Project title:	Understanding the ecology and epidemiology of *Pythium violae* to enable disease management in carrot crops.
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Project leader:	Dr John Clarkson, Warwick Crop Centre, University of Warwick, CV35 9EF
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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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PhD Student

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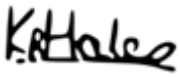
Signature:  Date: 26/09/2017

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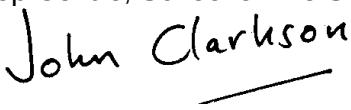
Signature:  Date: 26/09/2017

CONTENTS

GROWER SUMMARY

Headline

- A method to capture oospores from soil and improve detection of *Pythium violae* has been developed.
- Artificial inoculation of pot-grown carrots in the glasshouse with *P. violae* consistently resulted in the formation of small, stubby and stunted carrots with some typical cavity spot lesions observed on roots.
- Artificial inoculation in the field resulted in high cavity spot incidence on roots.

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 from 1960 and has been reported widely across the globe (Hiltunen & White, 2002). Typical symptoms on carrot are dark, sunken elliptical lesions which 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 led to the discovery that the oomycete *Pythium* was the causal agent (Groom & 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 & White, 2002). In the UK, *P. violae* is now thought to be the most significant cause of cavity spot (White, 1986; Groom & Perry, 1985), although *P. sulcatum* is also known to be associated with the disease (White, 1988; Lyons & 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 & 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 & 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 & 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 & White, 2002). Representative sampling is challenging as 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 & 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 inoculum levels needed 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 & 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 Three:

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

    i) Continue collection and characterisation of multiple isolates of *Pythium;* produce phylogenetic trees to establish the genetic variation within *Pythium* species.
    ii) Develop, refine and test a new oospore capture method for quantification of *P. violae* in larger soil samples.
    iii) Develop *P. violae* inoculation systems for carrot seedlings and mature plants in glasshouse, and field experiments.

Summary

Objective 1 i) *Pythium* isolate collection and characterisation

Cavity spot infected carrots were collected from grower sites throughout the country between October 2014 and April 2015. 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 (Hales & Clarkson, 2016). Since then, further isolates were obtained in 2016/17 and results from a 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).

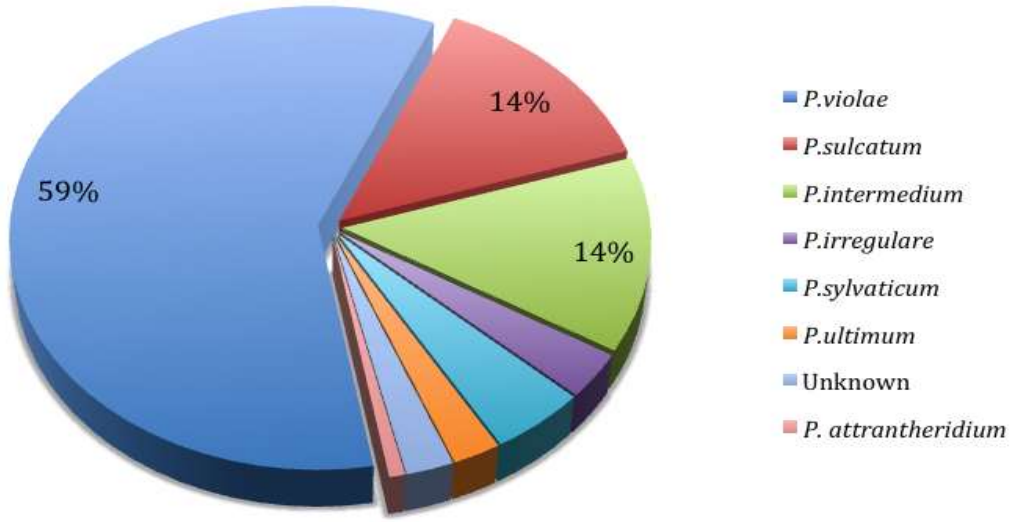


Figure 2: Relative proportions of different *Pythium* species identified from 125 isolates from carrot based on sequence of the ITS regions of the rDNA.

Objective 1 ii) Refining/testing new 'oospore capture' method

A new method for separating *P. violae* oospores from sand/soil by sucrose centrifugation and filtration was developed and refined. Soil/sand samples were suspended in water and sonicated to release oospores before adding to a saturated sucrose solution where oospores become suspended. These are then captured on a filter which can then be used for detection by DNA extraction and PCR. This new method enabled extraction of 50% more oospores than a previous 'standard' method. Combined with the *P. violae* specific qPCR, this method should allow more accurate quantification of oospores in soil and assessment of *Pythium violae* dynamics.

Objective 1 iii) Artificial inoculation

Seedling experiments

Sand and liquid-based *P. violae* inoculum were used to inoculate carrot seedlings in a controlled environment. *P. violae* oospore inoculum produced in V8 liquid culture did not result in any seedling mortality when applied either at sowing or to seedlings in autoclaved sand. However, when applied to seed or seedlings in non-autoclaved sand, up to 50% seedling mortality occurred, including in uninoculated control treatments. This suggested that seedling death was not due to *P. violae* and likely due to a contaminant. Similarly, a *P. violae* solid oospore inoculum showed high levels of seedling mortality including the uninoculated control treatments. Given the variability of this approach and recurrent problems with contamination, a new method is required to enable reliable infection of seedlings with *P. violae*.

Glasshouse experiments

Sand-based solid *P. violae* inoculum was used to initiate infection of carrots in pot-based glasshouse experiments, carried out jointly as part of AHDB project FV 391a. Inoculation of the growing media using this solid substrate at different rates resulted in some seedling death, reduced seedling size and a decrease in growth of foliage. However, at harvest, the principal effect of *P. violae* inoculation was the formation of small, stubby and stunted carrots with a much-reduced weight compared to the uninoculated control plants. These infected carrots were also characterised by a long hairy brown tap root with increased lateral root formation, many of which were collapsed. Typical cavity spot lesions were also observed in a large proportion of these stubby carrot roots (Fig. 3). *P. violae* could also be consistently isolated from the infected tap roots and cavity spot lesions, confirming that these symptoms were due to the inoculation. Generally, there was no clear effect of oospore concentration on the severity of any of these symptoms associated with *P. violae* inoculation. This method therefore shows promise for infection of carrots with *P. violae* but needs refining in order to reduce variability in disease levels and increase the number of cavity spot lesions.

Figure 3: Typical cavity spot lesions on carrot roots caused by *P. violae* following artificial inoculation.

Field experiment

A preliminary field experiment was carried out whereby macrocosms (concrete pipes sunk in the ground) were filled with a soil / sand mix, artificially inoculated with the same *P. violae* solid substrate inoculum as used in the glasshouse experiments and carrots sown. In this situation, there was no effect of pathogen inoculation on either seedling survival or subsequent carrot growth but at harvest, a large proportion of the carrots (up to 40%) were affected by typical cavity spot symptoms (Fig. 4). The sand based solid *P. violae* inoculum therefore shows promise for inducing cavity spot on a field scale.

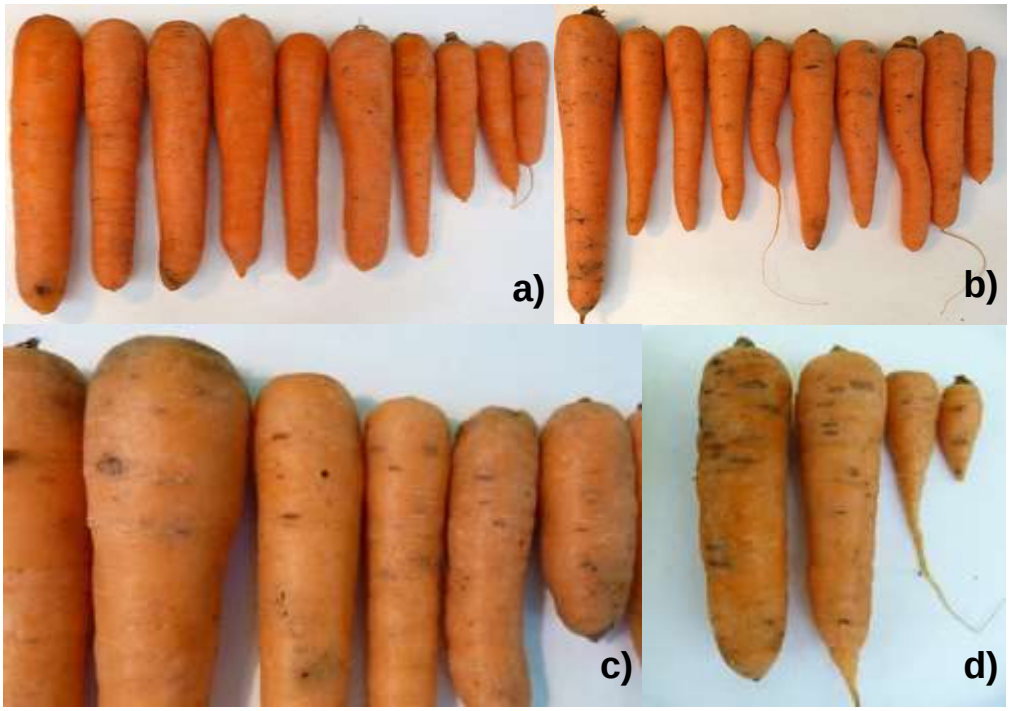


Figure 4: Carrots harvested from field macrocosms a) uninoculated control and b) inoculated with 50 *P. violae* oospores g^{-1}; close up view of carrots to show typical cavity spot lesions for carrots inoculated with c) 30 *P. violae* oospores g^{-1} and d) 50 *P. violae* oospores g^{-1}.

Financial Benefits

If artificial inoculation with *P. violae* can be refined to reduce variation, this approach may allow much more reliable testing of new control products and approaches, hence resulting in considerable financial benefits associated with a reduction in the number of failed field trials.

Action Points

None at this time.

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 1960s 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 which result in an unmarketable crop (Fig. 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 & 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 & 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 & 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 (Fig. 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 & 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 & 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 & 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 is concerning as it's 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 & 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 & 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 & 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 & 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 & 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 & 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 & Rouxel, 1988; Groom & 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 previously found 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 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*.

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 Three:

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

    i) Continue collection and characterisation of multiple isolates of *Pythium;* produce phylogenetic trees to establish the genetic variation within *Pythium* species.
    ii) Develop, refine and test a new oospore capture method for quantification of *P. violae* in larger soil samples.
    iii) Develop *P. violae* inoculation systems for carrot seedlings and mature plants in glasshouse, and field experiments

Objective 1 i) Isolate collection and characterisation

Introduction

A survey of the *Pythium* species causing cavity spot in the UK was carried out and isolates identified using molecular DNA sequencing. Genetic variation was investigated using phylogenetic analysis of different gene sequences.

Materials and methods

Cavity spot infected carrots were collected from growers' sites in Nottinghamshire, Lincolnshire, Cambridgeshire, Yorkshire, Shropshire and Norfolk between October 2014 and April 2015. Approx. 80 *Pythium* isolates were obtained from these samples and identified through PCR and sequencing of the ITS regions of the rDNA (Hales & Clarkson, 2015). Since then further isolates were obtained and identified in 2016/17 to give a total of 125. As well at ITS sequencing, all isolates have been further characterised through 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%) (Fig. 6).

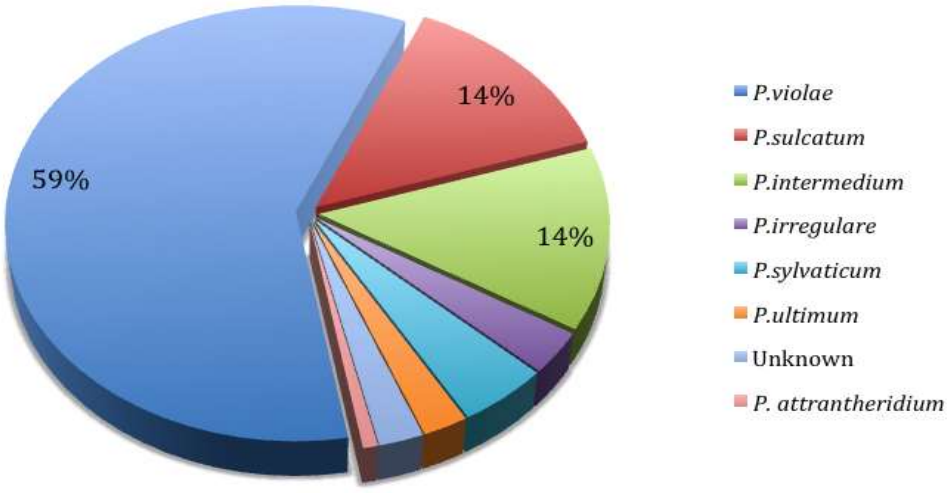


Figure 6: Relative proportions of different *Pythium* species identified from 125 isolates based on sequence of the ITS regions of the rDNA.

Phylogenetic trees were produced based both on sequences of the ITS regions of the rDNA and part of the COXII gene resulting in one and three clades for *P. violae* respectively (Figs. 7, 8). Hence there is some within-species variation for this pathogen.

ITS

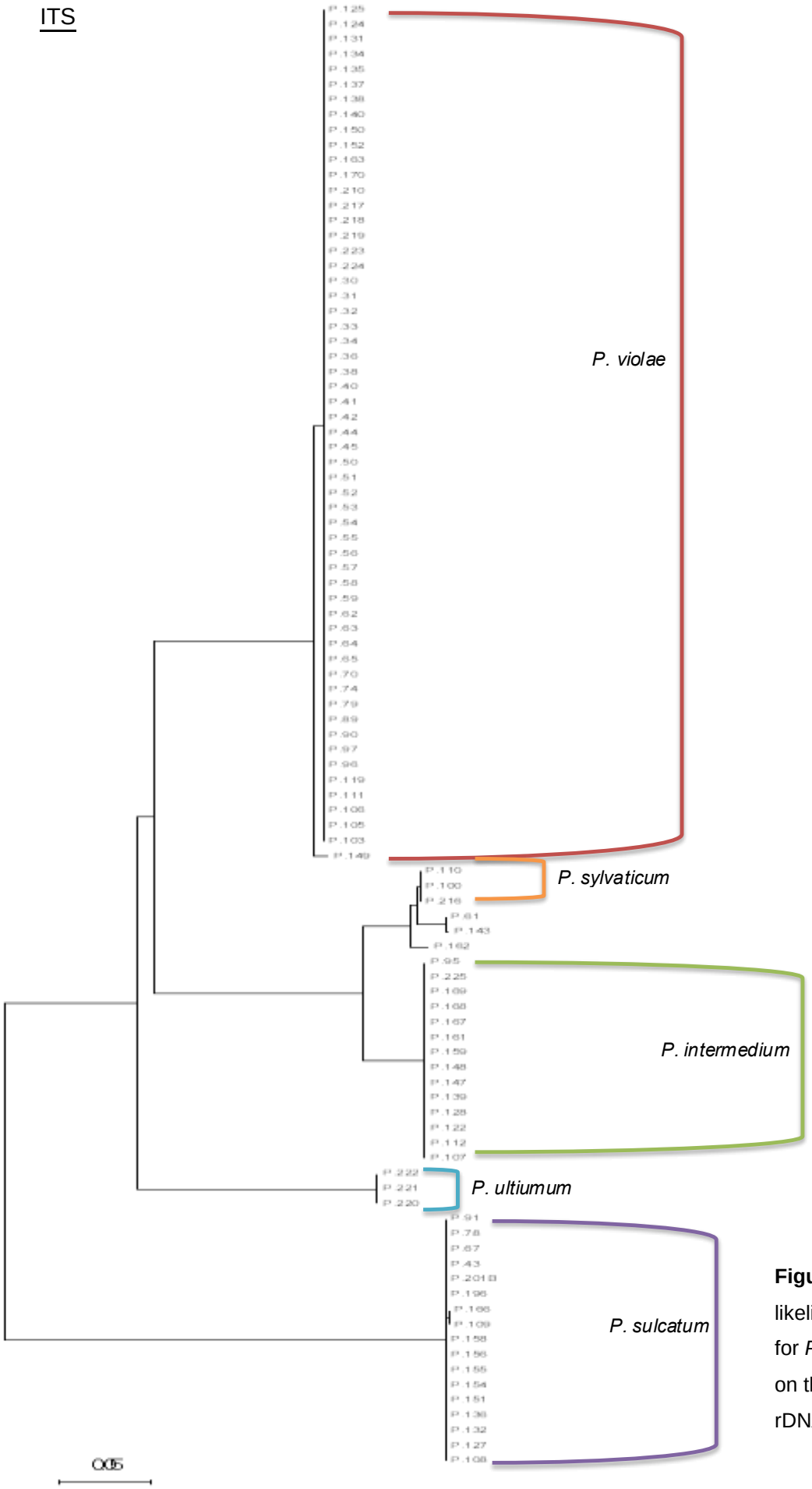


Figure 7: Maximum likelihood phylogenetic tree for *Pythium* isolates based on the ITS region of the rDNA gene.

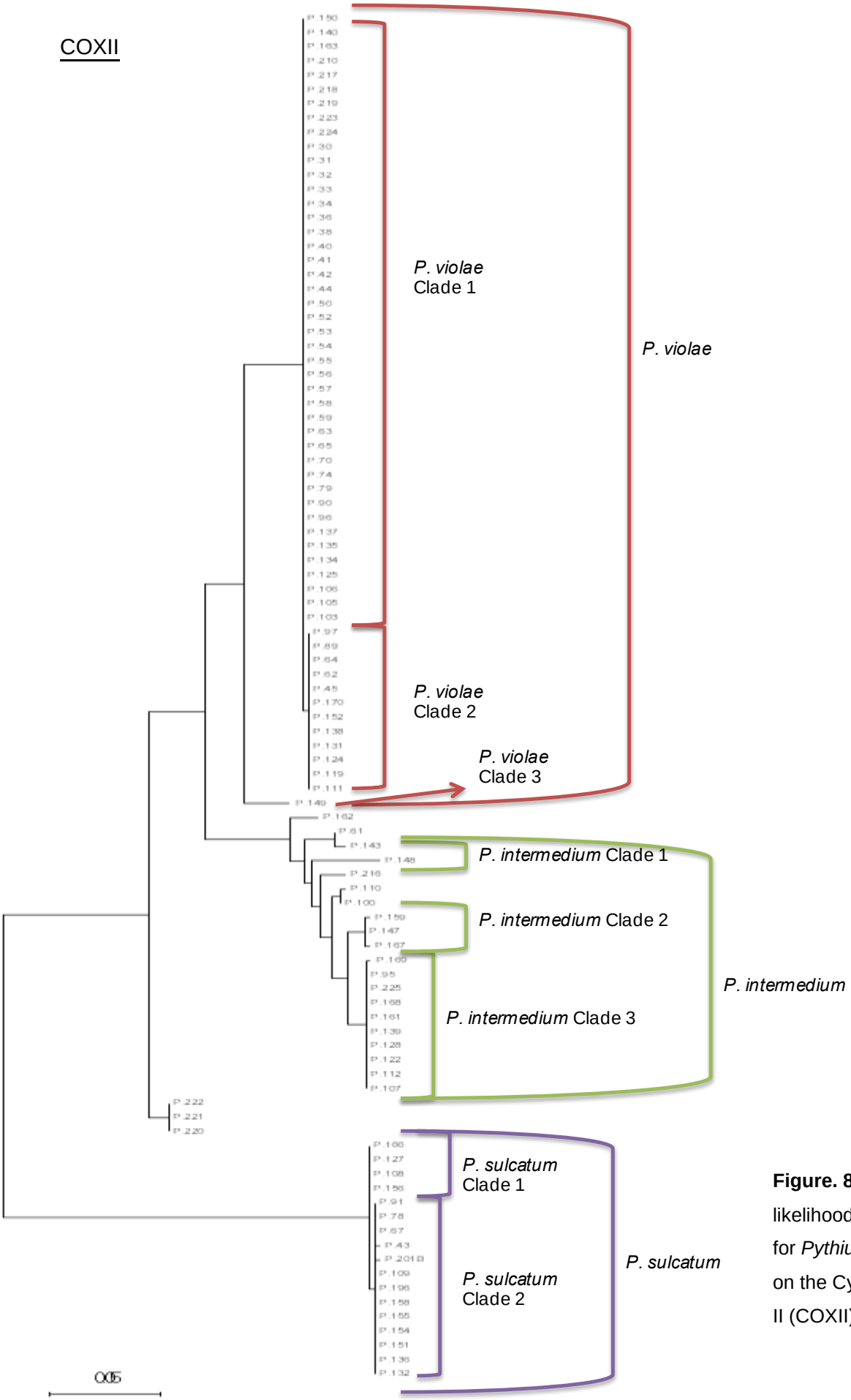


Figure. 8: Maximum likelihood phylogenetic tree for *Pythium* isolates based on the Cytochrome Oxidase II (COXII) gene.

Objective 1 ii) Refining and testing an oospore capture method for quantification of *P. violae*

Introduction

A new method for *P. violae* oospore capture from soil was developed based on sucrose centrifugation and filtration to allow 10 g of soil to be tested for presence of by the pathogen by PCR (Hales & Clarkson, 2015). When this method was further tested, it was found that less than 50% of spores were being retrieved from samples. The method was therefore refined by an initial step of suspending the sand in SDW followed by sonication, before the addition of the sucrose following an approach by Wang and Chang (2003). The efficacy of this method was then tested using inoculum samples and soil spiked with known numbers of oospores.

Materials and Methods

Samples (50 g) containing *P. violae* oospores were taken from a sand based solid substrate inoculum produced in flasks as previously described in Hales and Clarkson (2015). Each of these samples was thoroughly mixed and further sub-samples taken for spore capture and enumeration as described below.

To obtain an initial 'standard' quantification of the number of oospores in each sample, two 1 g portions were manually shaken in 10 ml SDW, vortexed for 1 min and oospores counted in using a 1 ml Sedgewick-Rafter counting under the microscope at x100 magnification. Three replicate counts for each 1 g sample were carried out.

P. violae oospores were captured from the sand inoculum using a sucrose flotation method. Samples (10 g) were added to a 50 ml sterile falcon tube and 45 ml saturated sucrose solution (75% w/v) added. The tube was manually shaken for 2 min (or shaken on a soil shaker, setting 7 for 2 min). The suspension was then centrifuged (Sorvall, UK) at 3500 rpm for 5 min and the supernatant filtered through a 5 μm filter (Cadisch Precision Meshes Ltd., UK) with a bottle-top filter unit (Nalgene, UK) to capture *P. violae* oospores. The filter was agitated for 5 min in 5 ml 1% Tween-20, rinsed with 1 ml SDW and oospores in the resulting suspension counted at x100 magnification as described before.

This method was then further refined based on work by Wang and Chang (2003) by introducing an initial step whereby the samples were first suspended in SDW and sonicated before adding to the sucrose solution. *P. violae* sand inoculum samples (10 g) were

suspended in 55 ml SDW in a 100 ml conical flask, vortexed for 1 min, sonicated for 5 min after which 600 µl of 1% Tween-20 was added and the suspension stirred for 5 min. The suspension was then added to a saturated sucrose solution (73 ml, 75% w/v) in a 250 ml centrifuge bottle (the conical flask rinsed out with a further 15 ml SDW which was also added to the sucrose), and the bottle inverted several times to mix. The entire suspension was then centrifuged at 2500 rpm for 5 min and the supernatant filtered through a 5µm nylon filter with a bottle-top filter unit to capture oospores.

Further sucrose extractions of the same sand inoculum sample were also carried out to capture any remaining oospores. SDW (40 ml) was added to the pellet and then vortexed for 1 min to re-suspend the whole sand sample. Sucrose solution (53 ml, 75%) was then added and the bottle inverted to mix. The suspension was then again centrifuged at 2500 rpm for 5 min and the resulting supernatant filtered through a second 5 µm filter as previously described. This was repeated a total of five times, reducing the volume of SDW / sucrose solution each time as follows: 1st extraction 55 ml / 73 ml; 2nd extraction 40 ml / 53 ml; 3rd extraction 40 ml / 53 ml; 4th extraction 20 ml / 26 ml; 5th extraction 20 ml / 26 ml. Each of the five 5µm filters resulting from these extractions was shaken in a soil shaker in 5 ml 1% Tween-20 for 5 min after which they were rinsed with 1 ml SDW before being transferred to a second tube where the process was repeated. A final wash in a third tube was then carried out to ensure all spores were washed off from the filter. The three 5 ml washes from each of the five filters were retained separately, and the number of oospores counted as before. Three replicate counts were conducted for each filter wash.

The efficacy of this approach for capturing oospores from soil was also tested by adding known concentrations of oospores (10, 50, 100, 500, 1000 oospores g^{-1}) to a light sandy loam field soil (Wick Series; Wellesbourne, UK). The same method was used with a slight adaptation to reduce the amount of soil residue being trapped on the 5 µm filter. After the centrifugation step, the supernatant was filtered through a 5 µm nylon filter as before, but the final 40ml (approx.) supernatant was retained. This was then transferred to a 50 ml Falcon tube and again centrifuged at 3000 rpm for 2 min which allowed any remaining soil particles to be better removed. The remaining supernatant was then passed through the same 5 µm filter. For the further sucrose extractions, the pellet from the 50 ml falcon tube was re-suspended in 40 ml SDW and the suspension added to the appropriate 250 ml centrifuge bottle before vortexing. The method was then repeated as above. Oospores captured from the filters were concentrated into 1.2 ml liquid and counted as described before.

Results

P. violae oospores were successfully captured from both the sand inoculum and spiked soil samples by sucrose flotation and subsequently trapped on filters. The initial enumeration method (shaking 1 g of sand inoculum in 10 ml SDW) was used as a reference count for comparison. Using the initial sucrose flotation approach, less than 50% of the spores were captured after two or three re-extractions of the same sample (Table 1). However, using the improved method where the inoculum was first suspended in SDW and sonicated before adding to the sucrose, oospore capture ranged between 121.6% and 157.5% compared with the original standard counting method with an average of 145% (Table 2). The number of spores captured declined with each sucrose extraction, with approx. 50% of the spores captured after the first sucrose extraction, and less than 10% after the fifth. The majority of spores (>90 %) were washed off after the first of the three filter washes. Enumeration of oospores captured from spiked soil samples as described above are still ongoing.

Table 1. Percentage oospore capture (compared with standard method) from five *P. violae* sand inoculum samples. Oospores from samples 1 (a,b) and 2 (a,b) were captured from two sucrose extractions of the same sand sample. Oospores from sample 3 were captured from three sucrose extractions of the same sand sample. Each nylon filter was washed three times to ensure maximum capture.

Sucrose extraction		**1**			**1 Total**	**2**			**2 Total**	**3**			**3 Total**	**Grand Total (%)**
Filter wash		**1**	**2**	**3**		**1**	**2**	**3**		**1**	**2**	**3**		
Sample no.	1a	11.8	7.4	2.9	**22.1**	19.3	3.4	2.1	24.8	-	-	-	-	**46.9**
	1b	12.7	5.2	2.7	**20.6**	15	3.3	1.7	20	-	-	-	-	**40.6**
	2a	5.2	5.9	3.4	**14.5**	7.1	2.1	1	10.2	-	-	-	-	**24.7**
	2b	14	8.6	3.4	**26.0**	5.8	1.9	1.5	9.2	-	-	-	-	**35.2**
	3a	14.8	3.7	2.1	**20.6**	12.1	2.2	0.9	15.2	7.9	1.6	0.4	**9.9**	**45.7**

Table 2. Percentage oospore capture from five *P. violae* sand inoculum samples. Oospores from each sample were captured with five sucrose extractions, and each filter washed three times to ensure maximum capture.

Sucrose extraction		**1**			**1 Total**	**2**			**2 Total**	**3**			**3 Total**	**4**			**4 Total**	**5**			**5 Total**	**Grand Total (%)**
Filter wash		**1**	**2**	**3**		**1**	**2**	**3**		**1**	**2**	**3**		**1**	**2**	**3**		**1**	**2**	**3**		
Sample no.	**1**	52.6	3.8	0.3	**56.7**	45.3	1.5	0.2	**47.0**	27.4	0.8	0.1	**28.3**	16.2	2.5	0.5	**19.2**	5.6	0.6	0.0	**6.3**	**157.5**
	2	51.7	2.6	0.2	**54.5**	43.8	1.8	0.2	**45.8**	23.1	3.4	0.5	**27.0**	9.7	2.1	1.0	**12.8**	5.8	0.5	0.0	**6.4**	**146.5**
	3	43.8	1.5	0.6	**45.9**	62.5	2.0	0.4	**65.0**	25.7	0.6	0.2	**26.4**	6.5	1.4	0.3	**8.2**	2.4	0.1	0.1	**2.5**	**148.0**
	4	66.0	2.3	0.2	**68.5**	29.1	3.0	0.4	**32.5**	12.1	1.9	0.1	**14.1**	2.6	2.9	0.2	**5.7**	0.4	0.3	0.1	**0.8**	**121.6**
	5	50.5	3.0	0.3	**53.8**	44.4	2.1	0.6	**47.1**	32.6	1.1	0.1	**33.8**	12.3	0.5	0.1	**12.9**	6.0	0.3	0.3	**6.6**	**154.2**

Objective 1 iii) Testing artificial inoculation methods for *P. violae*

Seedling experiments

Materials and Methods

A *P. violae* sand based inoculum in which many oospores could be produced was developed previously and used to inoculate carrot seedlings which resulted in varying levels of damping-off (Clarkson *et al*., 2017). Given this variation, a further seedling test was carried out to evaluate the effect of *P. violae* oospores produced in a liquid inoculation on carrot seed germination and damping off in autoclaved and non-autoclaved sand when applied at sowing or to seedlings three weeks after sowing. V8 Juice broth (V8B) was selected as the liquid growth medium as it has been reported to support growth of *Pythium* species as well other oomycete species (Sutherland & Cohen, 1983; Pettitt, 2002). V8 juice (1 L) was stirred with 20g $CaCO_3$ for 45 minutes and the mixture centrifuged at 9000 rpm for 30 min at 20^{o}C. For experiments, a 10% (v/v) V8B was prepared by combining 100 ml of the clarified supernatant with 900 ml distilled water and autoclaving at 121^{o}C for 15 min. Cholesterol (Sigma-Aldrich, UK) from a stock solution (15 mg ml^{-1} in 95% ethanol) was added to V8B to achieve a final concentration of 30 mg L^{-1} (V8B+C). Agar plugs of mycelium (2 x 6 mm) from the actively growing edge of a *P. violae* culture (isolate HL) grown on CMA were used to inoculate 20 ml aliquots of V8B+C in flat cell culture flasks (50ml, VWR, UK) with vented lids. After incubation at 15^{o}C for 7 weeks in the dark, mycelial mats were removed, washed twice in 20 ml SDW, blotted dry on sterile filter paper and weighed. Mycelial mats from three culture flasks were transferred to a sterile bulbous flask (MSE, UK), and homogenized (MSE Homogeniser) on a low setting for 8 min in 45 ml SDW and the process repeated for mats from a further two sets of three flasks. The homogenates for the total of nine mycelial mats were combined and the homogeniser blade and flask washed with SDW to give a total volume of 150 ml homogenate. *P. violae* oospore concentration was then determined by removing a 200 µl sample, vortexing for 20 s and counting in a Fuchs-Rosenthal haemocytometer. Diluted oospore inoculum (10 ml) was added to either autoclaved or non-autoclaved sharp sand (300 g, Westland, UK) contained in clear plastic boxes at concentrations 1 x 10^3, 1 x 10^4 and 1 x 10^5 oospores ml^{-1} to give final concentrations of 3, 30 and 300 oospores g^{-1} and carrot seed (cv. Nairobi) sown (20 seeds per box). Oospore inoculum at the same final concentrations was also used to inoculate carrot seedlings grown in the same system three weeks after sowing by pipetting the spore suspension around the plants. *P. violae* oospores produced in solid inoculum were also prepared as previously described (Hales & Clarkson, 2015) and used to amend sand to the same final concentrations before sowing (Table 3). For each treatment, there were four replicate boxes which were placed in a randomized block design over four shelves in a

controlled environment room at 15°C. Untreated control treatments (no *P. violae* inoculum) were also set up. Seedling germination and disease symptoms were assessed weekly for 10 weeks. The number of seedlings which damped off as a percentage of those which germinated in each box was recorded.

Table 3. *P. violae* oospore treatments evaluated in the seedling tests.

Inoculum type	**Sand type**	**Inoculation time**	**Concentration (oospores g^{-1})**
Liquid	Non-autoclaved	Sowing	Control
Liquid	Non-autoclaved	Sowing	3
Liquid	Non-autoclaved	Sowing	30
Liquid	Non-autoclaved	Sowing	300
Liquid	Autoclaved	Sowing	Control
Liquid	Autoclaved	Sowing	3
Liquid	Autoclaved	Sowing	30
Liquid	Autoclaved	Sowing	300
Liquid	Non-autoclaved	Germination	Control
Liquid	Non-autoclaved	Germination	3
Liquid	Non-autoclaved	Germination	30
Liquid	Non-autoclaved	Germination	300
Liquid	Autoclaved	Germination	Control
Liquid	Autoclaved	Germination	3
Liquid	Autoclaved	Germination	30
Liquid	Autoclaved	Germination	300
Solid	Non-autoclaved	Sowing	Control
Solid	Non-autoclaved	Sowing	3
Solid	Non-autoclaved	Sowing	30
Solid	Non-autoclaved	Sowing	300

Results

The *P. violae* oospore inoculum produced in liquid V8B did not result in any seedling mortality when applied either at sowing or to seedlings in autoclaved sand (Table 4). However, when applied to non-autoclaved sand, seedling mortality ranged between 27.6% and 54.2% over all treatments, regardless of inoculum concentration. However, seedlings also died in uninoculated control treatments with percentage mortality ranging between 44.1% and 51.4% suggesting that seedling death was not due to *P. violae*. Similarly, the *P. violae* solid oospore inoculum showed high levels of seedling mortality (60.5% to 75%) including the uninoculated control treatments.

Table 4. Effect of different concentrations of *P. violae* oospores on carrot seedling mortality when applied at sowing or to seedlings as liquid or solid inoculum in autoclaved / non-autoclaved sand

Inoculation Method	Sand type	Inoculation	Oospores g^{-1}	% seedlings killed
Liquid	Autoclaved	Seedlings	3	0
			30	0
			300	1.3
			0	0
	Autoclaved	At sowing	3	0
			30	0
			300	0
			0	0
	Non-autoclaved	Seedlings	3	46
			30	47.8
			300	38.2
			0	44.1
	Non-autoclaved	At sowing	3	27.6
			30	52.9
			300	54.2
			0	51.4
Solid	Non-autoclaved	At sowing	3	75
			30	66.3
			300	60.5
			0	72.1

Mature plant experiments

Two glasshouse experiments were conducted joint with AHDB project FV391a, with the aim of inducing cavity spot symptoms in glasshouse grown carrots by inoculating the growing medium with different concentrations of *P. violae* oospores produced on a solid sand-based substrate.

Materials and Methods

Large pots were filled with a mixture of compost and sharp sand and inoculated with solid *P. violae* inoculum (isolate HL) at a range of oospore concentrations. Carrot seed (cv. Nairobi) was sown directly into the pots and thinned 6-7 weeks later to five carrot seedlings per pot. 16 replicate pots were prepared for each oospore concentration in a randomised block design. Two experiments were set up (Fig. 9); and in each experiment, carrot seedling emergence and damping off was recorded for the first six weeks. Plants were harvested when carrots reached maturity (21/23 weeks). The number of cavity spot lesions was then recorded for each carrot as well as carrot weight, and a selection of roots were taken for plating onto CMA to assess colonisation by *P. violae*. All data recorded were subjected to statistical analysis using ANOVA with angular transformation of percentage seedling emergence, damping off, and cavity spot incidence and $\log_{10}$ transformation of number of cavity spot

lesions. For detailed description of experimental set up and assessment of symptoms, see Final Report for AHDB project FV391a (Clarkson *et al*., 2017).

Figure 9. Pot-grown carrots inoculated with *P. violae* in two experiments 6 and 13 weeks after sowing.

Results

Full results are presented in the Final Report for AHDB project FV391a (Clarkson *et al*., 2017) and a summary is presented here. In the two experiments, carrot seedling germination was similar between control and inoculated treatments (73-88%) but some post-emergence damping off was observed (5-7%) in the *P. violae* treatments. Seedling weight was significantly reduced in the 50 and 75 oospores g^{-1} concentrations compared to the control in both experiments ($P < 0.001$, $P < 0.05$ for experiments 1 and 2 respectively; Fig. 10ab). In experiment 1 carrot top-growth was reduced at 6-9 weeks post sowing in inoculated treatments compared to controls, with some visual evidence that this was greater with increasing oospore concentration (Fig. 11). However, this was not observed in experiment 2 nor by the end of both experiments at harvest, where there was no significant difference in top-growth weight between any of the treatments and the control (data not shown).

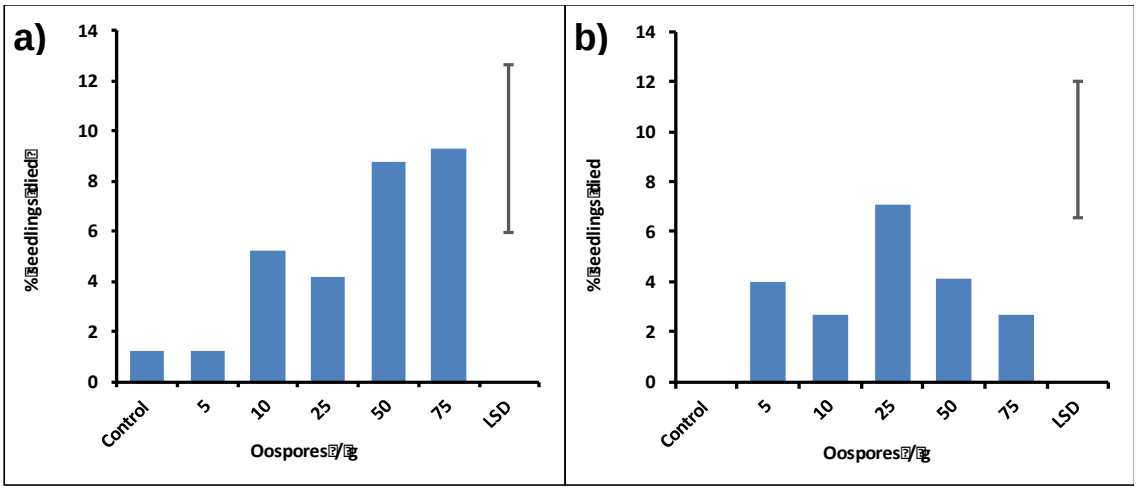


Figure 10: Effect of different concentrations of *P. violae* solid sand-based inoculum on mean percentage damping off of carrot seedlings in a) experiment 1 and b) experiment 2 (angular transformed data). Bars represent the least significant difference between treatments (LSD) at the 5% level.

Figure 11: Effect of different concentrations of *P. violae* solid sand-based inoculum on carrot foliage growth after 6 weeks in experiment 1. From L to R; untreated control, 5, 10, 25, 50 and 75 oospores g^{1}.

At harvest, all inoculated treatments showed marked stunting of the roots. They were small, stubby with long hairy brown tap roots with increased lateral root formation, many of which were collapsed. This observation was reflected in carrot weight, with a significant reduction in weight of roots in inoculated treatments compared to controls in both experiments ($P < 0.001$; Fig. 12ab), but there was no apparent effect of different oospore concentrations.

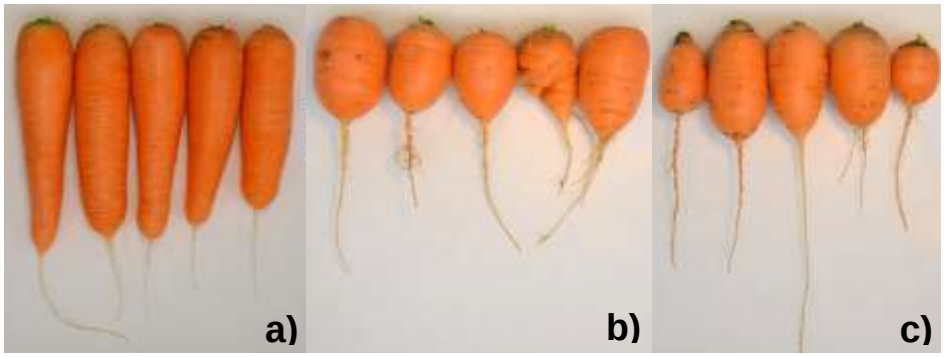


Figure 12: Effect of *P. violae* solid sand-based inoculum on carrot root growth (experiment 1); a) control, b) 25 oospores g^{-1}, c) 50 oospores g^{-1}.

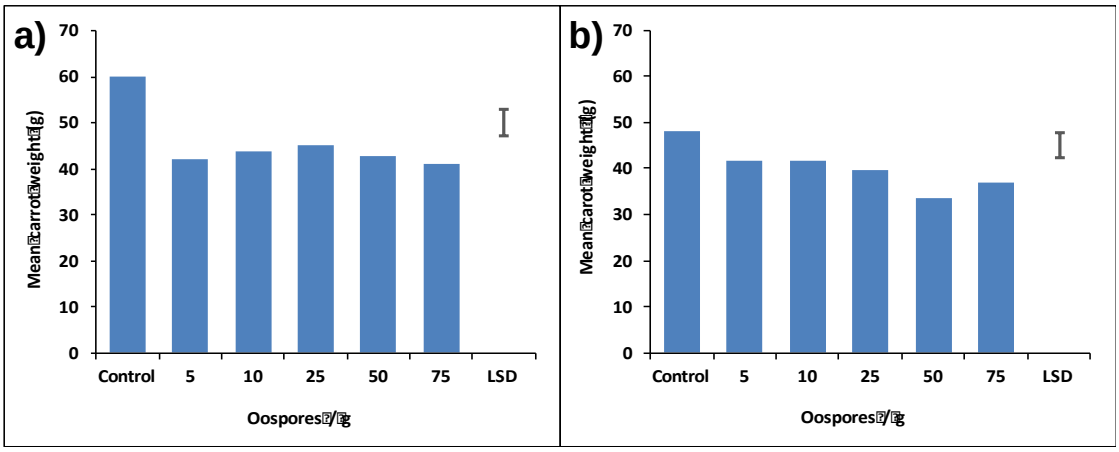


Figure 13: Effect of different concentrations of *P. violae* solid sand-based inoculum on carrot weight in **a**) experiment 1; **b**) experiment 2. Bars represent the least significant difference between treatments (LSD) at the 5% level.

Typical cavity spot symptoms were observed in all *P. violae* treatments (Fig. 14) with 18-26% incidence in experiment 1 and 4-13% in experiment 2 with no apparent effect of oospore concentration (Fig. 15ab). No cavity spot symptoms were observed in uninoculated control treatments. However, the effect of *P. violae* inoculation on the incidence of cavity spot compared to the untreated control was only statistically significant for experiment 1 ($P < 0.001$), and just outside the 5% level of significance for experiment 2 ($P = 0.063$). Maximum number of cavity spot lesions ranged from 4-6 and 2-5 in experiments 1 and 2 respectively (data not shown).

Figure 14: Cavity spot lesions on carrot roots from *P. violae* inoculated treatments.

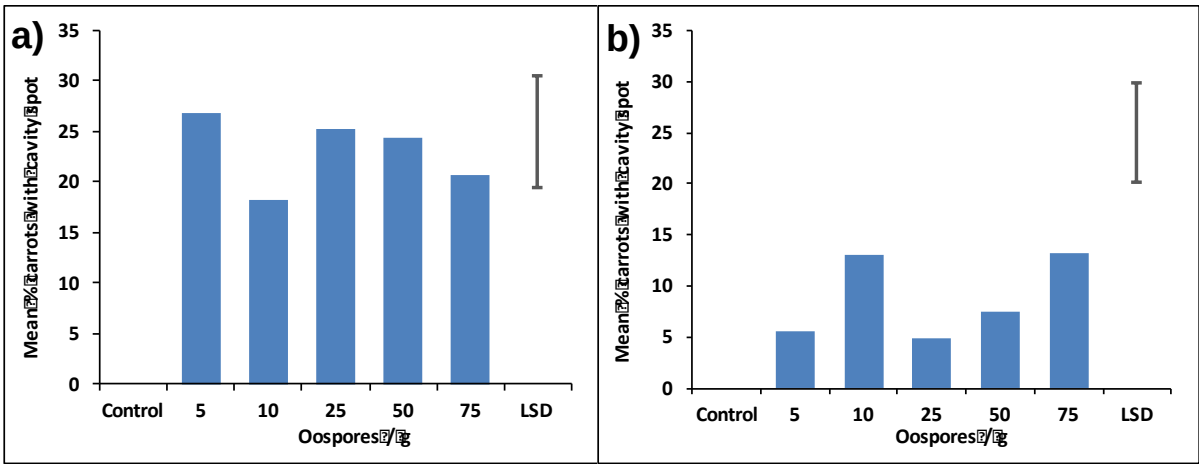


Figure 15: Effect of different concentrations of *P. violae* solid sand-based inoculum on cavity spot incidence in **a,** experiment 1 and **b** experiment 2 (angular transformed data). Bars represent the least significant difference between treatments (LSD) at the 5% level.

A high proportion of carrot tap root pieces from the inoculated carrots (Expt. 1 29-58%; expt. 2. 12-15%) yielded *P. violae* colonies on agar (Fig. 16ab) demonstrating that the stunting of carrots was due to high levels of pathogen infection. No *P. violae* was isolated from any of the root pieces from uninoculated control treatments. *P. violae* was also successfully isolated from selected cavity spot lesions (Fig. 18c). For a detailed description of results, please refer to Final Reprt FV391a.

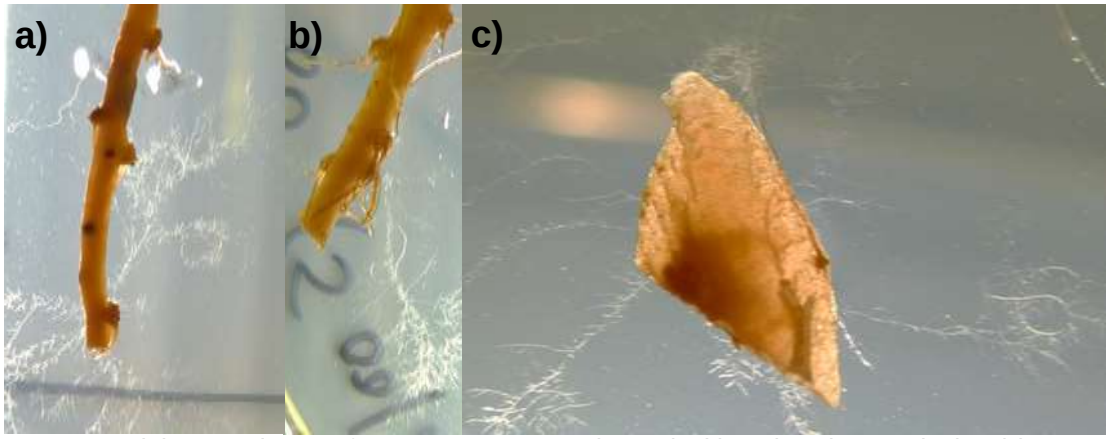


Figure 16: *P. violae* growing out from carrot tap root pieces (a, b) and cavity spot lesion (c) on agar.

Field experiments

Materials and Methods

A cavity spot field trial was carried out in collaboration with AHDB project FV 391a from May 2016 to February 2017 in 24 'macrocosm' plots located in the Wellesbourne quarantine field (Fig. 17). The macrocosms comprised sunken concrete tubes filled with a mixture of sandy silt loam (Wick series; Wellesbourne, UK) and horticultural sand (Westland, UK). *P. violae* isolate HL sand inoculum was produced in 1 L flasks as previously described, and 5 kg batches raked into the top 10 cm of the macrocosms to give final oospore concentrations of 5, 10, 20, 30 and 50 oospores g^{-1}, with four replicates in a randomised block design. Each macrocosm was sown with approximately 280 carrot seeds (cv. Nairobi). Carrots were harvested eight months after inoculation and both carrot yield and cavity spot symptoms recorded. For detailed description of experimental set up and assessment of symptoms, see Final Report for AHDB project FV391a.

Figure 17: Macrocosms used for *P. violae* inoculation of carrots (July 2016).

Results

Carrots grew well in the field macrocosms and all *P. violae* inoculated treatments resulted in typical cavity spot lesions forming on roots (Fig. 18). Cavity spot incidence ranged between 24% and 39% roots affected and there was no apparent effect of inoculum concentration although the 50 oospore g^{-1} treatment had a much greater incidence of cavity spot (39%) compared to the other treatments (Fig. 19). There were very few or no cavity spot-type lesions in the control treatments.

Figure 18: Carrots harvested from field macrocosms inoculated with P. violae showing typical cavity spot symptoms.

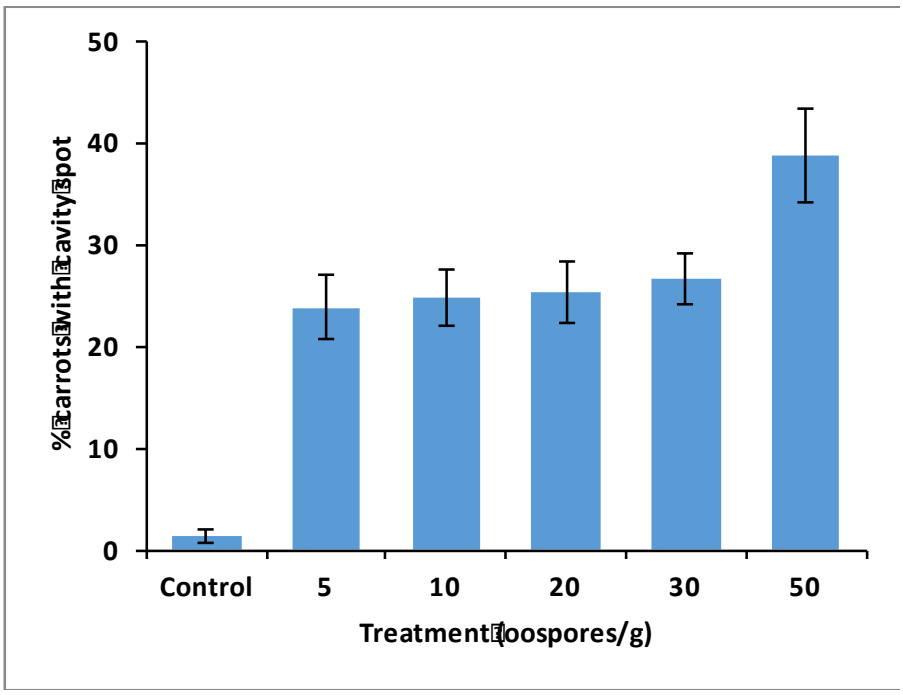


Figure 19: Effect of different concentrations of *P. violae* solid sand-based inoculum on percentage of carrots with cavity spot in the field.

Discussion

Objective 1 i) *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* accounted for 14% of isolates each. These results confirm previous research suggesting that *P. violae* was the main pathogen causing cavity spot in the UK (Lyons & White, 1992; White, 1988; White, 1986; Groom & Perry, 1985; Cooper *et al*., 2004). This part of the work has therefore provided a larger and more contemporary 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. The genetic analysis showed that different *Pythium* species can easily be distinguished from one another using ITS gene sequence while sequences from another housekeeping gene, COXII, indicated intraspecific variation within *P. violae*, as well as *P. intermedium* and *P. sulcatum*. A second housekeeping gene, NADH, has also just been sequenced and also revealed variation within different *Pythium* species. This information will be used to investigate how genetic variation may relate to biological variation such as pathogenicity and spore production.

Objective 1 ii) Refining and testing an oospore capture method for quantification of *P. violae*

The sucrose-based oospore capture method previously developed (Hales & Clarkson, 2016) was fully tested, but found to capture less than 50% of the *P. violae* oospores in sand inoculum samples compared to a standard extraction and counting method. In other work which quantified oospore production over time (as part of project FV391a) it was found that oospores were very difficult to separate from mycelium suggesting that the spores may not be detaching from mycelium just using agitation. The method was therefore adapted to make use of both sonication to detach the oospores from mycelium, as well as water and Tween to separate the oospores from the sample before adding to the sucrose. This was largely based on a paper by Wang and Chang (2003) which investigated extraction of oospores of *P. myriotylum* from soil by sucrose centrifugation with this method in turn being based on that of Vandergaag and Frinking (1997). The improved method increased oospore capture beyond the standard approach with approx. 50% more spores obtained from the samples. Previous reports of spores adhering to soil particles has been documented in the literature (Kuczynska & Shelton, 1999).

As the oospore capture method has now been defined, further work is currently underway to investigate its efficacy using soil samples spiked with different numbers of oospores. However, the number of *P. violae* oospores in field soil is largely unknown. Previous research in AHDB project FV 5f (Pettitt, 2002) using a soil plating procedure suggested that *P. violae* oospore populations can range between 0 and 30 oospores g^{-1} dry soil, and can rise to between 80 and 200 oospores g^{-1} dry soil in heavily infested soils. In this project, soil samples from cavity spot infected carrot fields were acquired in 2015 and were found to contain 57 oospores g^{-1} dry soil for a field three months after harvest of a heavily infected carrot crop, and 280 oospores g^{-1} dry soil from a field soil collected from around cavity spot infected carrots. From these estimations, the oospore capture method is therefore currently being tested on soil spiked with concentrations of 0, 10, 50, 100, 500, and 1000 *P. violae* oospores g^{-1}.

Objective 1 iii) Testing artificial inoculation methods for *P. violae*

Seedling experiments

The seedling tests with *P. violae* inoculum again proved inconclusive and difficult to interpret and seedling death in the control treatments in non-autoclaved sand suggested another causal agent, mostly likely resident in the sand. This potential contamination problem along with the previous variable results with solid sand based inoculum highlights the difficulty in working with *P. violae* and assessing the viability of inoculum. Oospores do not germinate readily *in vitro* and hence further work in FV 391b is aiming to address this problem by developing approaches to measure oospore viability and / or stimulate germination. Further seedling experiments will now investigate a millet-based inoculum based on research by El-Tarabily *et al*. (2004)

Mature plant experiments

This is the first report of successful artificial inoculation of mature carrots with a *P. violae* solid substrate inoculum. Inoculation with *P. violae* resulted in some seedling death, reduced seedling size, an initial decrease in growth of foliage and the formation of stubby, stunted carrot roots with some typical cavity spot lesions as well as brown and hairy taproots.

Damping off, although commonly associated with many *Pythium* species (e.g. *P. ultimum*), has not been generally recorded with *P. violae*. However, previous work in this PhD project (Hales & Clarkson, 2016) has observed varying levels of damping off in carrot seedlings using *P. violae* solid inoculum at a range of oospore concentrations. Furthermore, Pettitt (2002), used a suspension of *P. violae* oospores in water (3 x 10^6 spores ml^1) to inoculate carrot

seedlings sown in a sand contained in small modules. This resulted in up to 31% roots becoming colonised with *P. violae* as measured by plating onto agar media but no mortality was reported. (Howard *et al*., 1978) also reported damping off and root die back in carrot seedlings as being caused by at least 11 different *Pythium* species (but not *P. violae*) including *P. sulcatum* which is also associated with cavity spot in the UK (Hales & Clarkson, 2016).

One of the major of effects of *P. violae* inoculation using the solid substrate inoculum was on carrot plant growth with the formation of stubby, stunted carrots for all the treatments. In addition, tap roots extending from the bottom of these carrots were brown and hairy compared to untreated control treatments. This was a somewhat surprising result as *P. violae* has not generally been associated with stunted or malformed carrots although Pratt and Mitchell (1973) previously reported that *P. sulcatum* was consistently isolated from carrots with 'forked and stunted' taproots and with 'brown root symptoms' in North America.

As well as a decrease in root weight, artificial inoculation with the *P. violae* solid substrate inoculum resulted in typical cavity spot lesions on carrots. Lesions have been reported previously by White (1986) who inoculated sterile soil with a *P. violae* maizemeal inoculum. Two other studies used millet seed colonised with different *Pythium* spp. associated with cavity spot (but not *P. violae*) as inoculum which generally resulted in high levels of typical cavity spot symptoms (El-Tarabily *et al*., 1996) (El-Tarabily *et al*., 2004) although severity of symptoms varied between different *Pythium* species.

Finally, a high incidence of cavity spot was also observed in carrots grown in the inoculated field macrocosms which, along with the results from the pot tests, suggests that the solid substrate inoculum is a good approach for infecting carrots with *P. violae*.

Conclusions

- Over 100 *Pythium* isolates were collected from cavity spot infected carrots obtained from different locations. *P. violae* was identified as the predominant species present.
- A new oospore capture method was devised and refined which is suitable for efficient extraction of oospores from soil samples.
- Inoculation of carrot seedlings with either liquid or solid *P. violae* inoculum was very variable and prone to contamination.
- Cavity spot was induced in mature pot-grown carrots following soil amendment with a solid sand based inoculum of *P. violae*. Symptoms included a decrease in growth of foliage, formation of small, stubby and stunted carrots with brown tap roots and typical cavity spot lesions.

- Cavity spot was induced following inoculation of field macrocosms with *P. violae* and resulted in high incidence of cavity spot.

Knowledge and Technology Transfer

- Presentation: SCI Young Researchers in Crop Sciences Conference, Syngenta, 14th July 2016.
- Poster: AHDB Crops Studentship Conference, Stratford Upon Avon, 16th/17th November 2016.
- Presentation: Carrot Advisor Experience Exchange Network (CAEEN), Thetford, 24th November 2016.
- Presentation: 38th International Carrot conference, Bakersfield, California, 19th - 22nd March 2017.
- Poster: Molecular Biology of Plant Pathogens (MBPP), Durham, 29-30th March 2017
- Poster: Postgraduate Research Showcase 2017, University of Warwick, 7th June 2017
- Presentation: British Society for Plant Pathology (BSPP) Presidential Meeting 2017, Nottingham, 11th-13th September 2017.

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