, 2010).

Aims of the PhD project

The overall aim of this PhD project is to develop an understanding of cavity spot disease of carrots, by studying the biology, ecology and epidemiology of the main causal agent *Pythium violae*.

Objectives in Year Two:

1. Develop effective tools for *P. violae* research:
   i) Continue collection and characterisation of multiple isolates of *Pythium;* conduct pathogenicity tests and whole genome sequencing to help understand the genetic basis of pathogenicity.
   ii) Develop a more robust and accurate PCR test for *P. violae* suitable for use with quantitative PCR
   iii) Develop a *P. violae* inoculation system for seedling and mature plant trials

2. Investigate *P. violae* dynamics, ecology and interactions with soil microbiota:
   i) Assess the dynamics of *P. violae* on carrot crops throughout the year.

Summary

Objective 1i) *Pythium* isolate collection and characterisation

From October 2014 through to April 2015, cavity spot infected carrots were collected from grower sites throughout the country. Approx. 80 *Pythium* isolates were obtained from these samples and the species identified through PCR and DNA sequencing of the internal transcribed spacer regions (ITS) of the rDNA (see Annual Report 2015 for details). Since then further isolates have been obtained and results from a current total of 125 isolates indicated that *P. violae* was the predominant species associated with cavity spot lesions, comprising 59% of isolates followed by *P. sulcatum* (14%) and *P. intermedium* (14%) (Fig. 2).

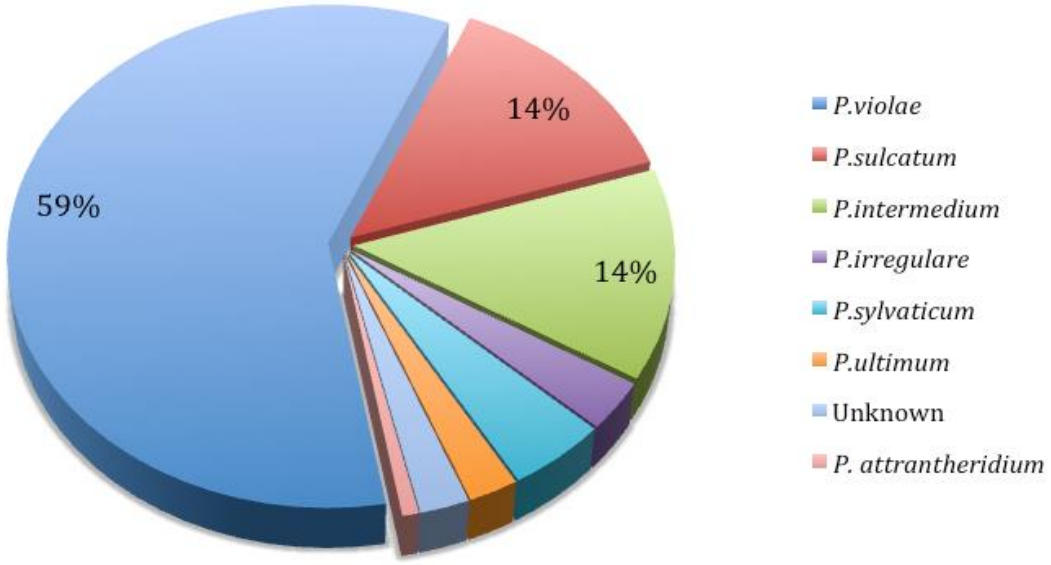

Figure 2. Relative proportions of different *Pythium* species identified from 125 isolates based on sequence of the ITS regions of the rDNA.

Objective 1ii) Improvement in soil sampling and detection

A new method based on 'oospore capture' from soil by sucrose centrifugation and filtration was developed to allow 10 g of soil to be tested for *P. violae* by PCR (see Annual Report 2015 for details). Following this, a number of different PCR primer pairs have been developed and tested under a range of conditions, and found to be specific to *P. violae.* The latest primer pair, AT_ITS FOR/REV1 was specific and suitable for qPCR.

Objective 1 iii) Artificial inoculation

Production of a sand-based *P. violae* inoculum was developed and tested in carrot seedling experiments previously (see Annual Report 2015 for details) and this year further repeat experiments were carried out as well as pot-based mature plant trials as part of project FV 391a. Although results have been variable, damping off has been induced in seedlings (Fig.

3), while reduced yield and cavity spot symptoms were observed in the mature plant trials (Fig. 4).

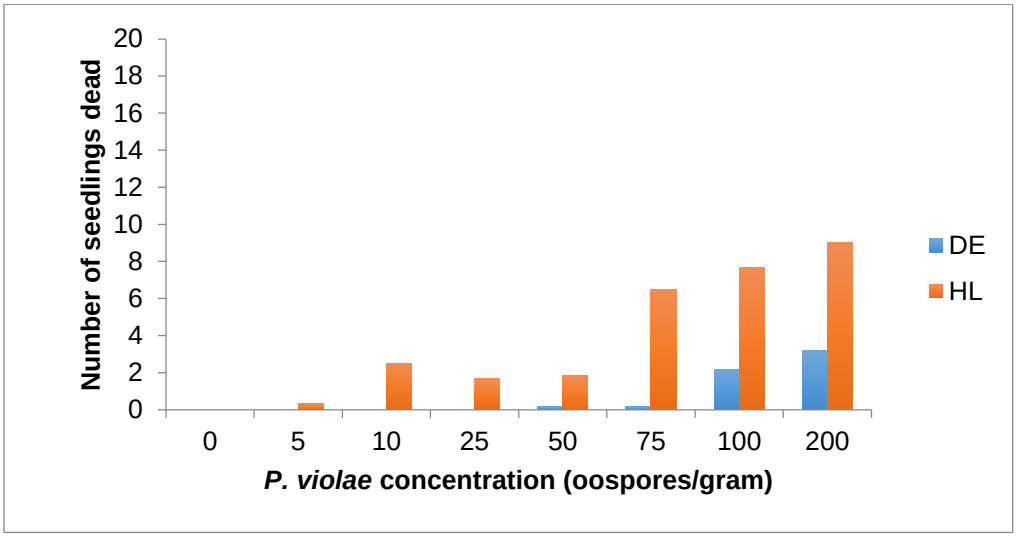

Figure 3. Carrot seedling death from damping off at 10 weeks post sowing for two *P. violae* isolates (DE, HL) at concentrations of 0-200 oospores/gram growing media.

Figure 4. Cavities produced on roots from pot-based mature plant trial after artificial inoculation with a range of *Pythium* concentrations as part of FV 391a.

Financial Benefits

Financial benefits have yet to be established – further details on this are expected at the end of year 3 of the project.

Action Points

Experiments are still underway to establish proof of concept, so no action points at present.

SCIENCE SECTION

Introduction

Cavity spot disease of carrot

Cavity spot is the most important disease problem for carrot growers and regularly results in losses of £3-5 million per season (Martin, 2013). The disease was first recognised in the UK in 1960's and has been reported widely across the globe, from the USA and Canada to Israel and Australia (Hiltunen and White, 2002). Typical symptoms on carrot are dark, sunken elliptical lesions that result in an unmarketable crop (Figure 5).

Figure 5. Symptoms of cavity spot.

It wasn't until the 1980's that Lyshol *et al*. (1984) found that the fungicide metalaxyl was able to reduce the severity of cavity spot. This led to the discovery that the oomycete *Pythium* was the causal agent (Groom and Perry, 1985). A range of *Pythium* species have since been associated with the disease in different parts of the world including *P. violae*, *P. sulcatum*, *P.*

ultimum and *P. irregulare*. In the UK, *P. violae* is now thought to be the most significant causal agent of cavity spot (White, 1986; Groom and Perry, 1985; Cooper *et al*., 2004) although *P. sulcatum* is also known to be commonly associated with the disease (Cooper et *al*., 2004; White, 1988; Lyons and White, 1992). White (1986) introduced five *Pythium* species individually to carrots grown in sterilised soil and observed that *P. violae* resulted in the highest percentage of carrots with cavities. Groom and Perry (1985) placed plugs of 28 actively growing *Pythium* colonies on to freshly lifted carrots and of the seven that induced sunken lesions, all were identified as *P. violae*. White (1988) found the most common species identified from isolating from cavity spot lesions were *P. violae* (261 times) and *P. sulcatum* (61 times). Hiltunen and White (2002) reviewed the range of species causing cavity spot in different counties. In California, Israel and Canada, *P. violae* was the major cause of disease, alongside *P. ultimum, P. irregulare* and *P. sulcatum* respectively, which is similar to the situation in the UK. In the Netherlands and Australia, *P. sulcatum* is reported to be the main causal agent. Although *P. violae* is the major *Pythium* species causing cavity spot in the UK, it is still unclear whether the proportion of different the *Pythium* species causing disease varies between different fields or carrot growing areas. However, reliable identification is now much easier as sequencing techniques are routinely used to distinguish *Pythium* species (primarily using the Internal Transcribed Spacer (ITS) region of the rDNA (Capote *et al*., 2012).

The symptoms of cavity spot can vary significantly, from small clean and dry looking shallow lesions to large dark lesions (Figure 5). It is unclear whether this variation is caused by environmental factors or is related to the species or isolate of *Pythium* causing the infection. A number of factors have been associated with disease development including rainfall (soil moisture) and temperature (Barbara and Martin, 2007; Martin, 2013). The early infection process however is little understood and information regarding infection routes and the effect of inoculum concentration, environmental factors and other microorganisms in the rhizosphere on disease development is sparse.

Control of cavity spot

In the absence of resistant carrot cultivars, the fungicide metalaxyl has been the primary means of trying to manage cavity spot. Since the first report of this fungicide's utility in combating disease (Lyshol *et al*., 1984), control has largely improved (Hiltunen and White, 2002), but more recently, results have been variable and defining the most appropriate time of application is proving challenging (Gladders, 2014). Some of this variability in control may be due to the enhanced degradation of the active molecule by microbes in the soil (Davison and McKay, 1999). New fungicide treatments have been tested recently (Gladders, 2014) but results were disappointing and demonstrating efficacy was hampered by low disease levels in

many of the trials. The dependency on metalaxyl as the single fungicide is concerning as its long-term sustainability is questionable.

Pythium violae

As indicated above*, P*. *violae* is thought to be the principal plant pathogen associated with cavity spot in the UK and is in the class Oomycota, making it distinct from 'true fungi'. The genus *Pythium* contains a large number of species, most of which are plant pathogens (Hendrix and Campbell, 1973). *P. violae* can infect many plant species including wheat, alfalfa, broccoli, celery and cucumber, although it does not cause disease in all of these hosts (Schrandt *et al*., 1994). It may also utilise a wide variety of weed hosts (Barbara, 2010; Kretzschmar, 2010). The ability of *P. violae* to exploit a wide range of hosts may therefore explain why even long rotations between carrot crops may sometimes be ineffective as a management strategy.

P. violae has been characterised morphologically and found to be slower-growing than many other *Pythium* species, while producing larger oogonia with a thicker oospore wall. Paul *et al*. (2008) found that *P. violae* oospore size ranged from 22-32 µm (average = 27 µm). Oospores are long-term survival structures, which can persist for a number of years in soil (Hall *et al*., 1980; Stanghellini and Burr, 1973). They are resistant to desiccation and are thought to be one of the primary sources of inoculum (van der Plaats-Noterinl, 1981). *P. violae* also differs from other *Pythium* species in that zoospores have never been seen. However, a recently published paper studied the phylogenetics of flagella genes in oomycetes and constructed phylogenies based on two genes involved with zoospore ontology (Robideau *et al*., 2014). It revealed a stop codon in one of these genes for *P. violae* hence potentially offering some insight into why *P. violae* does not appear to produce zoopores. This distinction from other *Pythium* species may also provide the genetic basis for a specific diagnostic test.

Detection of P. violae

Detection and isolation of *P. violae* both from the soil and from carrots can be difficult as it has a very heterogeneous distribution, and secondary infections can occur on carrots (Hiltunen and White, 2002). Klemsdal (2008) published specific PCR primers for five *Pythium* species associated with cavity spot in Norway including *P. violae* based on ITS sequences. Although the test was validated in the UK (Barbara and Martin, 2007), it did not prove to be useful as a predictive tool for the disease either before carrots were grown or before strawing down (Barbara, 2010). However, the PCR test is potentially useful in monitoring the dynamics of *P. violae*, although representative sampling is challenging as generally 0.25 g of soil is used for DNA extraction.

Early infection

In *Pythium* species, the production of oospores provides structures that can survive harsh conditions and cause primary infections when conditions improve (Stanghellini and Burr, 1973; Hall *et al*., 1980). The infection process of *P. violae* has been investigated by Suffert and Montfort (2007) who found that oospore inoculum in the soil is responsible for primary infections but that cavity spot lesions on carrots could also cause secondary infection on healthy roots, which was actually more efficient in forming lesions than oospore inoculum. This highlighted the need to understand how the pathogen travelled between carrots and whether this occurred due to direct physical contact between carrot lateral roots (Suffert and Lucas, 2008). Carrots infected with *P. violae* were planted next to healthy carrots, with either a 'buffer zone' between non-infected and transplanted infected roots, allowing the spread of the pathogen only through soil, or in direct physical contact through lateral roots. It was found that root-root contact did not increase *P. violae* transfer between infected and non-infected roots, and hence lateral roots were not the most prevalent means of secondary infection. Studies by Kretzschmar (2010) however showed that *P. violae* was detectable in the lateral roots of carrots even if they are not essential for plant to plant spread.

Further investigation of early infection events by *P. violae* in carrots is needed to understand under what conditions oospores germinate, at what stage, and by what mechanisms the pathogen colonises carrot roots. The amount of inoculum required for colonisation, infection and disease development as well as the optimum environmental conditions also need to be better defined.

Artificial inoculation

Cavity spot research has been hampered by a lack of effective research tools including the ability to artificially inoculate carrots. A number of methods have been investigated with only limited success. Cavity spot lesions can develop when mature carrot roots are wounded and an agar plug of *P. violae* is placed onto the damaged area (Montfort and Rouxel, 1988; Groom and Perry, 1985; White, 1986) but methods that are more realistic to field conditions (soil-based inoculum) have been harder to develop successfully. Suffert *et al*. (2008) used barley grains inoculated with plugs of *P. violae* to produce artificial inoculum and although this method proved successful in the short term, there were issues surrounding the longevity of the inoculum, with a decline in infection potential over time. Furthermore, the amount of inoculum was difficult to standardise as the amount of pathogen growth on the barley grains can vary. This lack of ability to consistently produce and quantify inoculum as well as inconsistency of infection rates means there is no robust plant assay that is reliable and predictable. Research by Kretzschmar (2010) used a variation of the method above, where wheat grain inoculated with *P. violae* agar plugs was used as inoculum. The results of this were disappointing, with the number of roots with lesions and the number of lesions per root only slightly higher in

inoculated pots compared to controls. A standardised protocol for production of *P. violae* inoculum and infection of carrots therefore needs to be developed.

P. violae *dynamics*

Little is known regarding the dynamics of the different *Pythium* species in soil. However, research in a DEFRA project (Anon., 2009) followed the dynamics of *P. violae* using a semi-quantitative PCR over four years. Results suggested that *P. violae* was usually undetectable in soil pre-planting, but increased from low levels in April in newly sown carrot crops to reach a peak in late August/September as the plants matured, before disappearing from the soil at an unpredictable and variable rate. It is unlikely that *P. violae* fails to survive in the soil as it produces long-lived oospores, and hence the failure to detect the pathogen pre-planting and post harvest may be due to issues with sampling or the sensitivity of the PCR test.

Effects of environmental factors on disease development

Despite a large amount of research, little correlation has been found between any environmental factors and disease development. A large field-based study was undertaken by Martin (2013) where thirty commercial carrot production sites were monitored for water input, soil moisture and soil temperature and carrots sampled to assess cavity spot disease. During the three-year study, the only correlation with disease found was with water input, but this varied from year to year, with the tentative relationships that were observed in year 1 and 2 not apparent in year 3. No correlation could be found between soil temperature and disease in any year, and no firm conclusions could be drawn. In contrast to this multi-field study, an experimental approach was undertaken by Barbara (2010) to examine the effect of irrigation on disease levels. Poly-tunnels were erected over a carrot crop and over-head irrigation established at 7.5, 15, 30, 45 and 60mm of water/week in different treatments. *P. violae* levels were monitored by PCR and roots sampled monthly to assess cavity spot lesions. Overall, development of cavity spot was very low but it was shown that growth of *P. violae* and the appearance of disease were dependent on soil moisture levels, and inputs of approximately 30 mm water/week seemed to be the lower limit for pathogen growth and disease development. However, no increase in cavity spot was found at, or above 45mm water/week.

Effect of soil microbiota on cavity spot

One theory of why cavity spot development is so unpredictable is that the soil microbiota in the rhizosphere, or more generally in the soil, may be important in suppression of *P. violae*. It has been found previously that incorporation of crop plants high in glucosinolate compounds gives partial control of some other soil-borne pathogens. Barbara (unpublished) showed that incorporation of a “hot” mustard crop (high in glucosinolates) virtually eliminated cavity spot disease development in a subsequent carrot crop. The application of Perlka (a fertilizer with

an antimicrobial effect) in the same trial however, resulted in increased disease and it was concluded that this might be due to the pasteurization of the soil reducing competition from other organisms, allowing increased proliferation of *P. violae.*

Aims

The overall aim of this PhD project is to develop an understanding of cavity spot disease of carrots, by studying the biology, ecology and epidemiology of the main causal agent *Pythium violae*.

The project is split into two main objectives:

1. To develop effective tools for *P. violae* research through a) improved molecular detection of *P. violae* in soil and b) development of artificial systems to induce cavity spot disease and study of early infection events.

2. To investigate *P. violae* dynamics, ecology and interactions with soil microbiota through a) understanding the year round dynamics of *P. violae* and effect of abiotic factors and b) investigating the ecology of *P. violae* and interaction with soil microbiota.

Objectives in Year Two:

1. Develop effective tools for *P. violae* research:

i) Continue collection and characterisation of multiple isolates of *Pythium;* conduct pathogenicity tests and whole genome sequencing to help understand the genetic basis of pathogenicity.
ii) Develop a more robust and accurate PCR test for *P. violae* suitable for use with quantitative PCR
iii) Develop a *P. violae* inoculation system for seedling and mature plant trials

2. Investigate *P. violae* dynamics, ecology and interactions with soil microbiota:

i) Assess the dynamics of *P. violae* on carrot crops throughout the year.

Objective 1 i) Isolate collection and characterisation, pathogenicity testing and whole genome sequencing

Isolate collection and characterisation

Materials and methods

From October 2014 through to April 2015, cavity spot infected carrots were collected from growers sites throughout the country. Approx. 80 *Pythium* isolates were obtained from these samples and the species identified through PCR and sequencing of the ITS regions of the rDNA (see Annual Report 2015 for details). Since then further isolates have been obtained to give a total of 125 and have been further identified by sequencing of the cytochrome oxidase II (COXII) gene.

Results

P. violae was the predominant species identified, comprising 59% of isolates followed by *P. sulcatum* (14%) and *P. intermedium* (14%) (Figure 6).

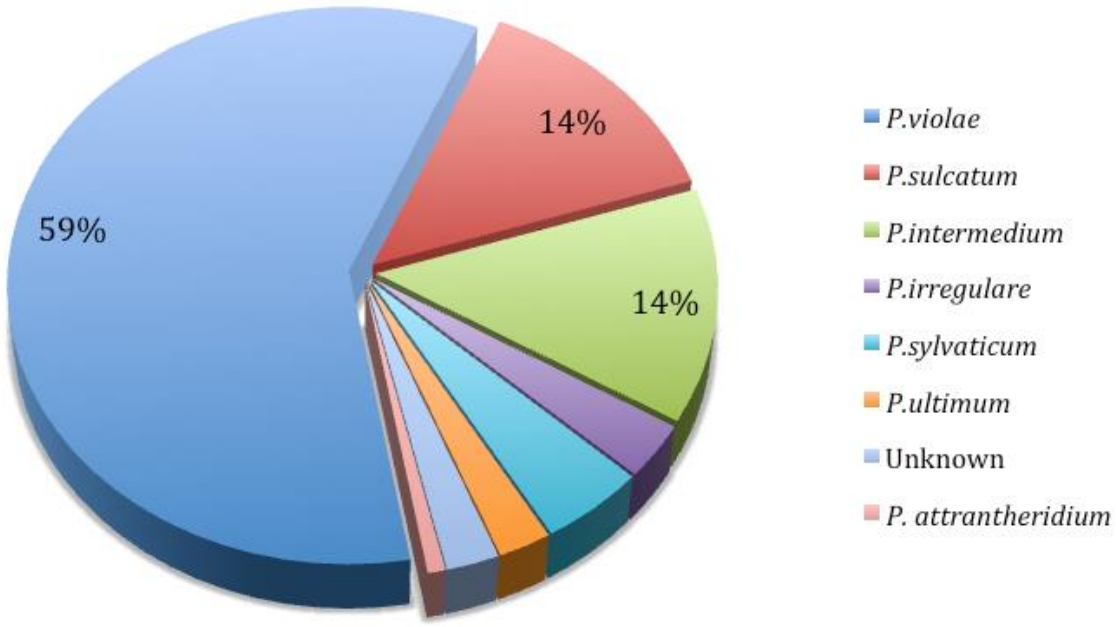

Figure 6. Relative proportions of different *Pythium* species identified from 125 isolates based on sequence of the ITS regions of the rDNA.

Phylogenetic trees were produced for both the ITS regions of the rDNA and the COXII gene resulting in one and three clades for *P. violae* respectively (Figures 7 & 8).

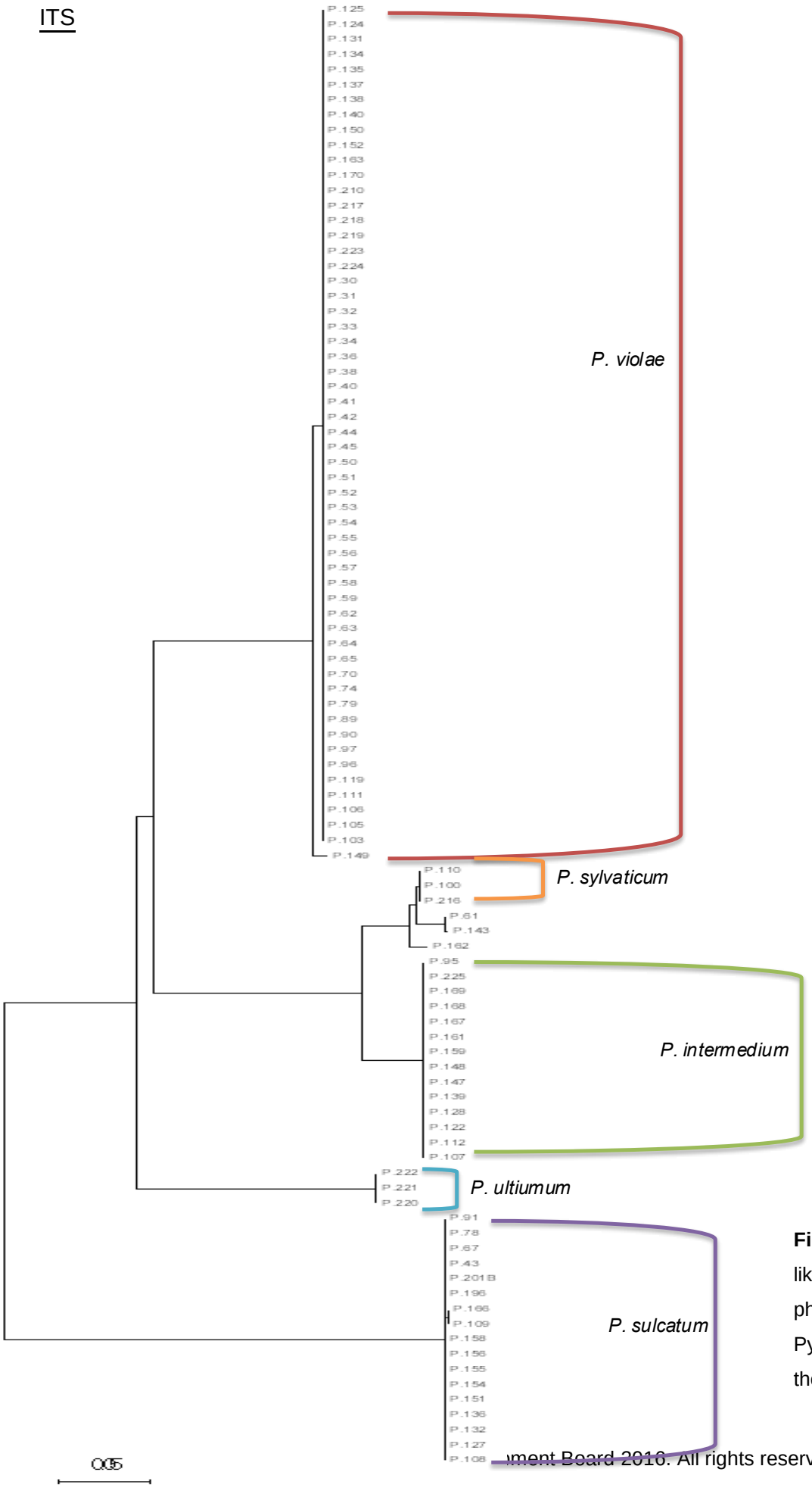

Figure 7. Maximum likelihood tree from phylogenetic analysis of Pythium isolates based on the ITS region of the rDNA

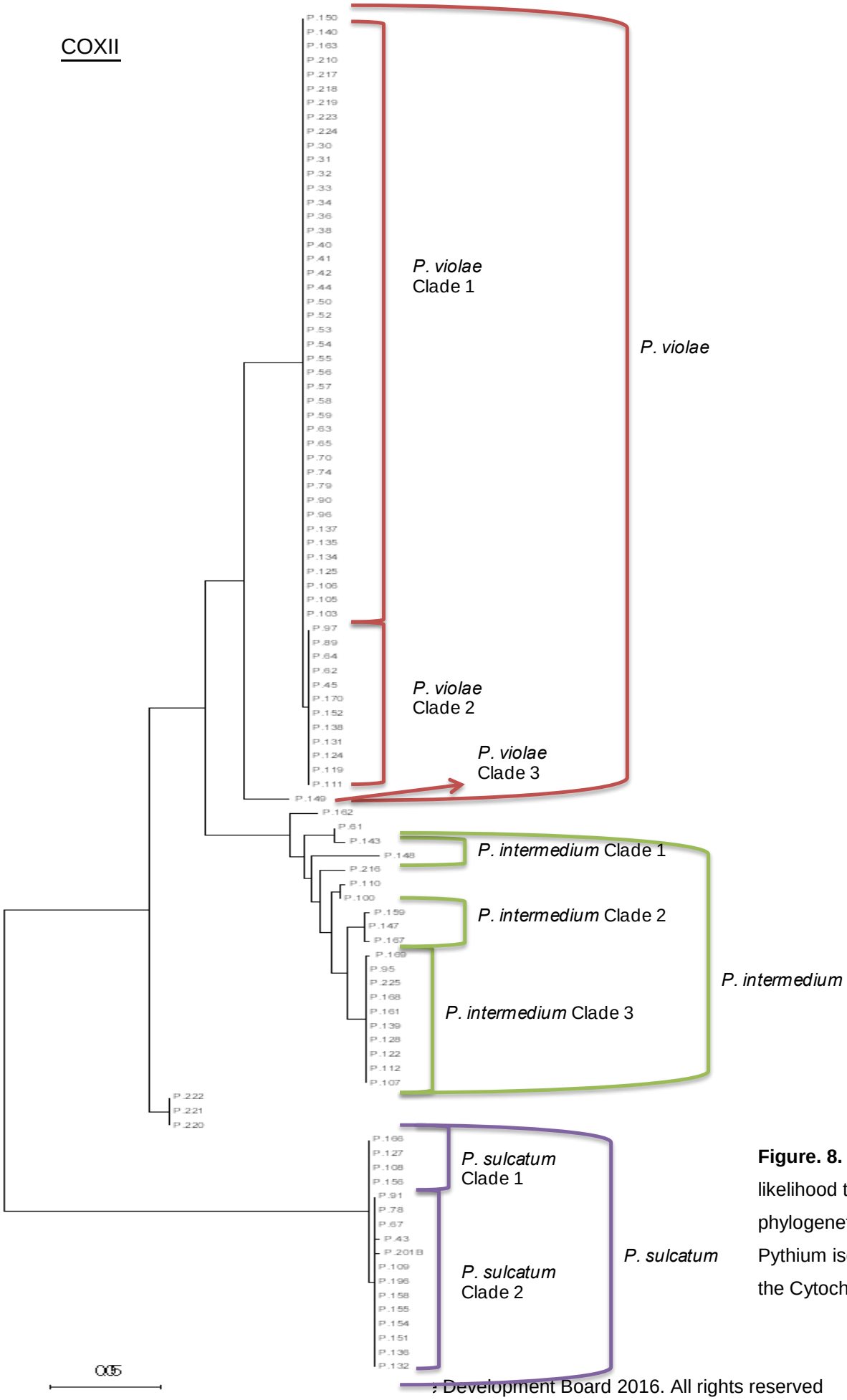

Figure. 8. Maximum likelihood tree from phylogenetic analysis of Pythium isolates based on the Cytochrome Oxidase II

Pathogenicity testing

Materials and methods

A pathogenicity test was carried out to assess the ability of ten *Pythium* isolates (*P. violae* x4, *P. intermedium* x3, *P. sulcatum* x3) to produce cavity spot lesions on fresh carrot roots from a commercial carrot field in Nottinghamshire. Isolates were selected based on original collection location and results from previous preliminary experiments. Carrot roots were washed in water to remove loose soil, and surface sterilised by submerging in 70% ethanol for 30 seconds after which the carrot was removed and the crown sprayed with 70% ethanol. The roots were left to air dry for 30 minutes. Two 5 mm agar plugs of *Pythium* mycelium from the edge of an actively growing colony of each isolate were then placed on carrot roots and incubated in darkness at 20ºC in boxes lined with damp paper towel. A total of nine carrots were inoculated for each isolate with three replicated boxes of three carrots. After five days, agar plugs were removed and cavities photographed every two – three days for 16 days.

Results

Photographs will be analysed with ImageJ version 1.49v (Schneider, C.A *et al*, 2012) in order to accurately measure lesion area. At the time of writing this analysis is still being carried out and hence results are not available. Figure 9 shows a selection of the lesions observed.

Figure 9. Lesions on carrot roots following inoculation with a range of *Pythium* isolates after incubation for 21 days at 20ºC.

Whole genome sequencing

Materials and Methods

Based on results of previous pathogenicity testing, two isolates of *P. violae* (HL / P10 and DE / P4) were selected for whole genome sequencing. These were a strongly pathogenic isolate (HL / P10) and a less pathogenic isolate (DE/P4). After a further preliminary pathogenicity test (results not shown) one isolate of *P. intermedium* and one isolate of *P. sulcatum* both with high pathogenicity were also selected for whole genome sequencing. All four isolates were obtained between 2013 and 2015 from cavity spot infected carrots in England (Table 1). DNA extraction was performed on freeze-dried mycelium and library preparation and sequencing using an Illumina MiSeq machine at East Malling Research.

Table 1. Information for isolates used for whole genome sequencing

Isolate code	***Pythium* code**	**Date isolated**	**Location**
HL	P10	October 13	Lincolnshire
DE	P4	October 13	Norfolk
BScreed	P67	October 14	Nottinghamshire
Alla2 2B	P107	January 15	Nottinghamshire

Adapter sequences and low-quality data were removed using fastqc-mcf. The sequencing depth and genome size was estimated following *k*-mer counting using KMC (Gurevich *et al*., 2013). *De novo* assembly was performed using SPAdes and analysed using Quast *(*Parra *et al*., 2007). The genome was assembled into contigs (>500bp and >10X coverage).

Results

All four isolates were assembled; the assembly statistics can be seen in Table 2.

Table 2. Assembly statistics for isolates used in whole genome sequencing

Isolate	**Assembled genome size (Mb)**	**Number of contigs**	**N_{50} (kb)**	**Largest scaffold (kb)**	**G+C content (%)**
P10	50599620	6216	38171	668133	52.41
P4	51739281	6050	37410	668139	52.43
P67	57493839	38179	3434	82409	55.99
P107	46835703	35777	2457	99287	54.83

Objective 1 ii) Developing specific primers for PCR detection and quantification

Oospore capture and PCR

Materials and Methods

A new method based on 'oospore capture' from soil by sucrose centrifugation and filtration was developed to allow 10 g of soil to be tested for *P. violae* by PCR (see Annual Report 2015 for details). Following this, a number of different PCR primer pairs were developed and tested under a range of conditions for specificity to *P. violae*. Previously, two specific primer pairs (OCM1 1128 F/R and CSViol FOR/REV, based on the flagellum gene OCM1 and the cellulose synthase gene respectively) were identified but had not been tested with quantitative PCR. However, further analysis of these primer pairs with qPCR revealed they were not sensitive enough to use with soil samples (results not shown). Following this, further primers (AT_ITS FOR1 / REV1) based on the ITS regions were designed and tested.

Results

The new primer pair AT_ITS FOR1 / REV1 was found to be very specific to *P. violae*, with only a small amount of non-specific binding occurring (Figure 10; see Appendix 1 for primer details).

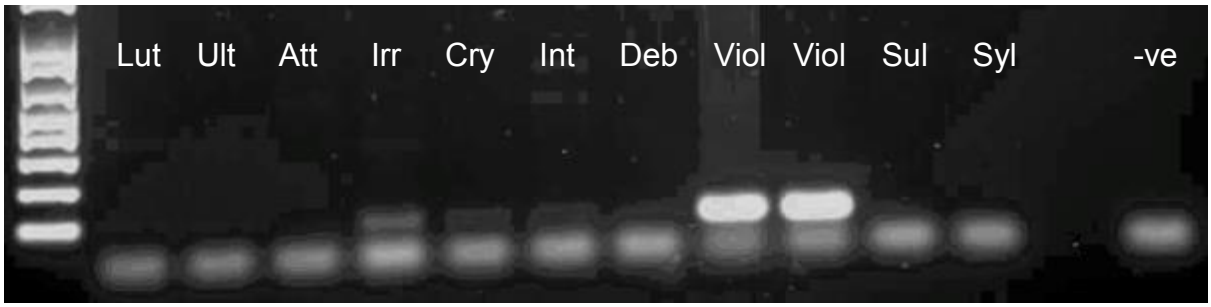

Figure 10. Gel electrophoresis of PCR products showing amplification of *Pythium* species using AT_ITS FOR/REV primers. Sul = *P. sulcatum*, Ult = *, P. ultimum,* Lut = *P. lutarium,* Irr = *P. irregulare,* Syl = *P. sylvaticum,* Int = *P. intermedium*, Viol = *P. violae,* Att = *P.attrantheridium,* Deb = *P. debaryanum*, Cry = *P. cryptoirregulare*.

Quantitative PCR

Materials and Methods

Due to previous success with a TaqMan-based qPCR assay, the same approach was adopted for primer pair AT_ITS FOR1 / REV1 (see Appendix 1) using a TaqMan probe 5' (6FAM)CGGAGGAGGAACGAAGGTTGGTCTTGT(TAMRA)-3'. This was tested for a range of *Pythium* species including *P. violae*, *P. lutarium*, *P. ultimum*, *P. attrantheridium*, *P. irregulare*, *P. cryptoirregulare*, *P. intermedium*, *P. debaryanum*, *P. sulcatum* and *P. sylvaticum*. All non-target DNA samples were diluted to 10 ng/ul. Initially, different primer concentrations were tested and 0.2 µM was found to be optimum (data not shown). A 10-fold dilution series of *P. violae* DNA (isolate P10) was made from 1 ng/ul to 0.1fg/ul. Reactions were set up in triplicate and the total reaction volume of 10 µl was made up as in Table 3.

Table 3. Ingredients for qPCR reaction

Reagent (concentration)	**µl /10µl reaction**
Probe mix (x2)	5
Forward primer (4µM)	0.5
Reverse Primer (4µM)	0.5
Probe (1µM)	1
DNA	1
Water	2

The samples were loaded into a microwell plate and the real-time PCR reaction run on the LightCycler® 480 Real-Time PCR system (Roche). Cycling conditions consisted of initial denaturation at 95°C for 3 minutes followed by 45 cycles of denaturation at 95°C for 5 seconds and annealing and extension at 67°C for 30 seconds. During the 45 cycles of denaturation, annealing and extension, quantification occurred.

Results

Amplification using the TaqMan approach and primers AT_ITS FOR1 / REV1 was highly specific to *P. violae* with very minimal detection of other *Pythium* species tested. The highest level of amplification was detected for *P. irregulare* and was equivalent to 0.00005% of a corresponding *P. violae* sample. The assay was extremely sensitive with a detection limit of at least 1 fg *P. violae* DNA (1×10^{-6} ng), efficiency of 01.984 and error of 0.00784 (Figure 11).

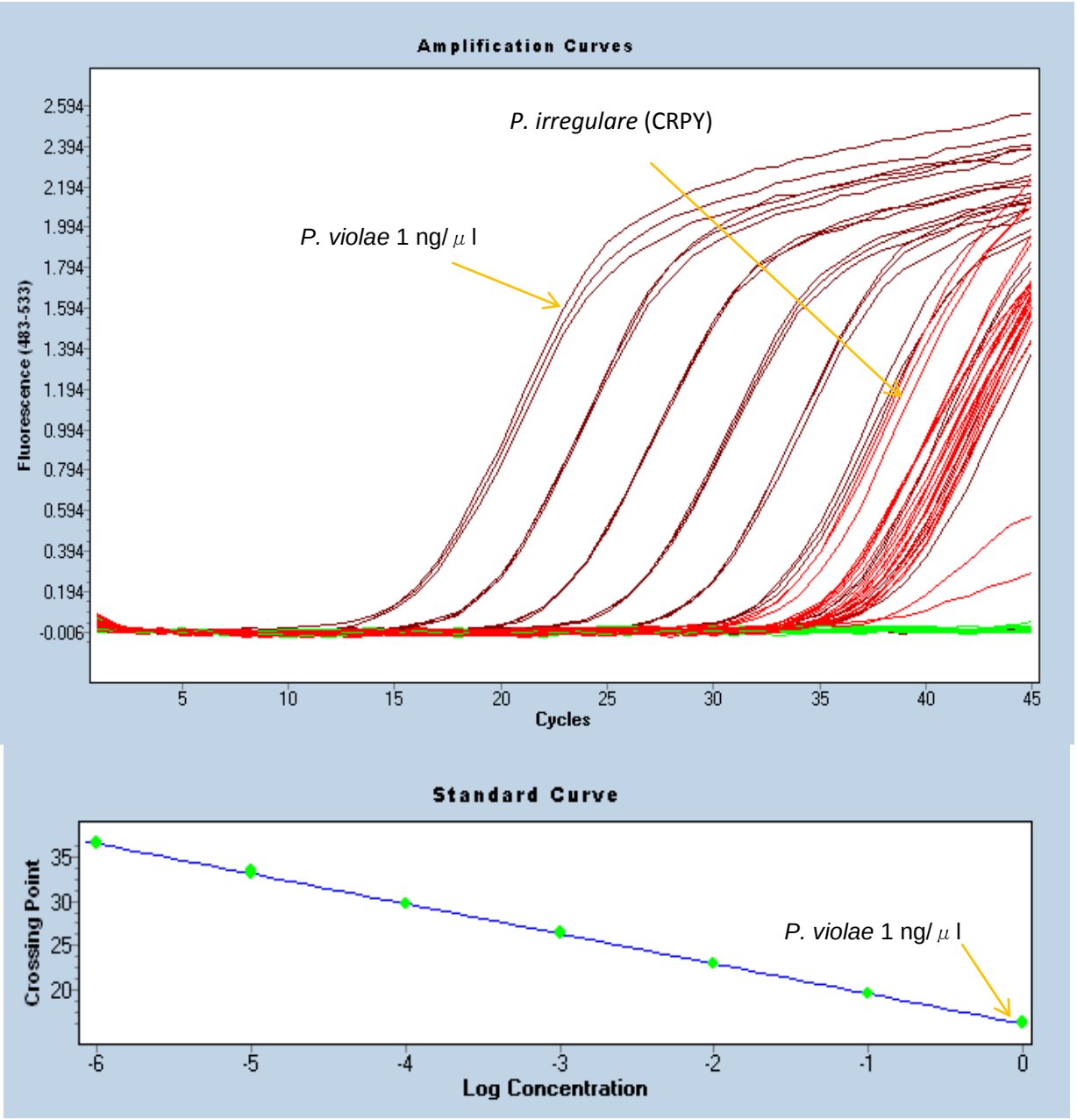

Figure 11. TaqMan assay for quantification of *P. violae*. Amplification curves and dilution series from 1 ng/ul to 1 fg.ul are shown. Amplification of *P. violae* from 1 ng/µl to 1 fg/µl (brown lines) and other non-target *Pythium* species at 10 ng/µl (bright red lines) are shown. Non-target Pythium species include: *P. lutarium, P. ultimum, P. attrantheridium, P. irregulare, P. cryptoirregulare, P. intermedium, P. debaryanum, P. sulcatum* and *P. sylvaticum*.

Objective 1 iii) Artificial inoculation

Seedling tests

Materials and Methods

A sand based inoculum in which many oospores could be produced was developed previously and used to inoculate carrot seedlings which resulted in damping-off (see Annual Report 2015 for details). Further seedling tests were carried out to test the effect of eight inoculum concentrations on carrot germination and seedling damping off. Inoculum concentrations tested ranged from 0 to 300 oospores / gram. Inoculum of two *P. violae* isolates (P10 and P4), was incubated at 14°C in the dark and mixed with appropriate weights of sand (sharp sand, J. Arthur Bowers) to produce the desired oospore concentration for each treatment. Five replicate experiments were set up over the course of 14 months using different concentrations and ages of *P. violae* inoculum (Table 4). Each replicate experiment was arranged in a randomized design as appropriate. Boxes were weighed and watered every two weeks to bring back to original weight (Figure 13).

Table 4. Experiments set up to test the effect of *P. violae* oospore concentration and age on carrot seed germination and seedling damping off between June 2015 and August 2016

Experiment number	Inoculum age (weeks)	Temp °C	Isolate	Replicate boxes / concentration	Oospore concentrations (oospores/g)
1	12	14	P10, P4	4	0, 5, 10, 25, 50, 75, 100, 200
2	20	10, 14	P10	1	0, 5, 10, 25, 50, 75, 100, 200
3	13	14	P10, P4	12	0, 5, 10, 25, 50, 75, 100, 200
4	12	14	P10, P4	12	0, 10, 25, 50, 75, 100, 200, 300
5	10, 32, 64	14	P10	4	0, 25, 50, 100, 200, 300

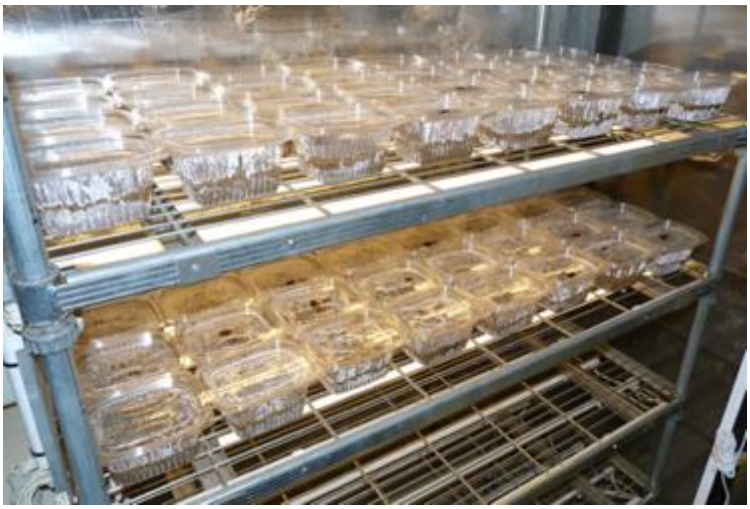

Figure 13. Seedling experiments on shelving in controlled environment

Results

Overall, varying degrees of damping off due to *P. violae* was observed in different seedling experiments, but none to the level expected, and no clear relationship between *P. violae* oospore concentration and disease or germination was observed. This was an unexpected result given the preliminary trial, which showed a clear dose-dependent level of damping off. The adjustments to treatments made over the course of the different experiments attempted to clarify what factors may be affecting damping off; however changes to temperature, inoculum level and inoculum age did not provide clear results.

In experiment 1, very little damping off was observed, with disease only affecting carrot seedlings treated with the highest inoculum level (200 oospores/g) for *P. violae* isolate P10 (results not shown). Experiment 2, was set up to attempt to establish if a lower temperature (10°C) would be more conducive to disease development using *P. violae* isolate P10 than the standard temperature of 14°C. Here, a high level of damping off was seen at both temperatures (over 75%) in the four highest inoculum concentrations (50, 75, 100 and 200 oospores/g, results not shown). Given the promising results from this experiment, a repeat test (experiment 3) was set up for both isolates but resulted in a lower level of damping off (less than 50% for P10 and 4% for P4 at the highest inoculum level of 200 oospores/g; Figure 14). A further experiment was set up (experiment 4) as above, but including the higher inoculum level of 300 oospores/g. This experiment resulted in less than 1% damping off in all levels with both isolates (results not shown). It was then hypothesised that age of the inoculum may play a significant role in the ability of oospores to infect carrot seedlings and hence experiment 5 tested three different ages of inoculum on disease development. This experiment however was terminated early (5 weeks post sowing) due to an unknown factor causing complete death of all seedlings in all boxes (including controls). However, results up to this time, indicated that only seedlings in the boxes with the youngest inoculum (10 weeks old) showed signs of damping off (results not shown).

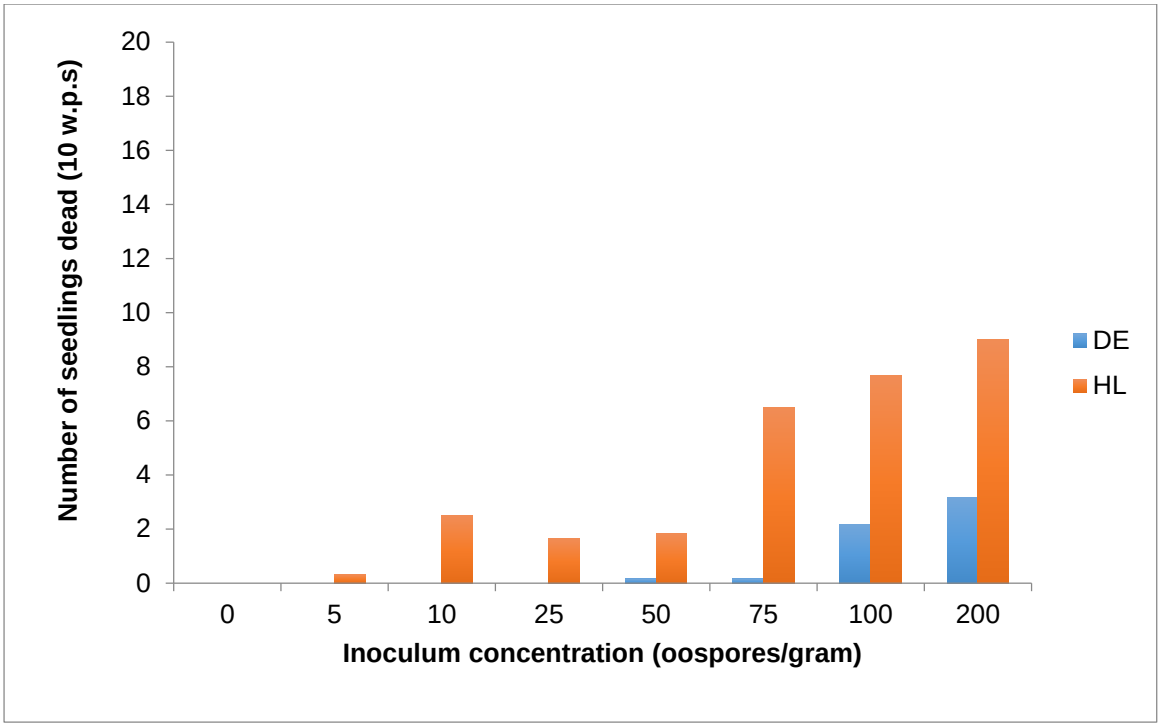

Figure 14. Carrot seedling death from damping off at 10 weeks post sowing for two *P. violae* isolates (DE, HL) at concentrations of 0-200 oospores/gram growing media.

Mature plant tests

Materials and Methods

As part of the current PhD project, assistance was given to set up two glasshouse experiments which were conducted for Project FV 391a in which carrot seeds (cv. Nairobi) were planted in large pots containing a range of six *P. violae* isolate P10 inoculum concentrations from 5 to 75 oospores/g. Pots were placed in a randomised design in a glasshouse with set temperatures of 18°C day and 16°C night. The highest level of inoculum was selected based on the initial seedling tests whereby it would induce some damping off but allow survival of the majority of plants. Seedling emergence and damping off were monitored, and yield and cavity spot symptoms were scored post harvest.

Results

Increasing oospore inoculum levels decreased carrot seedling emergence compared to the untreated control, as well causing an increase in damping off (results not shown). At harvest (22 weeks after sowing), all inoculated treatments resulted in a failure of carrot roots to form properly compared to the control, which resulted in a reduced root weight. Inoculated carrots were stunted with long thin tap roots and lateral roots appeared collapsed and darker than uninoculated control roots. Cavity spot symptoms were also observed on the stunted roots (Figure 15). *P. violae* was successfully re-isolated from tap roots, lateral roots and cavities,

indicating that the pathogen was responsible for the observed symptoms. Full details of results will be presented in the annual report for FV 391a.

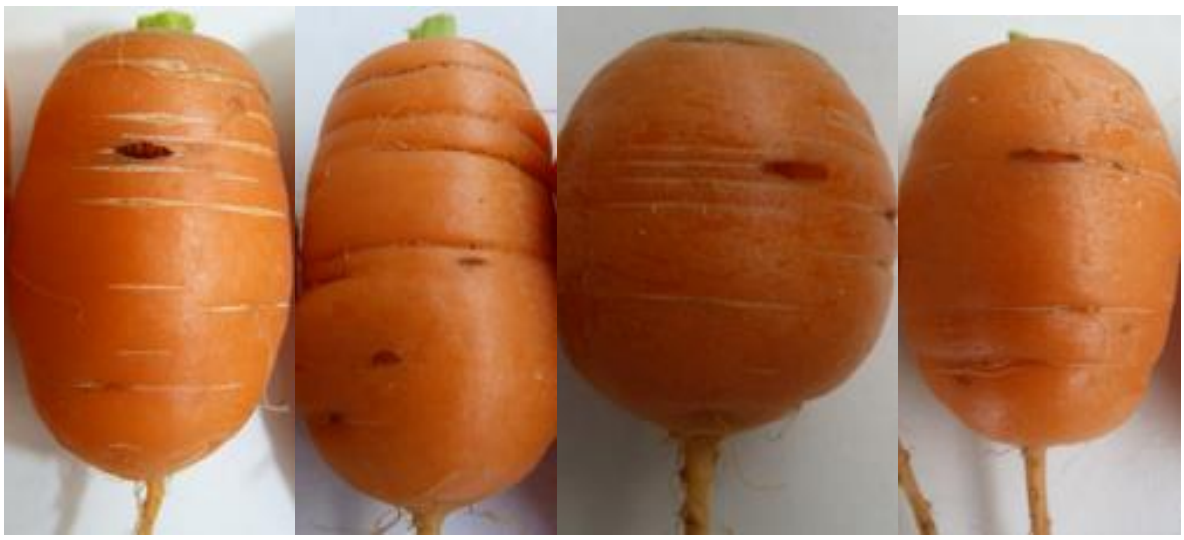

Figure 15. Cavities produced on roots from mature pot trial after artificial inoculation with a range of *Pythium* concentrations.

Objective 2 i) Monitoring *P. violae* dynamics in a commercial carrot field

Materials and Methods

Previous studies on *P. violae* dynamics (Barbara and Martin, 2007) using PCR have only monitored pathogen levels during the carrot growing season. To gain an understanding of *P. violae* dynamics year round, a commercial field site was identified where samples could be collected and analysed continually from January 2015 to January 2016 (see Annual Report 2015 for details of experimental design and sampling procedure). Table 5 below details the sampling dates.

Table 5. Dates of sampling at field site being monitored at a carrot growing site.

Date	**Action**
14/01/15	Soil sample taken of whole field – baseline measure
02/03/15	Soil sampling
31/03/15	Soil Sampling
21/04/15	Soil sampling
08/06/15	Carrot sampling
13/07/15	Carrot sampling
10/08/15	Carrot sampling
29/09/15	Carrot sampling
23/11/15	Carrot sampling
12/01/16	Carrot sampling

Carrots were pulled up with soil still attached around the outside, and placed in plastic bags and transported to Warwick Crop Centre the same day. On arrival, carrots were laid out to dry overnight, after which soil from the outside of the carrot was rubbed off, collected and stored at -20^{o}C. The carrots were then washed and assessed for cavity spot (number of carrots with cavities and the number of cavities per carrot).

Results

Over the sampling times, carrots developed cavity spot, with first signs developing in August (Figure 16) with progressively more severe symptoms developing over time (Figures 17 & 18). By January, many of the lesions were well developed (Figure 19).

Figure 16. Examples of cavities from carrots from monitoring field in Yorkshire collected on 10/08/15.

Figure 17. Examples of cavities from carrots from monitoring field in Yorkshire collected on 29/09/15.

Figure 18. Examples of cavities from carrots from monitoring field in Yorkshire collected on 23/11/15.

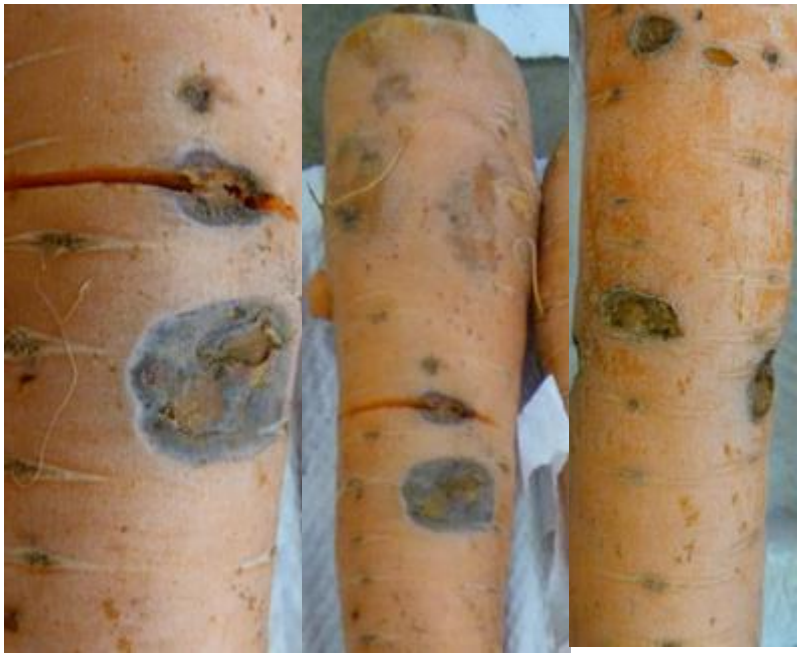

Figure 19. Examples of cavities from carrots from monitoring field in Yorkshire collected on 18/01/16. Cavities are darker and more expanded than those seen previously.

Discussion

Objective 1i) *Pythium* isolate collection and characterisation

Amongst the large collection of *Pythium* isolates obtained from cavity spot diseased carrots, *P. violae* was identified as the most prevalent species, accounting for 59% of isolates while *P. sulcatum* and *P. intermedium* were the second major pathogens identified, accounting for 14% of isolates each. These results confirm previous research where *P. violae* was identified as the main pathogen causing cavity spot in the UK (Lyons and White, 1992; White, 1988; White, 1986; Groom and Perry, 1985; Cooper *et al*., 2004). This part of the work has therefore already provided a larger survey of the incidence of different *Pythium* species associated with cavity spot than has previously been carried out and more formal analysis will now investigate whether certain species are more prevalent in particular regions or in individual carrot growing fields. Further isolates from new locations will be collected in the future with the aim of also assessing within-field variation and distribution of different *Pythium* species.

The ITS sequence data showed that different *Pythium* species can easily be distinguished from one another while sequences from another housekeeping gene, COXII, indicated variation within *P. violae*, as well as *P. intermedium* and *P. sulcatum*. This information will be used alongside further housekeeping genes that will be sequenced, to investigate how genetic variation may relate to biological variation e.g. pathogenicity.

Objective 1i) Pathogenicity testing

The *Pythium* isolates collected were all obtained from carrots displaying a wide range of cavity spot symptoms. In order to assess how species / isolates can be related to the type of symptom observed, initial pathogenicity tests were conducted to see how lesions developed. Variation in the lesion size has yet to be analysed and results will be presented in the next report.

Objective 1i) Whole genome sequencing

The genomes of two selected *P. violae* isolates assembled well and are undergoing further analysis. The genomes will be used to identify common pathogenicity/effector genes and comparisons made between the two isolates as well as closely related species. The *P. intermedium* and *P. sulcatum* isolates have yet to be assembled satisfactorily and further analysis will continue.

Objective 1 ii) Developing specific primers for PCR detection and quantification

The development of new *P. violae* specific primers using the ITS regions of the rRNA gene proved difficult, and *Pythium* sequences for the flagella gene OCM1 (Robideau *et al.*, 2014) and the cellulose synthase gene (Blum *et al*., 2012) provided much more variation between species, so primer pairs based on this gene resulted in good amplification of *P. violae* and only very weak amplification of the other species. However, further development of these primers indicated they were not sensitive enough to detect the small amount of DNA found in soil samples. This result could be due to both the OCM1 gene (Robideau *et al*., 2014) and the cellulose synthase gene being single or low copy genes (Zerillo *et al*., 2013) whereas the ITS region (where previous primers has been designed) is a multi-copy gene. Primers based on the ITS region were re-designed, and the results of the qPCR are now very promising. The assay developed was shown to be highly sensitive with a detection limit of less than 1 fg/ul, and hence this assay is more likely to accurately detect and quantify the very low levels of *P. violae* DNA often found in soil samples. These primers will be tested on soil samples in the near future and will be useful for monitoring *P. violae* levels in the field and to detect the pathogen in roots of artificially inoculated plants.

Objective 1 iii) Artificial inoculation

Seedling tests

The mixed results from the seedling trial experiments have proved difficult to interpret. Many adjustments were made in an attempt to understand the observed variation in disease levels but changes to temperature, inoculum concentration and inoculum age did not provide conclusive results. There are likely to be multiple factors which affecting the ability of *P. violae* to cause disease, and further work will develop methods to determine if different batches of oospore inoculum vary in their viability and ability to germinate.

Mature plant tests

The results from the first pot trial were promising, with inoculated carrots consistently stunted and displaying cavity spot symptoms. In addition, *P. violae* was consistently isolated from roots and cavities. The results from a second experiment will establish the consistency of these findings.

Objective 2 i) Monitoring *P. violae* dynamics in a commercial carrot field

The on-going monitoring of *P. violae* and cavity spot disease in a commercial field will build on previous work by assessing the pathogen levels year-round and coupled with the improved sampling, oospore capture, DNA extraction and PCR protocols should result in a better understanding of *P. violae* dynamics. Carrot samples from August 2015 to January 2016 resulted in a clear progression of cavity spot incidence and severity and the associated soil samples will allow corresponding *P. violae* levels to be quantified. A further monitoring field has been established this year (2016-2017) at another commercial field site.

Conclusions

- 125 isolates of *Pythium* have been collected from cavity spot infected carrots obtained from different locations. *P. violae* was identified as the predominant species present (59% of isolates).
- New primers for use in conventional and quantitative PCR have been developed and show increased specificity to *P. violae* and high sensitivity.
- Inoculation of carrot seedlings and mature plants has resulted in disease development but with some variability.

Knowledge and Technology Transfer

- Oral presentation at Warwick Crop Centre Seminar Series, October 2015
- Poster presentation at UK Carrot and Onion UK Conference and exhibition, November 2015
- Invited oral presentation at Bayer (Crop Science) Potato and Onion Conference, January 2016
- Invited oral presentation for the British Carrot Growers Association / AHDB Seminar, March 2016
- Poster presentation and organiser of School of Life Sciences Postgraduate Symposium, April 2016 (prize won for best poster)
- Flash presentation and Poster at SCI Young Researchers in Crop Sciences Conference, July 2016 (prize won for best flash presentation and poster)
- On-going communications with multiple carrot growers who kindly provide assistance and advice.

Appendices

Appendix 1 i) Primer details and PCR conditions

Species	Target DNA	Expected product size (bp)	Initial denaturation temp (°C) & time	Denaturation temp (°C) & time	Annealing temp (°C) & time	Extension temp (°C) & time	Final extension temp (°C) & time	Cycle number	Primer name	Primer sequence (5' -> 3')	Reference
Fungi	ITS1	700-900	94 4 mins	94 30 secs	58 30 secs	72 45 secs	72 10 mins	36	ITS1 ITS4	TCCGTAGGTGAACCTGCGC TCCTCCGCTTATTGATATGC	White et al. 1990
Phytoph-thora spp.	NADH dehydrog-enase subunit 1	897	94 5 mins	94 1 min	52 1 min	72 1 min	72 10 mins	35	NADHF1 NADHR1	CTGTGGCTTATTTTACTTTAG CAGCATATACAAAAACCAAC	Kroon et al. 2004
Pythium spp.	Cyto-chrome oxidase subunit 2	563	94 5 mins	94 1 min	52 1 min	72 1 min	72 10 mins	35	FM58 FM66	CCACAAATTTCACTACATTGA TAGGATTTCAAGATCCTG	Martin 2000
P.violae	ITS1	352	93 2 mins	93 1 min	61 1 min	72 1 min	72 10 min	40	PviolF PviolR	ATGTGTGTGTGCGGGACT CCACTCCCCAAAGAGAGAAG T	Klemsdal et al 2008
P.violae	ITS1	81	94 5 mins	94 1 min	60 1min	72 30 sec	72 10 min	35	PviolNEWF PviolNEWR	TGGTGTTTCGGACGCTGCGC TG TCCGCACACACACATTGCTG	Taylor, unpublished
P.violae	OCM1	126	94 5 mins	94 45 secs	55 30 secs	72 30 secs	72 7 mins	30	Pviol OCM1 910F Pviol OCM1 910R	GACCATCCCTATCCCCTA ACCGTCGTCCCACCGTG	Hales, unpublished
P.violae	OCM1	100	94 2 mins	94 1 min	62 30 secs	72 30 secs	72 5 mins	30	Pviol OCM1 1128 F Pviol OCM1 1128 R	CGGTGTTGGGGACAGTGACC CCGGCAAGCCAGTGACGGTA	Hales, unpublished
P.violae	Cellulose Synthase	199	94 5 mins	94 1min	65 10 secs	72 30 secs	72 5 mins	30	CSVioL FOR/REV	CGAAATGCGCGTGTACTGAC AT CGAGCAGCAAGAGCGGTCCC A	Taylor, unpublished
P.violae	ITS1	81	95 3 mins	95 5 secs	69 10 secs	72 30 secs	72 mins	35	AT_ITS FOR1/REV1	TGGTGTTTTCGGACGCTGCG CTG TCCCGCACACACACATTGCT G	Taylor, unpublished

Appendix 1 ii) Primers developed and tested for specificity to *P. violae*. Primers were tested against a range of other *Pythium* species. Purple squares indicate a strong positive PCR band, blue squares indicate a faint PCR band, grey squares indicate a negative (no PCR band). *P. viol = P. violae, P. sul – P. sulcatum, P. int = P. intermedium, P. irr = P. irregulare, P. syl = P. sylvaticum, P. ult = P. ultimum, P. cry = P. cryptoirregulare, P. deb - P. debaryanum, P. att = P. attrantheridium.*

Primer details			***Pythium* species**								
Reference	**Primer name**	**Target DNA**	***P. viol***	***P. sul***	***P. int***	***P. irr***	***P. syl***	***P. ult***	***P. cry***	***P.att***	***P.deb***
Klemsdal et al, 2008	PviolF/R	ITS1							n/a	n/a	n/a
Taylor, unpublished	PviolNEWF/ R	ITS1							n/a	n/a	n/a
Hales, unpublished	Pviol OCM1 910F/R	OCM1							n/a	n/a	n/a
Hales, Unpublished	Pviol OCM1 1128F/R	OCM1									
Taylor, Unpublished	CSViol FOR/REV	Cellulose Synthase									
Taylor, Unpublished	AT_ITS FOR/REV1	ITS1									

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