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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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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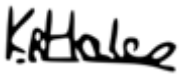
Signature:  Date: 12/03/2019

Report authorised by:

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Signature:  Date: 12/03/2019

CONTENTS

GROWER SUMMARY

This document is a summary of a PhD project. The full thesis is available through the University of Warwick.

Headline

- *P. violae* is the most common species identified from cavity spot lesions on carrots, but both *P. intermedium*, and *P. sulcatum* are also associated with the disease. Virulence between species and isolates within species was variable.
- 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 the formation of high levels of cavity spot (up to 40% incidence).
- To enable soil testing for *P. violae*, oospore capture, lysis and DNA extraction methods were optimised and a specific, and highly sensitive quantitative qPCR for the pathogen successfully developed. However, further work is required before this approach can be used routinely to quantify *P. violae* in field soil and relate to disease development.

Background

Carrot production

Carrots are produced across all temperate regions (Hiltunen & White, 2002) in over 64 countries worldwide (Nemecek et al., 2012). In Britain, approximately 11,500 hectares (ha) of carrots are planted each year, with over 800,000 tonnes of carrots produced (DEFRA, 2018).

Impact of cavity spot on commercial carrot production

Cavity spot disease of carrot has been a significant problem for growers for over 40 years and results in dark, sunken elliptical lesions which result in an unmarketable crop. Growers often suffer severe losses as even roots with only a small number of superficial lesions are rejected by the market. Losses to cavity spot vary from year to year, but are generally reported to be at least 10% (G. Poskitt, MH Poskitt Ltd., UK, personal communication). In some years, growers have reported losses up to 35% (Gladders, 2014). Overwintered crops lost to cavity spot lead to particularly high financial losses (Gladders, 2014). Calculations on the financial losses due to cavity spot are

difficult to ascertain. The unpredictable nature of the disease means growers have to implement emergency strategies for early harvesting: increasing labour for grading and making marketing alterations to attempt to salvage a crop (Martin, 2014). These all result in considerable costs and disruption from standard operations, which are challenging to accurately calculate. One estimate indicates that just in direct crop losses, cavity spot can cost growers approximately £3-5 million each season (Martin, 2014). However DEFRA have indicated that losses can rise to £20-30 million in particularly severe years when taking into account wider impacts (Gladders, 2014).

Cavity spot infection

Cavity spot is associated with several oomycete *Pythium* species, and the main causal agent appears to vary from country to country. It is important to be able to identify particular *Pythium* spp. associated with cavity spot in different locations, because they vary in host range (Schrandt *et al.*, 1994; Davison & McKay, 2001), and sensitivity to fungicides such as metalaxyl (White, 1988; Hiltunen & White, 2002). The diversity of *Pythium* spp. in a complex may affect the severity of cavity spot (Suffert & Guibert, 2007) but there has been limited research into the distribution of different *Pythium* spp. associated with cavity spot in the UK and how this may relate to pathogenicity. *Pythium* produces white/transparent hyphae which are fragile, thin and generally less than 6 µm wide (Figure 1.4a) (van der Plaats-Niterink, 1981; Agrios, 2005) as well as oospores. Oospores serve as long-term survival structures. *P. violae* has been described as the most important *Pythium* species associated with cavity spot in the UK (Hiltunen & White, 2002) although a more recent, thorough survey has not been conducted.

P. violae host range

P. violae causes cavity spot disease on mature carrot roots, but can colonise roots of other plants asymptomatically (Kalu *et al.*, 1976; Dewan & Sivasithamparam, 1988; Schrandt *et al.*, 1994; Chavarriaga *et al.*, 2007; Barbara, 2010a; Kretzschmar, 2010). Carrot is the only crop in which it is described as causing disease of economic importance. Alternative plant hosts can be colonised by the pathogen, and so can support mycelial growth and/or replenish oospores in the soil (Dissanayake *et al.*, 1997; Dhingra & Netto, 2001; Kretzschmar, 2010). Therefore, these alternate hosts can contribute to an increase in inoculum level, and subsequently an increase in disease incidence when a susceptible crop such as carrot is planted (Davies & Nunez, 1999; Johansson *et al.*, 2006). It has been previously suggested that successive carrot crops can increase the likelihood of cavity spot development (Rubens & Halford, 1983; Lyshol *et al.*, 1984). These influences need to be understood to create effective management strategies (discussed in Section 1.4).

P. violae oospore survival and detection

Understanding cropping history, as well as the susceptibility of crops to *P. violae* colonisation, is fundamental to managing the soil-borne inoculum. Oospores of *P. violae* are known to survive for several years in the soil (Hendrix & Campbell, 1973; Mitchell, 1978; van der Plaats-Niterink, 1981) and are thought to be the primary inoculum source for plant infection (Hendrix & Campbell, 1973). Detection and quantification of *P. violae* in the soil is therefore vital for understanding the biology, epidemiology and population dynamics of the pathogen, which in turn will inform effective management practices (Pavon *et al.*, 2007). However, difficulties in detection have hampered the understanding of *P. violae* dynamics and assessment of disease risk. It is thought that the inoculum levels required for cavity spot development are very low (1 colony forming unit (CFU) in 40 g soil) (Hiltunen & White, 2002) and initial attempts to use pre-season *P. violae* levels (as measured by a PCR test) to predict final cavity spot severity in the crop have failed and it is not clear if lack of a relationship between initial inoculum levels and subsequent disease could be due to inadequate sensitivity of detection (Barbara, 2010b). A reliable capture method allowing both specific and sensitive detection of *P. violae*, as well as a reliable measure for quantifying the pathogen from soil samples is needed to enhance understanding of *P. violae* epidemiology.

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).

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.

Aims

The overall aim of this research was to develop effective tools for understanding the biology and epidemiology of *Pythium* spp. causing cavity spot disease in carrots, in order to aid the development of new management approaches. The specific objectives were to:

1. Identify the current *Pythium* species associated with cavity spot in the UK and investigate the phylogeny and pathogenicity of a range of isolates.
2. Develop an artificial inoculation system for *P. violae* to reliably induce cavity spot disease in carrots.
3. Develop molecular tools to effectively capture, detect and quantify *P. violae* from field soil.

Summary

Aim 1: Identify the current *Pythium* species associated with cavity spot in the UK and investigate the phylogeny and pathogenicity of a range of isolates

- A survey of the *Pythium* species associated with cavity spot lesions in carrot production areas throughout England was carried out with 178 isolates collected. Several *Pythium* spp. were isolated from roots with a range of symptoms, but the main causal species was *P. violae* (56*%)* followed by *P. intermedium* (16%) and *P. sulcatum* (11%). *P. violae* accounted for 61% of isolations from the carrot variety Nairobi. Genetic analysis revealed both between- and within-species variation.

- Inoculation of carrot roots with agar plugs of isolates representing *P. violae, P. intermedium* and *P. sulcatum* resulted in cavity spot lesions but showed shown variation in lesion size. *P. violae* and *P. sulcatum* isolates were the most aggressive and produced large, dark lesions within 72 hours which expanded during the next six days. However, lesions were smaller with less discolouration of the tissue following inoculation with *P. intermedium* isolates. Inoculation of different carrot cultivars / lines with a *P. violae* isolate resulted in a range of lesion sizes with the third largest lesions occurring on cv. Nairobi.

Aim 2: Develop an artificial inoculation system for *P. violae* to reliably induce cavity spot disease in carrots.

- An optimised protocol for production of *P. violae* oospores was developed using a solid medium comprised of horticultural sand and an oat-based substrate. This inoculum was then used to try and initiate damping-off type symptoms in carrot seedlings grown in coarse sand as a means of developing a quick pathogenicity assay. However overall, considerable variation in damping-off symptoms were observed in carrot seedlings and across five experiments, no clear relationship was observed between *P. violae* oospore concentration, percentage seed germination or post-emergence disease levels. However, this initial study established that seedling mortality generally occurred at levels > 75 oospores g^{-1}, and the greatest increase in mortality was between 50-75 oospores g^{-1}.
- In glasshouse experiments to develop an inoculation system for mature carrots, amending a compost/sand growing medium with different levels of this inoculum initially resulted in some seedling mortality, reduced seedling size and decreased growth of carrot foliage. When harvested, carrot roots were severely stunted with brown / hairy tap roots and also had typical cavity spot lesions. In field experiments, all inoculated treatments resulted in characteristic cavity spot lesions. This is the first report of successful artificial inoculation of mature carrot plants grown both in pots in the glasshouse and under field conditions using a *P. violae* oospore inoculum. Further details and discussion of this work is in AHDB final project reports for FV391a/b.

Aim 3: Develop molecular tools to effectively capture, detect and quantify *P. violae* from field soil.

- An oospore capture method was developed that allowed efficient retrieval of *P. violae* oospores from sand inoculum samples; however, the capture of oospores from spiked soil samples was less efficient. Nonetheless, this approach has considerable potential benefits for use in the future allowing much larger soil samples to be processed. The Taqman qPCR for quantification of *P. violae* DNA was highly sensitive and specific and is a significant improvement compared with previous non-specific molecular detection tools.
- The combination of the capture and DNA detection did not result in reliable quantification of *P. violae* in a commercial carrot-growing soil unless levels were very high. Time constraints limited any further evaluation of the oospore capture method, but there are a number of potential improvements that could be made. Despite these problems, the combination of efficient oospore capture and Taqman qPCR assay for *P. violae* represents a major advance in pathogen detection and quantification, which will be a valuable tool for further research.

Financial Benefits

- Further development and use of the *P. violae* artificial inoculation system developed in this project could lead to significant savings in the future as it should ensure reliable and consistent disease levels for testing of crop protection products, potentially resistant carrot lines or other approaches to cavity spot control.
- Substantial progress has been made towards the capture, detection and accurate quantification of *P. violae* oospores in field soil. This will be a valuable tool for further research in understanding of *P. violae* infection and dynamics, and in the future could be used to assess disease risk prior to sowing or strawing down.

Action Points

- Growers need to be aware that cavity spot can be caused by several *Pythium* species with *P. violae* being the most prevalent and aggressive. Currently there is no evidence to suggest that control methods should differ between *Pythium* species.
- Different carrot cultivars showed huge variation in response to a single *P. violae* isolate; further work on the use of cultivars in breeding programmes could explore this potential.
- The sensitivity of the individual *Pythium* isolates and species to metalaxyl has yet to be explored.

SCIENCE SECTION

Introduction

Carrot production and economic importance

Carrots (*Daucus carota* L., Kingdom Plantae, Order Apiales, Family Apiaceae) are produced across all temperate regions (Hiltunen & White, 2002) in over 64 countries worldwide (Nemecek *et al.*, 2012). The amount of land dedicated to carrot production has increased threefold since 1965 (Simon, 2000). In Britain, approximately 11,500 hectares (ha) of carrots are planted each year, with over 800,000 tonnes of carrots produced (DEFRA, 2018). Carrots are Britain's major root vegetable crop (BCGA, 2018) with a retail sales value of approximately £290 million, and the country is self-sufficient in supplying carrots for the fresh market and for processing over 11 months of the year. Carrots are grown and harvested year-round using a variety of techniques. Early sown carrots (January-April) are protected from frost with fleece or plastic, and harvested from June onwards, while later sown crops (May-June) are stored in the ground from November/December through to May. These are kept under straw to protect from the cold weather and to prevent regrowth in the spring. The main areas where carrots are grown commercially are in Scotland, Yorkshire, Lancashire, Nottinghamshire, Cambridgeshire, Shropshire, Norfolk and Suffolk. The differing natural climates in these areas helps provide a year-round crop (BCGA, 2018). Roots of high quality are necessary for both the fresh and processing market, as visible signs of disease on roots make them unmarketable, and stunted, forked or malformed roots are unsuitable for the processing machinery (R. Hobson, Hobson Farming Ltd., UK; I. Holmes, Strawsons Ltd., UK, personal communication).

Cavity spot disease of carrot

1.1.1 Impact on commercial carrot production

Cavity spot disease of carrot has been a significant problem for growers for over 40 years. It is frequently cited as the most important disease in carrots, but research progress has been slow and there is increasing pressure to provide control measures. Over the years, a large amount of industry-funded research has been conducted, but effective control is still elusive (Hiltunen & White, 2002; Kretzschmar, 2010).

Cavity spot disease of carrots results in sunken lesions on the carrot root. Growers often suffer severe losses as even roots with only a small number of superficial lesions are rejected by the market. Losses to cavity spot vary from year to year, but are generally reported to be at least 10% (G. Poskitt, MH Poskitt Ltd., UK, personal communication). In some years, growers have reported losses up to 35% (Gladders, 2014). In extreme cases, total crop loss can occur, as harvest and grading become uneconomical even at a relatively low disease threshold (I. Holmes, Strawsons Ltd., UK, personal communication) (Hiltunen & White, 2002). The above ground growth of carrots shows no visible symptoms of cavity spot disease (Vivoda *et al.*, 1991), and therefore growers do not know the disease level until harvest, by which point all inputs have been applied, and economic losses are highest (Hermansen *et al.*, 2007). Overwintered crops lost to cavity spot lead to particularly high financial losses (Gladders, 2014).

Calculations on the financial losses due to cavity spot are difficult to ascertain, as cavity spot lesions are often graded out during the packing process, and may not be distinguishable from other visible lesions or blemishes to the untrained eye (R. Hobson, Hobson Farming Ltd., UK, personal communication). Furthermore, due to the unpredictable nature of the disease, growers have to implement emergency strategies for early harvesting: increasing labour for grading and making marketing alterations to attempt to salvage a crop (Martin, 2014). These all result in considerable costs and disruption from standard operations, which are challenging to accurately calculate. One estimate indicates that just in direct crop losses, cavity spot can cost growers approximately £3-5 million each season (Martin, 2014). However DEFRA have indicated that losses can rise to £20-30 million in particularly severe years when taking into account wider impacts (Gladders, 2014).

1.1.2 Cavity spot symptoms and infection

Cavity spot is caused by the oomycete *Pythium* (see Section 1.2.4) and typical symptoms are dark sunken elliptical lesions which form on the carrot root, but these can also be circular or irregular (Figure 1.1) (Hiltunen & White, 2002). Lesions form in a horizontal plane across the breadth of the root, and can appear over the entire carrot surface (Perry & Harrison, 1979), though they are often are more concentrated on the upper half of the root (Guba *et al.*, 1961; Vivoda *et al.*, 1991). Lesions are always sunken, and mostly shallow, only penetrating between 1-5 mm into the root (Vivoda *et al.*, 1991). Cavity spot lesions often change in appearance over time: initially they are light, sometimes described as pale olive colour or are surrounded by a light yellow halo (Hiltunen & White, 2002; El-Tarabily *et al.*, 2004) and are generally less than 20 mm in length. Over time, as the roots mature, the lesions can enlarge, with reports of lesions increasing to 40 mm in length and 7 mm deep (Perry & Harrison, 1979). They usually darken over time, becoming dark brown

or black (Hiltunen & White, 2002). As no foliar symptoms are visible, roots must be harvested and washed to accurately determine disease severity (McDonald, 1994).

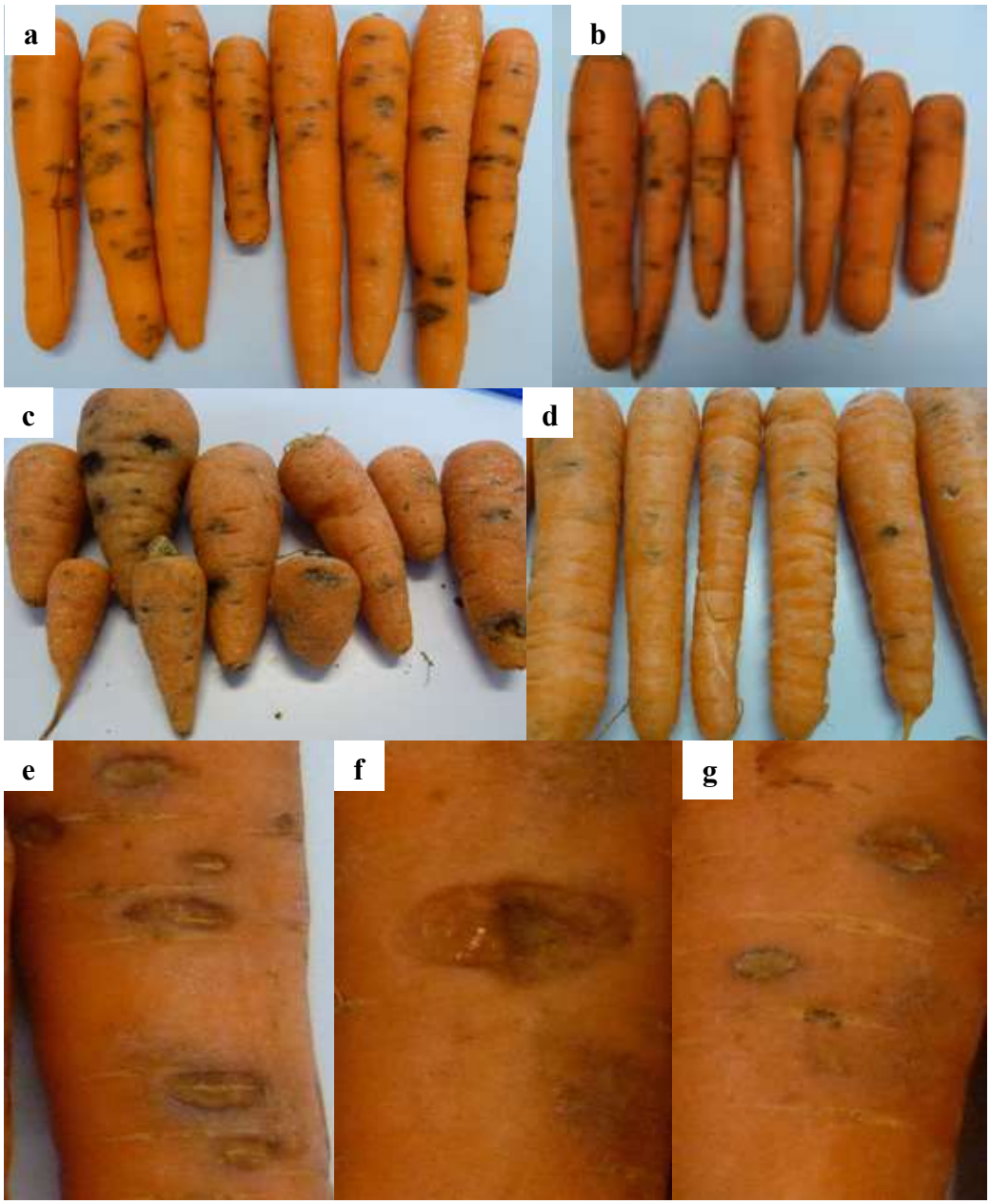


Figure 0.1 Symptoms of cavity spot. **a-d: Carrots from commercial carrot crops:** Roots from Yorkshire (Oct. 2014), lesions are dark, expanded and concentrated on the upper half of some roots (a); roots from Nottinghamshire (Oct. 2014), lesions are lighter in colour with some darkening/expansion (b); roots from Nottinghamshire (Dec. 2014), lesions are less dense, but irregular in shape, dark and heavily sunken (c); roots from Nottinghamshire (Feb. 2015), lesions are light in colour, shallow, mainly concentrated on the upper half of the root (d). **e-g: close-up cavity spot lesions:** cavity spot lesions from: Yorkshire (Oct. 2017) showing elliptical sunken lesions with soft, dark edges (e); a large heavily sunken lesion becoming irregular in shape (f), small shallow lesions with a dark outline (g).

Cavity spot lesions generally extend approximately 10-12 cells deep (Hiltunen & White, 2002) and their formation has been studied by Perry and Harrison (1979). First, hyphae penetrate the root surface and infect the phloem, but leave the epidermis (or periderm) slightly darkened, but intact (Figure 1.2). Next, the outer cells of the phloem collapse and this spreads to neighbouring cells,

until the periderm ruptures and the pericycle cells disintegrate, leaving a cavity. The wound periderm forms a layer beneath the lesions and suberin and lignin are deposited in the cell walls (Perry & Harrison, 1979). If the lesion is not sealed, secondary fungi and bacteria infect the cavity, and this is commonly what leads to expansion and darkening of the lesion (Perry & Harrison, 1979; Campion *et al.*, 1997; El-Tarabily *et al.*, 2004). It is unclear whether the variation seen in cavity spot symptoms are caused by biotic/abiotic factors in the field, or related to the specific species/isolate of *Pythium* causing the infection. Young carrot plants are thought to be affected by *Pythium* species through destruction of small feeder roots (Stanghellini & Burr, 1973), but it is unclear how and when initial infection occurs in mature carrot roots. Perry and Harrison (1979) observed that cavity spot lesions are occasionally associated with fine lateral roots, but these are not necessary for disease spread between roots (Suffert & Lucas, 2008).

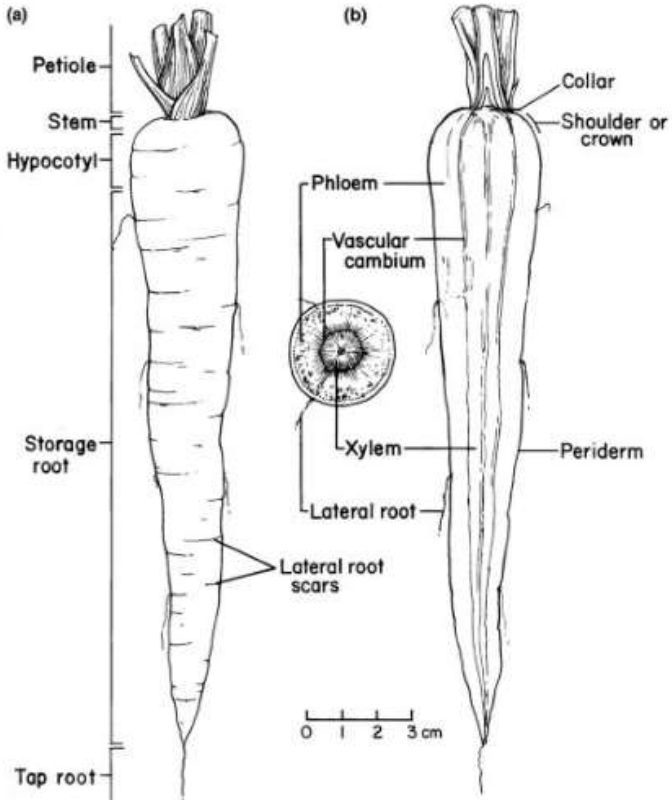


Figure 0.2 Carrot root anatomy exterior (a) and interior (b) as described by Kjellenberg (2007); reprinted from Rubatzsky *et al.* (1999).

1.1.3 Cavity spot: a historical perspective

Cavity spot disease was first described on carrot and parsnip roots growing in Massachusetts, USA in the 1960s (Guba *et al.*, 1961). A causal organism was not identified at this point, and it was concluded the disease had a physiological origin (Hiltunen & White, 2002). In the UK, symptoms of cavity spot were observed on carrots growing in peat/mineral soils in Eastern

England from 1960 onwards (Baker, 1972). Through the 1960 and 1970s, cavity spot was attributed to a range of factors, including calcium deficiency induced by high potassium levels, and high soil ammonium levels (Hiltunen & White, 2002). However, further experiments studying both of these factors failed to find a causal link (Perry & Harrison, 1979; Scaife *et al.*, 1981; Soroker *et al.*, 1984; Vivoda *et al.*, 1991).

It was also observed that cavity spot disease incidence was greater in wetter seasons (Guba *et al.*, 1961), as well as in water logged and compacted soils (Perry & Harrison, 1977). This was the first time a pathogen was thought to be associated with the disease, however further indication of pathogen involvement was not considered until 1984, when Lyshol *et al.* (1984) observed that cavity spot disease incidence could be reduced with fungicides which had action against oomycetes. Although the causal agent was not isolated, when carrots were grown in pots with field soil from a site with cavity spot history, the carrots had similar lesions to the field site (Green & Makin, 1985). It was hypothesised that a soil-borne pathogen, most likely an oomycete, was responsible. Isolations of cavity spot lesions onto agar suitable for oomycete growth (French Bean Agar with antibiotic and antifungal compounds) resulted in growth of *P. violae*, and subsequent inoculation of carrot roots with agar plugs of a range of *P. violae* isolates resulted in sunken lesions morphologically similar to cavity spot (Groom & Perry, 1985). This work therefore identified *Pythium* as the causal agent of cavity spot disease.

1.1.4 *Pythium* species associated with cavity spot

A number of different *Pythium* species (spp.) have since been associated with cavity spot, and the main causal agent appears to vary from country to country (Table 1.1) It is important to be able to identify particular *Pythium* spp. associated with cavity spot in different locations, because they vary in host range (Schrandt *et al.*, 1994; Davison & McKay, 2001), and sensitivity to fungicides such as metalaxyl (White, 1988; Hiltunen & White, 2002). *Pythium* as a genus is often easily outcompeted by other fungi (Zamski & Petretz, 1995) and growth rates vary between *Pythium* spp.. For example, *P. violae* and *Pythium sulcatum* are relatively slow growing *Pythium* spp., and may be less easily isolated than faster *Pythium* spp. such as *Pythium intermedium*. This can lead to misidentification of the *Pythium* spp. that caused the initial lesion (Hiltunen & White, 2002; Kageyama, 2014). Therefore, it is important that appropriate isolation techniques are used to optimise *Pythium* growth.

Table 0.1 The *Pythium* species associated with cavity spot in different countries. Black squares indicate commonly associated, grey squares indicate less frequently associated, white squares indicate no association. Adapted from Hiltunen and White (2002). *P. vio = P. violae, P. sul= P. sulcatum, P. int = P. intermedium, P. ult = P. ultimum, P. col = P. coloratum, P. syl = P. sylvaticum*.

Country	***Pythium* species implicated in causing cavity spot**							Source
	P. vio	*P. sul*	*P. int*	*P. irr*	*P. ult*	*P. col*	*P. syl*	
America	■				■			(Vivoda *et al.*, 1991)
Canada	■	■		▒	▒		▒	(McDonald, 1994; Benard & Punja, 1995; Allain-Bouléa *et al.*, 2004)
Australia	▒	■			■	■		(El-Tarabily *et al.*, 1996; Davison & McKay, 1998)
Norway	▒	■	■				▒	(Hermansen *et al.*, 2007)
The Netherlands		■						(Wagenvoort *et al.*, 1989)
France	■	■						(Montfort & Rouxel, 1988; Guerin *et al.*, 1994)
Israel	■			■				(Shelvin *et al.*, 1987; White *et al.*, 1993)
UK	■	▒	▒	▒	▒		▒	(Perry & Groom, 1984; Groom & Perry, 1985; White, 1986, 1988)

Pythium

1.1.5 Oomycete taxonomy

Oomycetes are commonly known as ‘water moulds’ and many are known plant pathogens (Fry & Grünwald, 2010). Oomycetes have a number of similarities with true fungi (mycelial growth, nutrition via absorption, and reproduction via spores) which meant for many years they were thought of as ‘lower fungi’. However, evolutionary analysis has shown oomycetes to be phylogenetically distinct to true fungi, and more related to algae/green plants (Figure 1.3); (Rossman & Palm, 2006; Fry & Grünwald, 2010). Oomycetes produce oospores during sexual reproduction. These are thick-walled resting structures, formed by the fusion of an oogonium and an antheridium.

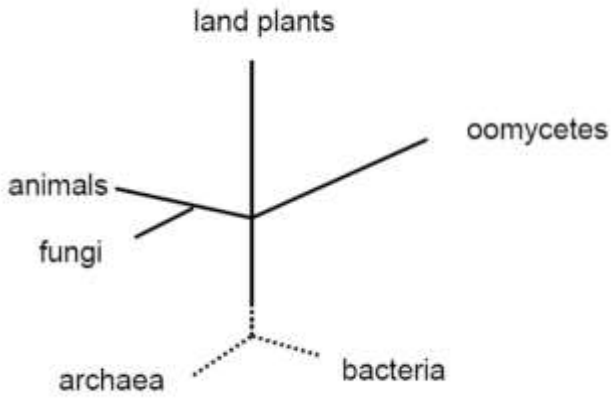


Figure 0.3 Schematic of the relationship between oomycetes, land plants, animals and fungi as described by Fry and Grünwald (2010); image adapted from http://home.planet.nl/~gkorthof/images/tree_of_life2.jpg.

1.1.6 Taxonomic classification and life cycle of *Pythium*

Pythium is a genus of oomycetes within the family Pythiacae and comprises 138 named species (Table 1.2) (Roskov *et al.*, 2018).

Table 0.2 The taxonomic hierarchy of the genus *Pythium* by Bisby *et al.* (2011) and named species *Pythium violae* known to be associated with cavity spot.

Kingdom	Chromista
Phylum	Oomycota
Class	Oomycetes (Peronosporea) [1]
Order	Pythiales (Peronosporales) [1]
Family	Pythiacae
Genus	*Pythium (Globisporangium)* [1]
Species	*Pythium violae (G. violae)* [1] (Chesters & Hickman, 1944)

[1] Reclassification according to **(**Roskov *et al.*, 2018).

Pythium produces white/transparent hyphae which are fragile, thin and generally less than 6 µm wide (Figure 1.4a) (van der Plaats-Niterink, 1981; Agrios, 2005). The hyphae are non-septate and highly branched, and the mycelium often forms coils on the surface of the agar (Figure 1.4b) (Ho, 2018). Oospores serve as long-term survival structures and usually require a resting period, after which they germinate, as described for sporangia.

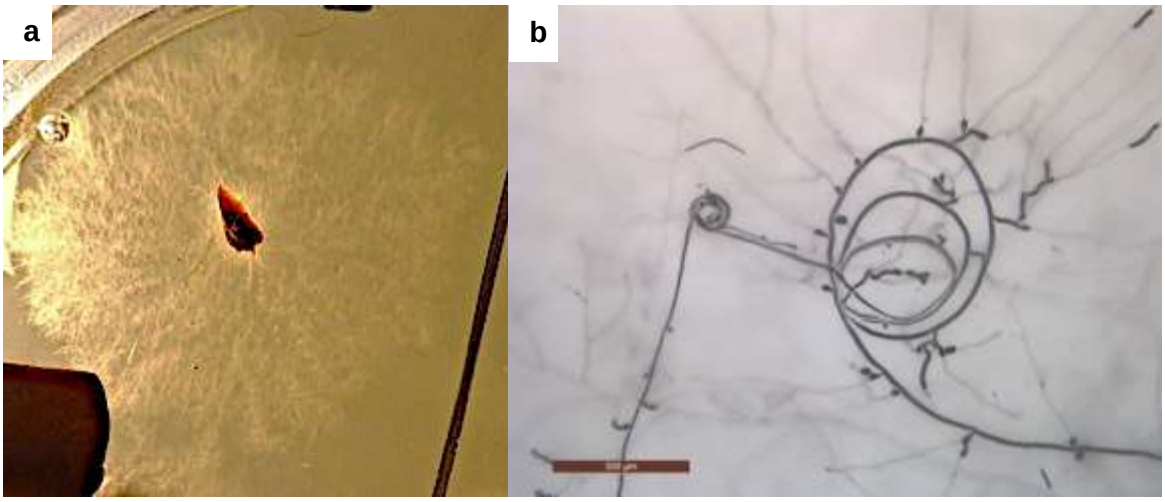


Figure 0.4 *Pythium violae* isolate P.10: mycelium growing on CMA + rifampicin (30mg L^{-1}) from a cavity spot lesion (a) and characteristic coil of mycelium growing on CMA (b).

1.1.7 *Pythium violae*

Pythium violae was first described by Chesters and Hickman (1944) and was originally isolated in England from *Viola* spp. but has since been reported worldwide (van der Plaats-Niterink, 1981). *P. violae* has been described as the most important *Pythium* species associated with cavity spot in the UK (Hiltunen & White, 2002) although a recent, thorough survey has not been conducted. *P. violae* is a comparatively slow-growing *Pythium* species and produces large, thick-walled aplerotic oospores (mean 27 µm diameter, walls up to 3 µm thick) (van der Plaats-Niterink, 1981; Hiltunen & White, 2002). The life cycle of *P. violae* is not well defined and unusually it has never been observed to produce zoospores (Hiltunen & White, 2002) and a recent study has found a stop codon in the flagellum reading frame for *P. violae*, implying loss of function (Robideau *et al.*, 2014). However, mycelial growth is sufficient for infection to spread from root to root (Suffert & Lucas, 2008).

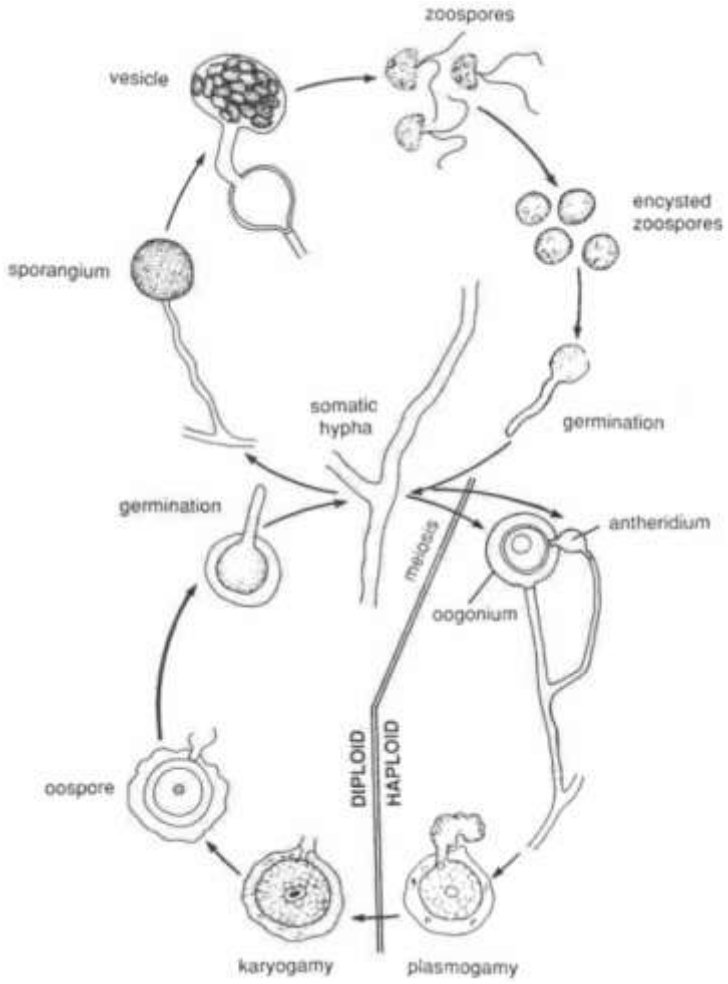


Figure 0.5 Life cycle of *Pythium* (Kendrick, 2000). Sexual reproduction occurs via fertilisation of the oogonium by the antheridium, and a diploid oospore is formed. Germination of the oospore produces mycelial growth, and oogonia can be formed from the hyphae. Asexual reproduction occurs via formation of sporangia from mycelial growth. *P. violae* has not been observed to produce zoospores.

1.1.8 *P. violae* host range and epidemiology

P. violae host range

P. violae causes cavity spot disease on mature carrot roots, but can colonise roots of other plants asymptomatically (Kalu *et al.*, 1976; Dewan & Sivasithamparam, 1988; Schrandt *et al.*, 1994; Chavarriaga *et al.*, 2007; Barbara, 2010a; Kretzschmar, 2010). Carrot is the only crop in which it is described as causing disease of economic importance. Weed species including black nightshade, prickly sow-thistle and knot-grass (Kalu *et al.*, 1976; Kretzschmar, 2010); crops including wheat, beetroot, alfalfa, cauliflower and cowpea (Dewan & Sivasithamparam, 1988; Schrandt *et al.*, 1994; Kretzschmar, 2010); and other plants families including marigold, clover and pine (Chavarriaga *et al.*, 2007; Kretzschmar, 2010) can all be alternative plant hosts that can be colonised by the pathogen, and so can support mycelial growth and/or replenish oospores in the soil (Dissanayake *et al.*, 1997; Dhingra & Netto, 2001; Kretzschmar, 2010). Therefore, these

alternate hosts can contribute to an increase in inoculum level, and subsequently an increase in disease incidence when a susceptible crop such as carrot is planted (Davies & Nunez, 1999; Johansson *et al.*, 2006). It has been previously suggested that successive carrot crops can increase the likelihood of cavity spot development (Rubens & Halford, 1983; Lyshol *et al.*, 1984). These influences need to be understood to create effective management strategies (discussed in Section 1.4).

P. violae oospore survival and detection

Understanding cropping history, as well as the susceptibility of crops to *P. violae* colonisation, is fundamental to managing the soil-borne inoculum. Oospores of *P. violae* are known to survive for several years in the soil (Hendrix & Campbell, 1973; Mitchell, 1978; van der Plaats-Niterink, 1981) and are thought to be the primary inoculum source for plant infection (Hendrix & Campbell, 1973). It has also been shown that *P. violae* mycelium can infect neighbouring carrot roots (Suffert & Montfort, 2007). However, mycelial growth is fragile and more sensitive to temperature changes (van der Plaats-Niterink, 1981), and therefore less likely to overwinter without a host.

Detection of *P. violae* in the field is currently only achieved once cavity spot lesions have developed on the carrot roots. However, once lesions have appeared, there is very little growers can do except harvest early and accept the losses. Detection and quantification of *P. violae* in the soil is therefore vital for understanding the biology, epidemiology and population dynamics of the pathogen, which in turn will inform effective management practices (Pavon *et al.*, 2007). However, difficulties in detection have hampered the understanding of *P. violae* dynamics and assessment of disease risk. It is thought that the inoculum levels required for cavity spot development are very low (1 colony forming unit (CFU) in 40 g soil) (Hiltunen & White, 2002) and initial attempts to use pre-season *P. violae* levels (as measured by a PCR test) to predict final cavity spot severity in the crop have failed (Barbara *et al.*, 2007). Furthermore, a pre-strawing PCR assay was tested in the autumn as a method of predicting cavity spot levels in strawed winter crops, but again, no correlation was found (Barbara, 2010b). Overall, it was concluded that other factors such as soil moisture and temperature were major factors affecting cavity spot development as levels of inoculum pre-planting may not define the disease severity later in the season (Hiltunen & White, 2002; Suffert *et al.*, 2008).

P. violae detection in soil has proved challenging and it is not clear if lack of a relationship between initial inoculum levels and subsequent disease could also be due to inadequate sensitivity of detection (Barbara, 2010b). Accurate detection is also further hampered by the patchy distribution of *P. violae* in soil (Phelps *et al.*, 1991), meaning that testing of small soil samples may not represent the pathogen load over the entire field. A reliable capture method allowing both specific

and sensitive detection of *P. violae*, as well as a reliable measure for quantifying the pathogen from soil samples is needed to enhance understanding of *P. violae* epidemiology.

1.1.8.1 Cavity spot disease dynamics / *Pythium* epidemiology

Growers commonly find it can be difficult to predict when and where cavity spot will occur. In research studies, disease is often observed to be randomly distributed across a field (Phelps *et al.*, 1991), and cavity spot lesions often appear in clusters on each carrot (Hiltunen & White, 2002; Suffert & Lucas, 2008). The dynamics of *P. violae* in relation to the carrot crop over time, and the development of cavity spot lesions, has not been established (Phelps *et al.*, 1991). Furthermore, it is still unclear whether *Pythium* infection occurs in seedlings or mature plants, and there are still many questions surrounding *P. violae* epidemiology, the role of the environment in infection, and the quantification of inoculum in soil during a season.

1.1.8.2 Factors influencing cavity spot disease epidemiology

The epidemiology of cavity spot is poorly understood. There has been a failure to identify environmental factors that are consistently associated with disease development despite a wide range of studies involving either monitoring of the disease in the field, or attempting artificial induction of the disease in pots (Hiltunen & White, 2002). One of the only environmental factors that has been correlated with cavity spot development is soil moisture. Even before *Pythium* spp. were identified as being the causal pathogens, increased rainfall had been associated with cavity spot disease (Guba *et al.*, 1961). Since then, increased incidence of cavity spot has been associated with soil moisture in experiments monitoring field crops, where there was increased cavity spot in wet seasons and in poorly drained fields (Hiltunen & White, 2002) and in pot experiments with varying watering treatments (Soroker *et al.*, 1984; Vivoda *et al.*, 1991). Vivoda *et al.* (1991) added a *P. violae* vermiculite inoculum into soil at 500 cfu g^{-1} and found that cavity spot incidence positively correlated with flooding, with more lesions developing on roots flooded for 24 or 48 hours than non-flooded roots. Martin (2014) conducted a large field-based study and monitored thirty commercial carrot production sites for cavity spot disease incidence and measured a range of environmental factors including total water input and soil moisture between 2010 and 2013. In both the 2010 and 2011 seasons, a ‘tentative relationship’ was observed between cavity spot incidence and total water input during July and August.

However, there have also been contradictory reports regarding the effect of soil moisture. Martin (2014) found that the relationship between cavity spot and soil moisture observed in the first two years of the study did not continue in the 2012 or 2013 seasons. Although the researchers recommended minimising total water input during August, they concluded that there were many

anomalies throughout the study, and no firm conclusions could be drawn (Martin, 2014). Another experimental approach using a multi-field study examined the effect of irrigation on disease levels (Barbara, 2010b). Polytunnels were erected over a carrot crop and overhead irrigation established at 7.5, 15, 30, 45 and 60 mm of water/week as 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 and patchy distribution, 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 45 mm water/week (Barbara, 2010b). It therefore appears there are multiple interactions between *P. violae* and its environment that affect its ability to colonise the carrot host, and a lack of understanding of this is impairing the ability to effectively research and develop management strategies.

Management and control of cavity spot

1.1.9 Fungicides

The work by Lyshol *et al.* (1984) where it was noted that oomycete fungicides reduced cavity spot not only played an important role in identifying the causal agent of the disease, but was also the first step in finding a suitable selective fungicide for control. Since this work, metalaxyl has been used routinely to control cavity spot, and is still currently widely used to help manage the disease (Hiltunen & White, 2002). It is most effective when applied as a spray or drench (Lyshol *et al.*, 1984; White, 1984) and when applied at a rate of 2.0 kg ha^{-1} as a spray after sowing, metalaxyl has been shown to reduce cavity spot incidence from 46% to 4% (Lyshol *et al.*, 1984). White (1984) reported similar results, where a combination of metalaxyl seed treatment and a soil drench reduced disease incidence from 42% to 3%.

Early application of metalaxyl, within 4 weeks of sowing, has been demonstrated to provide the best protection in carrot crops (Lyshol *et al.*, 1984; Gladders & McPherson, 1986; Gladders, 2014) with a first application being recommended at the first true leaf stage (Pettitt & Gladders, 2003). Multiple applications throughout the growing season have not been shown to increase effectiveness (Gladders & McPherson, 1986). Cavity spot is often most challenging for growers when it occurs in overwintered strawed crops as the high cost of strawing means that financial losses are greatest. However, studies investigating pre-strawing applications of metalaxyl have shown little or no beneficial effect (McPherson, 1995). Furthermore, the fact that early application is most effective, and subsequent applications of fungicide are not beneficial in reducing disease,

suggests that *Pythium* infects the root at an early stage, and remains asymptomatic until the plant is mature, or until environmental conditions favour infection and disease development.

Despite its widespread use, the control of cavity spot disease with metalaxyl has proved variable. The first experiments demonstrating efficacy of metalaxyl described poor disease control in around 10% of cases (Gladders & McPherson, 1986). It has also been reported that efficacy significantly declined over a 10 year period, from almost 100% control to 50-75% control (McPherson, 1995). Initially it was thought that this was due to differing sensitivities in *Pythium* spp. (White, 1988); however, it has since been established that there was enhanced microbial degradation of metalaxyl in soil (Davison & McKay, 1999). They reported that in fields where metalaxyl had been previously used, the fungicide had a half-life of less than 10 days, compared to 82 days for fields where metalaxyl had never been applied. This is a therefore a significant problem for UK growers and currently there is no alternative to metalaxyl that has similar control. The use of fosetyl-A1 and propamocarb that were initially identified as potential alternative compounds have shown inconsistent results (Hiltunen & White, 2002). The most recent study in 2014 found that none of the experimental products tested (including fungicides, and biological treatments) under field conditions gave significant control of cavity spot (Gladders, 2014). The continued reliance on metalaxyl and hence a single mode of action, as well the potential for withdrawal or non-renewal due to EU legislation, is a major concern for long-term sustainability and protection.

1.1.10 Cultural methods

Carrot growers use a combination of techniques to attempt to control cavity spot in addition to the use of fungicides. Growers first attempt to avoid disease by identifying fields which have not recently been used to grow carrots (Kretzschmar, 2010), or where carrots have never been planted ("virgin" land; R. Hobson, Hobson Farming, UK, personal communication). Rotation of at least six years is recommended to avoid inoculum build-up (Rubens & Halford, 1983; Lyshol *et al.*, 1984). Carrots are often grown on rented land in the UK, and therefore finding land which is of a suitable soil type, as well as an appropriate cropping history is challenging, and becoming more difficult year on year (R. Hobson, Hobson Farming Ltd., UK, personal communication).

1.1.11 Biological control and biofumigation

The use of biological controls has been investigated as a possible alternative to control of cavity spot disease with fungicides. *P. oligandrum* has been associated with suppression of a number of diseases (Hiltunen & White, 2002) and has been found in almost all fields cropped with carrot in the UK (White, 1993). Furthermore, it has been shown *P. oligandrum* is capable of over-growing and killing cultures of *P. violae* and *P. sulcatum* in laboratory tests on agar (White, 1993). However,

P. oligandrum populations are considerably reduced by metalaxyl application (Hiltunen & White, 2002) and fields with high levels of this antagonist are difficult to identify or manage as growers often use rented land (Hiltunen & White, 2002). A biological control agent would therefore need to be applied and have activity within the same yearly cycle as a carrot crop (Hiltunen & White, 2002). Another potential approach for cavity spot control is the use of biofumigation crops, but again this brings challenges in terms of use of rented land or fitting in with carrot rotations. Biofumigation involves growing specific crops with high glucosinolate levels, then crushing and incorporating them into the soil. If carried out under high soil moisture levels, the glucosinolate compounds convert to isothiocyanates, which are toxic to a range of soil microorganisms (Clarkson, 2014). Although early work by Barbara (Warwick Crop Centre) was encouraging (Clarkson, 2014), further work continued by Clarkson (2014) indicated that brown mustard, white mustard and radish sown in the autumn prior to the carrot crop, and incorporated in the spring had no effect on development of cavity spot. However, this may have been due to low biomass and low glucosinolate levels in the overwintered biofumigants.

1.1.12 Carrot plant resistance

Resistant carrot cultivars (cv.) would be of great benefit to the industry. Although cultivars vary in susceptibility, there are many other characteristics that are important for achieving high yield and marketable roots. Research to develop resistant cultivars has been conducted since the 1980's, and although cultivars such as cv. Nandor have consistently been shown to have higher levels of resistance than other cultivars (e.g. cv. Nanco) (Hiltunen & White, 2002), no complete resistance has ever been identified. This means there is no high-level resistance in any of the major cultivars grown in the UK (Hiltunen & White, 2002). In addition, given that only a few visible lesions make the root unmarketable, the level of resistance available is often insufficient to prevent economic loss. In the UK, Nairobi is the main carrot cultivar grown because it provides good consistency in yield and quality year on year. This makes the harvesting process easier: roots are cleaned and graded easily in the packhouse, and uniform roots are advantageous for processing. Furthermore, Nairobi roots are much less prone to breaking during harvest and processing than other cultivars (I. Holmes, Strawsons Ltd., UK, personal communication). These highly sought after characteristics have not been found to the same extent in other carrot varieties that may show a higher tolerance to cavity spot.

Challenges and opportunities for cavity spot control

Although carrot growers attempt to use a range of measures to control cavity spot disease, none are sufficient to reduce disease levels adequately to avoid major economic losses. Due to the unpredictable nature of the disease, some growers may just grow extra fields of carrots to ensure that orders can be met (G. Poskitts, MH Poskitt Ltd., UK personal communication). Alternative effective control products or measures are urgently required. A better understanding of *P. violae* biology, as well developing tools to enhance the ability to detect and quantify the pathogen in the soil will help build understanding of *P. violae* dynamics and epidemiology. Recent research to identify new actives for cavity spot control has proved difficult due to the unpredictable nature of cavity spot. This has meant that field experiments have failed because of little or no disease development (Gladders, 2014). The development of an artificial system to induce cavity spot disease is needed to establish a more efficient, reliable system to measure the effectiveness of new control measures.

Project aims and objectives

The overall aim of this research was to develop effective tools for understanding the biology and epidemiology of *Pythium* spp. causing cavity spot disease in carrots, in order to aid the development of new management approaches. The specific objectives were to:

1. Identify the current *Pythium* species associated with cavity spot in the UK and investigate the phylogeny and pathogenicity of a range of isolates.

2. Develop an artificial inoculation system for *P. violae* to reliably induce cavity spot disease in carrots.

3. Develop molecular tools to effectively capture, detect and quantify *P. violae* from field soil.

Aim 1: Identify the current *Pythium* species associated with cavity spot in the UK and investigate the phylogeny and pathogenicity of a range of isolates.

Objective 1: To identify the range of *Pythium* species causing cavity spot in English commercial carrot crops

Introduction

Attempts to understand the cause of cavity spot had a significant breakthrough when Lyshol *et al.* (1984) found that three fungicides known to control oomycetes, metalaxyl, fosetyl-A1 and propamocarb, could reduce the disease in commercial field experiments as well as glasshouse tests with naturally infested soil. This control was confirmed in further pot experiments by White (1984) and Green and Makin (1985), and in the field with metalaxyl by Gladders and Crompton (1984), Perry and Groom (1984) and Wheatley *et al.* (1984a, 1984b). The discovery of *Pythium* spp. as the causal agents occurred rapidly after Lyshol's findings. Groom and Perry (1985) isolated *P. violae* from cavity spot lesions, and then placed agar plugs of 28 different *Pythium* isolates on to freshly lifted carrots, and all seven that induced sunken lesions were identified as *P. violae*. Since then cavity spot has been shown to be caused by a range of *Pythium* spp. (Suffert & Guibert, 2007) with the predominant species varying around the world. Hiltunen and White (2002) reviewed the range of *Pythium* spp. thought to be the main causal agents of cavity spot disease in different countries.

In the UK, *P. violae* was thought to be the most significant causal agent of cavity spot (Groom & Perry, 1985; White, 1986; Cooper *et al.*, 2004) while *P. sulcatum* was also known to be commonly associated with disease (White, 1988; Lyons & White, 1992; Cooper *et al.*, 2004). White (1986) grew *Pythium* inoculum in autoclaved field soil mixed with maize meal (3% w/w) and introduced five *Pythium* spp. individually to carrots grown in sterilised soil. He observed that *P. violae* resulted in the highest percentage of carrots with cavity spot lesions. A subsequent study showed that the most common species identified following isolation from cavity spot lesions were *P. violae* and *P. sulcatum*. *P. dissotocum* with *P. intermedium* were found less frequently (White, 1988). Since then, other *Pythium* spp. (*P. sylvaticum, P. intermedium, P. ultimum, P. irregulare, P. aphanidermatum*) have been demonstrated to produce lesions on carrot, but are not regarded as primary pathogens (Lyons & White, 1992). Although *P. violae* has previously been identified as the major *Pythium* spp. causing cavity spot in the UK, it is still unclear whether the proportion of different *Pythium* spp. causing disease has changed over time, or varies between different

regions. A survey of the *Pythium* species causing cavity spot in the UK was carried out and isolates identified using DNA sequencing.

Materials and Methods

Samples of commercially grown carrots with symptoms of cavity spot were obtained from grower sites across England from October 2014 to February 2017 (Table 2.1). *Pythium* isolates were also obtained that originated from diseased carrots in The Netherlands.

Table 0.3 The number of *Pythium* isolates obtained from each region of the UK in this study.

Location (region)	Number of isolates
East Midlands	74
Yorkshire and the Humber	34
East of England	25
West Midlands	22
Confidential	1
UK: Unknown	8
The Netherlands	14

Diseased carrot root samples were washed in running tap water to remove visible soil particles and photographed before cavity spot lesions were excised with a scalpel in a class 2 laminar flow bench. Lesions were bisected and two lesions (four halves) were placed surface-side down in Petri dishes containing 20 ml corn meal agar amended with either two antibiotics and an antifungal compound (CMA, Table 2.2; 2014-2016) or one antibiotic (rifampicin 30mg^{-l}; 2016 onwards). The CMA was prepared according to manufacturer's instructions by adding 15.3g corn meal extract (Sigma-Aldrich, UK) to 900 ml deionised water and autoclaving at 121˚C for 15 minutes. Cultures were incubated for five days in the dark at 17˚C after which they were inspected under a light microscope for signs of *Pythium* colonies. Any typical mycelial growth was sub-cultured from the leading edge onto new CMA plates and incubated for five days as above. Isolates were then further sub-cultured, as required, until a clean, pure culture was obtained. *Pythium* isolates were stored on CMA slopes and as 5 mm^3 colonised agar plugs in sterile distilled water (SDW) at 4˚C (approx. 30 plugs in 10 ml of water in 20 ml universal tubes).

Table 0.4 Ingredients and weights of antibiotics/antifungals used in amended CMA.

Antibiotic/antifungal media ingredients	Quantity per 1 litre
SDW-H_20	1000 ml
Corn meal agar, granulated	17 g
Rifampicin (dissolved in 1 ml Methanol)	0.01 g / 0.03 g
Ampicillin (dissolved in 1 ml Methanol)	0.025 g
Pimaricin (dissolved in 500 µl DMSO, 500 µl Methanol)	0.003 g

*availability of Ampicillin and Pimaricin was restricted in 2016, and given suitable isolation was achieved with Rifampicin alone, use of Ampicillin and Pimaricin was dis-continued.

To identify the putative *Pythium* isolates, each was grown on CMA for approximately five days at 17˚C. Three 5 mm plugs from actively growing cultures of each isolate were then placed in a 50 ml Falcon tube or sterile Petri dish containing 20 ml of potato dextrose broth (PDB, 24 g^{-l}; Formedium, UK) and incubated at 17˚C for 7-14 days. Two Falcon tubes or Petri dishes were inoculated for each isolate. After incubation, cultures were centrifuged at 2190 x g for 10 minutes, excess liquid removed and the pellet of mycelium washed twice in SDW after which it was freeze-dried for 48-72 hours. DNA extraction was then performed using a rapid DNA extraction protocol from the freeze-dried material of each isolate (S. Rehner, personal communication). Approximately 2.5 mm^3 of lyophilised mycelium was transferred into 2 ml tubes containing a ceramic bead and silica beads (0.1 mm, BioSpec Products) and samples ground by placing in a Fast Prep® machine (MP BIO, Germany) speed setting 5.5 for 20 seconds three times. 300 µl of extraction buffer (Table 2.3) was added and tubes placed into a heat block at 100˚C for 10 minutes (at 5 minutes tubes were briefly removed and samples mixed). Tubes were centrifuged at 16,000 x g for 5 minutes, turned 180˚(so the pellet faces inwards) and centrifuged again for 5 minutes. 175 µl of the supernatant was then transferred to a clean tube and diluted 1:10 in sterile water for use as DNA template in Polymerase Chain Reaction (PCR).

Conventional PCR amplification (GeneAmp ® PCR system 9200) was performed using the ITS1 (TCCGTAGGTGAACCTGCGC) and ITS4 (TCCTCCGCTTATTGATATGC) primer pair, commonly used for fungal identification studies with expected product size of 700-900 bp (White *et al.*, 1990). Thermal cycling parameters consisted of 94˚C for 4 minutes; 35 cycles of 94˚C for 30 seconds, 58˚C for 30 seconds and 72˚C for 45 seconds; and 72˚C for 10 minutes. Each 20 µl PCR reaction contained 10 µl of Red Taq ReadyMix™ (Sigma-Aldrich, Germany), 7 µl of SDW, 1 µl of forward primer (5 µM), 1 µl of reverse primer (5 µM) and 1 µl of DNA. PCR products (4 µl) were loaded onto a 1.5% agarose gel containing GelRed (2 µl / 100 ml; Roche, UK) and gels visualised under UV light to confirm successful amplification. To identify the size of product, a 1Kb Plus DNA Ladder

(Invitrogen™) was also loaded. PCR products were purified using a Qiaquick PCR purification kit (Qiagen, Hilden) following the manufacturers guidelines and sequenced by GATC Biotech. Sequences were then used to conduct Basic Local Alignment Search Tool (BLAST) searches (Boratyn *et al.*, 2013) against publicly available databases (NCBI, 2018) for species identification.

Table 0.5 Reagents for extraction buffer used for rapid DNA extraction protocol.

Extraction Buffer	Volume/Weight	Action
Sodium metasilicate	21 g	Dissolve in 100 ml SDW, add another 94 ml
Citric Acid	0.5 g	-
2-butoxy ethanol	2.6 4 ml	-
1M Tris-HCl pH 7.0	13.5 ml	Sterilise via filtration

Results

In total a culture collection of 178 isolates was assembled and stored. The majority of cavity spot affected carrot samples were obtained from the East Midlands, predominantly Nottinghamshire (Figure 2.4), but collection spanned most of the major carrot growing regions in England. *Pythium* isolates were initially identified morphologically and amplification of the rRNA ITS region followed by BLAST analysis identified a total of 164 isolates of *Pythium* from the UK (Figure 2.5). Isolates of *Pythium* were identified from all UK regions that samples were taken from (Figure 2.3 a/b).

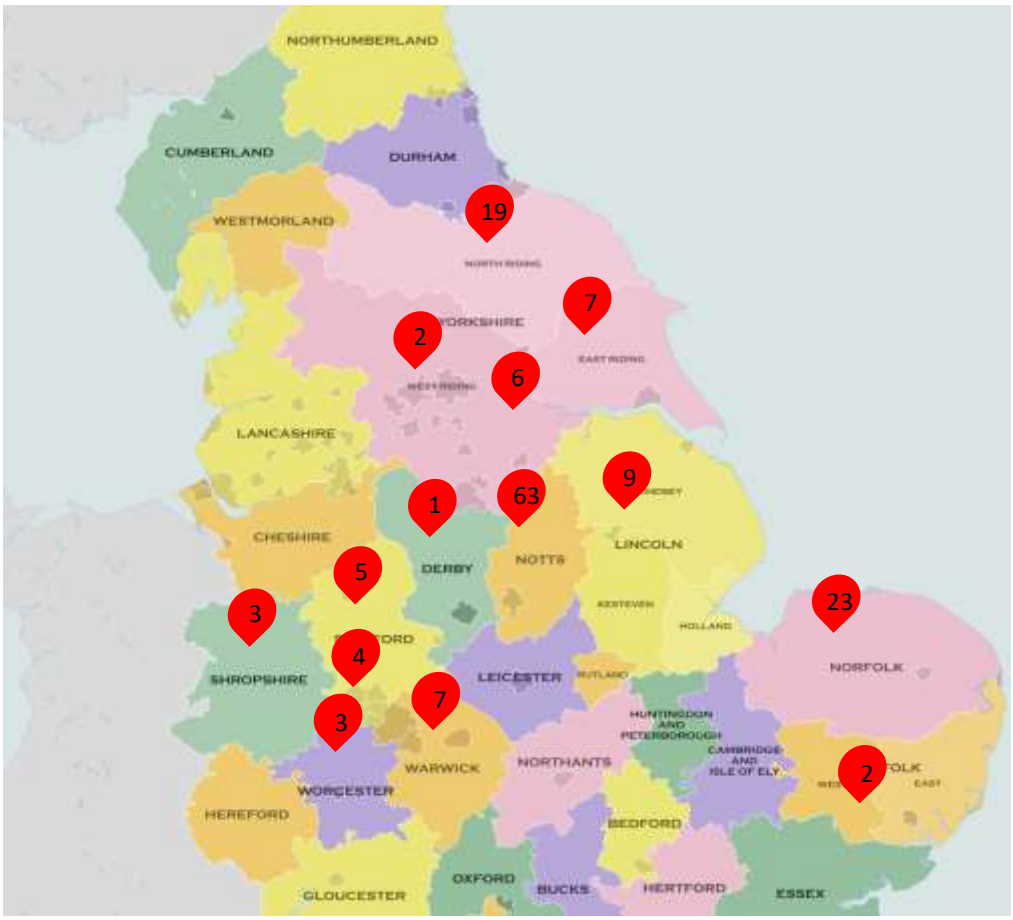


Figure 0.6 Map of *Pythium* isolates of known locations collected in UK by county (2013-2017; total 155). A total of 34 were collected from Yorkshire and the Humber, 74 from the East Midlands, 22 from the West Midlands, and 25 from the East of England. Nine UK collected isolates were of unknown or confidential locations.

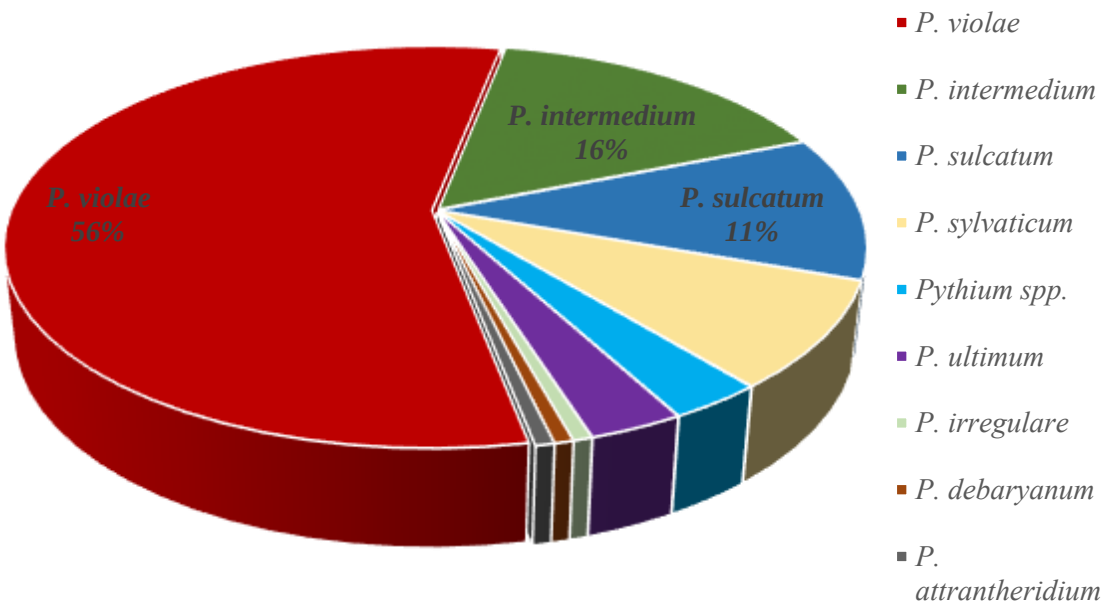


Figure 0.7 Relative proportions of different *Pythium* species identified from 164 UK isolates based on sequencing of the ITS regions of the rDNA.

P. violae was abundant in all UK locations where carrots were sampled, and was present in 80 fields from across 13 English counties. *P. violae* was found across all four regions sampled of the UK (Yorkshire and Humber, East Midlands, West Midlands and East of England) and accounted for 36%-80% of isolates in each region (Figure 2.6). In the East of England, 80% of the 25 isolates obtained were identified as *P. violae*, but no *P. sulcatum* was present from lesions in this region (Figure 2.6). In the West Midlands, *P. violae* comprised 36% of isolates of the 22 isolates collected, which was the same as for *P. intermedium* (Figure 2.6). The largest number of isolates were obtained from the East Midlands where the greatest number of *Pythium* spp. was also found (seven species, Figure 2.6). Yorkshire and the Humber had a lower proportion of *P. intermedium* from the carrots sampled than was found in the neighbouring East Midlands and West Midlands, and was the only region from which *P. ultimum* was isolated (isolated from a single field). *P. sylvaticum* was not isolated from any lesions from Yorkshire and the Humber, and was most commonly found in the East Midlands (Figure 2.6). *P. intermedium*, was identified across all of the regions sampled, with incidence ranging from 12% to 36% (Figure 2.6).

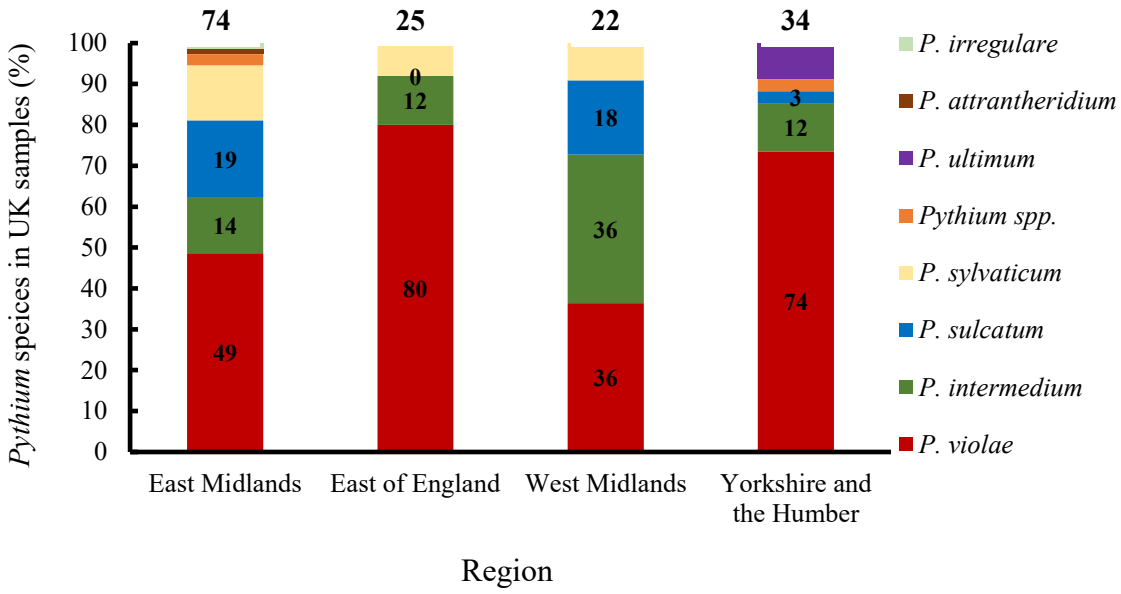


Figure 0.8 Percentage of different *Pythium* species isolated from samples of carrots with cavity spot lesions from commercial sites by region in England (total 155). The total number of samples collected for each region is shown above each percentage bar (bold).

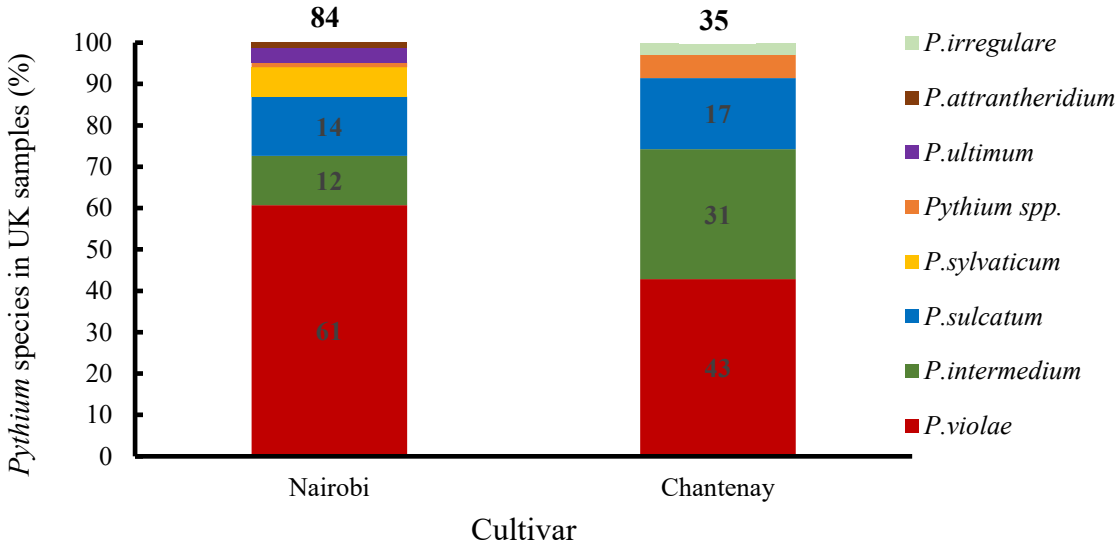


Figure 0.9 Percentage of different *Pythium* species isolated from two carrot varieties (Nairobi and Chantenay) from samples of carrots with cavity spot lesions from commercial sites in England (total 119). The total number of samples collected for each cultivar is shown above each percentage bar (bold).

Most of the carrots sampled in this study were either cv. Nairobi or Chantenay. A range of *Pythium* spp. were isolated from both varieties, with *P. violae* accounting for 61% and 43% in Nairobi and Chantenay, respectively. *P. intermedium* and *P. sulcatum* were found in both cultivars, but *P. sylvaticum* was only isolated from cv. Nairobi (Figure 2.7). A more intensively sampled field in the East of England which contained cv. Nairobi, as well as a more susceptible and less susceptible carrot cv. (names confidential) revealed that *P. intermedium* was only isolated from the less susceptible cultivar (one lesion), while *P. violae* was isolated from cv. Nairobi and *P. violae*, *P. intermedium* and *P. sulcatum* were isolated from the most susceptible cultivar. For eleven carrot fields, samples were taken over multiple time points from the season 2014-2015, and in each case, *P. intermedium* was isolated on the second or third sampling time, rather than the first (Table 2.6).

Table 0.6 Field name, sampling dates and identity of *Pythium* isolates collected from the same field across two or more time points**.**

***Pythium* no.**	**Field**	**Sampling 1**		**Sampling 2**	
		Species	**Date**	**Species**	**Date**
79, 167	BBE	*P. violae*	Oct.14	*P. intermedium*	Mar.15
40, 105	EG	*P. violae*	Oct.14	*P. violae*	Nov.14
65, 163	Fo*	*P. violae*	Oct.14	*P. violae*	Feb.15
63, 122	HHB	*P. violae*	Oct.14	*P. intermedium*	Dec.14
78, 112	Le	*P. sulcatum*	Oct.14	*P. intermedium*	Dec.14
130, 195	LT	*P. violae*	Dec.14	*P. intermedium*	Ap.15
124, 168	M10	*P. violae*	Dec.14	*P. intermedium*	Mar.15
74, 111	P S	*P. violae*	Oct.14	*P. violae*	Dec.14
59, 138	Rey	*P. violae*	Oct.14	*P. violae*	Jan.15
123, 162	Sb	*P. violae*	Dec.14	*P. irregulare*	Feb.15
62, 160	Yard	*P. violae*	Oct.14	*P. intermedium*	Feb.15

* A third sample was taken from Fo during March 2015, *P. intermedium* was isolated (P169).

Objective 2: To examine inter- and intra-specific variation of *Pythium* isolates through phylogenetic analysis of three housekeeping genes

Introduction

Genetic variation of the range of *Pythium* isolates was investigated using phylogenetic analysis of different gene sequences. The Cytochrome Oxidase Subunit II (CoxII) gene is an informative phylogenetic marker that has been used by Martin (2000) to identify and characterise oomycetes. Martin (2000) re-designed *Phytophthora* specific primer sets to examine the phylogenetic relationships of 24 *Pythium* spp. based on the partial sequencing of the mitochondrial CoxII gene. It was found that sequences were generally well conserved within each *Pythium* spp., but divergent among species. However, there were exceptions with *P. irregulare* and *P. ultimum* showing a high level of intraspecific variation. Due to this gene being mitochondrially encoded, it is considered to be more variable than nuclear DNA (Villa *et al.*, 2006). Partial sequences of the NADH dehydrogenase Subunit I gene (NADH) have been used as part of a phylogenetic analysis of *Phytophthora* species (Kroon *et al.*, 2004) but this marker has not been utilised for an analysis of *Pythium* spp..

Materials and Methods

Isolates identified as *Pythium* species (178) were further characterised by sequencing parts of two additional housekeeping genes, cytochrome oxidase subunit II (CoxII) and NADH dehydrogenase subunit II (NADH). For CoxII, the published primer pair with an expected product size of 563 bp FM58 (CCACAAATTTCACTACATTGA) and FM66 (TAGGATTTCAAGATCCG) was used. For NADH the published primer pair NADHF1 (CTGTGGCTTATTTTACTTTAG) and NADHR1 (CAGCATATACAAAAACCAAC) designed to amplify a 897 bp product from the genus *Phytophthora* (Kroon *et al.*, 2004), were re-designed. This new primer pair, with an expected product of 860 bp, NADHKHF1 (GCTGTAGCTTATTTTACTTTAGC) and NADHKHR1 (AAAAACTTTCCAACCTAATCTCA), was designed manually based on the NADH gene region obtained from whole genome sequences for *P. violae* (x2), *P. sulcatum* and *P. intermedium* (Appendix A). Self- and cross-hybridisation ability was tested by the Oligo Analysis tool (Eurofins, 2018) and primers synthesised by Sigma-Aldrich (Germany). Amplification of *P. violae* using NADH primers was initially tested against range of *Pythium* spp. (*P. violae* x5: P31, P42, P50, P96 and P149; *P. intermedium* x3: P107, P122, P128; *P. sulcatum* x3: P43, P78, P132). PCR reactions and sequencing for both CoxII and NADH were carried out as described in Section 2.2.2. For CoxII, PCR amplification was carried out with thermal cycling parameters of 94˚C for 5 minutes;

35 cycles of 94˚C for 1 minute, 52˚C for 1 minute and 72˚C for 1 minute; and 72˚C for 10 minutes. For NADH, thermal cycling parameters consisted of 94˚C for 2 minutes; 30 cycles of 94˚C for 45 seconds, 58˚C for 30 seconds and 72˚C for 1 minute; and 72˚C for 7 minutes. Sequences were aligned using the ClustalW algorithm (Thompson *et al.*, 1994) implemented in MEGA v7 (Kumar *et al.*, 2016).

Phylogenetic trees were constructed in MEGA using the Maximum Likelihood method and additional trees for each of the housekeeping genes constructed using Neighbour-Joining and Minimum Evolution methods for comparison. Bootstrap consensus trees were inferred from 1000 replicates and those above 50% were taken to represent the evolutionary history of the taxa (Felsenstein, 1985). Distances were computed using the calculated best model, and published ITS, CoxII and NADH sequences were used as references (Table 2.4). All trees were rooted using sequences obtained from a *Phytophthora cactorum* isolate from Somerset, UK (isolated from Strawberry cv. Elsanta in 2011) obtained from NIAB EMR. The ITS, CoxII and NADH sequences were then combined using MEGA to produce a concatenated Maximum Likelihood tree.

Table 0.7 Genbank accession numbers for ITS, CoxII and NADH sequences, origin, year of collection, reference, and species, used for taxonomic reference.

Gene	Accession no.	Origin	Host/Substrate	References	Species
ITS	AY598682	WI, USA	*Daucus carota*	Levesque and Cock (2004)	*P. sulcatum*
ITS	AY598688	UK	Soil	Levesque and Cock (2004)	*P. lutarium*
ITS	AY598634	OH, USA	Wheat roots	Levesque and Cock (2004)	*P. dissotocum*
ITS	AY598702	The Netherlands	*Phaseolus vulgaris*	Levesque and Cock (2004)	*P. irregulare*
ITS	AY598645	GA, USA	Soil	Levesque and Cock (2004)	*P. sulcatum*
ITS	AY598704	UK	*Tupipa sp.*	Levesque and Cock (2004)	*P. debaryanam*
ITS	AY598647	The Netherlands	*Agrostis stolonifera*	Levesque and Cock (2004)	*P. intermedium*
ITS	AY598706	Australia	Soil	Levesque and Cock (2004)	*P. violae*
ITS	AY598715	The Netherlands	*Daucus carota*	Levesque and Cock (2004)	*P. violae*
ITS	AY598657	The Netherlands	*Lepidium sativum*	Levesque and Cock (2004)	*P. ultimum var ultimum*
ITS	AY286014	Unknown	Unknown	Allain-Boule,N *et al* (unpublished)	*P. attrantheridium*
CoxII	DQ071380.1	Hokkaido	Soil	Villa *et al* (2006)	*P. intermedium*
CoxII	DQ071396	Gifu, Japan	*Daucus carota*	Villa *et al* (2006)	*P. sulcatum*
CoxII	DQ071397	Gifu, Japan	*Daucus carota*	Villa *et al* (2006)	*P. sylvaticum*
CoxII	DQ071400	Tottori, Japan	Garden pansy	Villa *et al* (2006)	*P. violae*
CoxII	JQ734266.1	CA, USA	*Daucus carota*	Lu,X.H., Rosenzweig,N. and Hao,J. - unpublished	*P. violae*
NADH	JQ734383.1	CA, USA	*Daucus carota*	Lu,X.H., Rosenzweig,N. and Hao,J. - unpublished	*P. violae*
NADH	JQ734355.1	CA, USA	*Daucus carota*	Lu,X.H., Rosenzweig,N. and Hao,J. - unpublished	*P. sulcatum*
NADH	JQ734357.1	CA, USA	*Daucus carota*	Lu,X.H., Rosenzweig,N. and Hao,J. - unpublished	*P. sylvaticum*
NADH	AB513108	Gifu, Japan	Unknown	Senda, M. and Kageyama, K. (2012) (unpublished)	*P. sylvaticum*
NADH	AB513092.1	Gifu, Japan	Unknown	Senda, M. and Kageyama, K. (2012) (unpublished)	*P. intermedium*
NADH	AB513090.1	Gifu, Japan	Unknown	Senda, M. and Kageyama, K. (2012) (unpublished)	*P. intermedium*

Results

Testing and development of primer pairs used for *Pythium* identification

Published primers ITS1 and ITS4, which amplify the rRNA ITS regions, and primers FM58 and FM66, which amplify a section of mitochondrial DNA CoxII region were used successfully for Pythium identification and characterisation. However, published primers for a part of the NADH dehydrogenase subunit 1 gene, previously used for *Phytophthora* species, produced inconsistent and weak amplification of the expected product when tested against 19 *Pythium* isolates, as well as some non-target amplification (data not shown). Therefore, three newly developed primer pairs were tested against a range of isolates from three *Pythium* spp. The primer pair NADHKHF1/R1 successfully amplified an ~860 bp product from all three *Pythium* spp. This primer pair was therefore used to characterise all the remaining isolates (Table 2.7).

Table 0.8 Amplification of a range of *P. violae, P. sulcatum* and *P. intermedium* isolates (*Pythium* numbers indicated below species) when tested against three primer sets for NADH dehydrogenase subunit 1 gene. Black squares indicate a bright band (strong amplification), grey squares indicate a weak band (poor amplification) and white square indicate no band (no amplification).

Primers	***P. violae***						***P. sulcatum***			***P. intermedium***		
	P10	P31	P42	P50	P96	P149	P43	P78	P132	P107	P122	P127
NADHKHF1/NADHKHR1	■	■	■	■	■	■	■	■	■	■	■	■
NADHKHF2/NADHKHR1	■	■	■	□	■	■	■	□	▒	□	■	■
NADHKHF2/NADHKHR2	▒										▒	▒

A phylogenetic analysis using the Maximum-Likelihood method was carried out using rRNA ITS, Cox II, and NADH dehydrogenase sequences to allow intra- and inter-specific genetic variation of *Pythium* spp. associated with cavity spot to be investigated. Individual analysis for ITS resulted in the clear separation of *Pythium* isolates into nine clades based on species identity with high bootstrap values, with very little within-species diversity present (Figure 2.8). CoxII and NADH dehydrogenase sequence analysis indicated within species variation resulting in four and five sub-clades for *P. violae* respectively (Figure 2.9 and 2.10). In general, sequence analysis of CoxII resulted in a greater number of sub-clades (*P. sulcatum* x4, *P. intermedium* x5), than the NADH gene analysis (*P. sulcatum* x1, *P. intermedium* x4). The Maximum Likelihood analysis of the

concatenated sequence data revealed sub-clades within *P. violae* (six clades), *P. sulcatum* (three clades) and *P. intermedium* (four clades) (Figure 2.11).

ITS

The phylogenetic analysis separated the *Pythium* isolates representing each species in the genus into nine clades, with high bootstrap values. All *P. violae* isolates clustered into a single clade (Clade 1), with only one isolate (P149) showing any genetic variation (Figure 2.8). Clades 2, 4, 7 and 9 contained *P. ultimum, P. sulcatum, P. irregulare and P. sylvaticum* isolates respectively, with little intraspecies variation. Clade 3 contained published sequences for *P. dissoctocum* and *P. lutarium*, as well as a number of Dutch isolates (P181-184, P186 and P199) which were not identified to species level. This clade also contains an historic *P. lutarium* isolate obtained from the Warwick culture collection (P173). Hence ITS sequences failed to resolve these species. *P. intermedium* and *P. attrantheridium* isolates grouped together in Clade 5 with the exception of one *P. intermedium* isolate, P5, (isolated from carrot from Norfolk in 2013), which formed its own clade (Clade 6) with high bootstrap support. Clade 8, sub-clade a, included isolates which were not identified to species level (P61, 126, 143) but were closely related to the representative sequence of *P. debaryanum* in Clade 8 sub-clade b.

Cytochrome Oxidase subunit II

Phylogenetic analysis of CoxII resulted in five major clades designated 1-5 (Figure 2.9). Intraspecific variation was observed within Clade 1 containing the *P. violae* isolates, forming three sub-clades (a-c ,with the majority grouping within sub-clade a). Sub-clade a and b were well resolved, with high bootstrap values, whilst sub-clade c contained two isolates indicating only a small amount of genetic variation from sub-clade b. As with the ITS alignment, isolate P149 did not fall within the main *P. violae* clade, but shared a common lineage (Figure 2.9). Isolates from the sub-clades of *P. violae* were from a range of locations and were not associated with carrot cv. or geographic region. Clade 2 contained all *P. sulcatum* isolates and was heterogenous, consisting of two main sequence types (sub-clades a and b). Within sub-clade b, b_1 contained five isolates in total, including the Dutch isolates of *P. sulcatum* (P180, 203-205), along with one UK isolate (P43). The remaining isolates in sub-clade b (b_2) and sub-clade a were from a range of locations and were not associated with carrot cv., or geographic region. Clades 3, 4 and 5 contained *P. intermedium* and *P. sylvaticum* isolates and were more closely related. As for the ITS sequencing analysis, isolate P5 fell outside of the main *P. intermedium* clade. This was also the case for P148, another *P. intermedium* isolate collected from Nottinghamshire (Clade 3). The main *P. intermedium* clade (Clade 5) consisted of two main sub-clades. All sub-clades were comprised of isolates from a range of carrot cvs., and originated from a range of counties. There was some

genetic variation within both sub-clade a and b with high bootstrap support. Clade 4 contained all *P. sylvaticum* isolates which grouped into three sub-clades. A small group of *P. sylvaticum* isolates from one field in Nottinghamshire from cultivar Laguna were grouped alone together in sub-clade c. This field contained carrots with cavity spot which presented with very large, expanded lesions.

Representatives of *Pythium* spp. in different clades were found in the same field; for example, *P. violae* isolates P74 and P111, as well as P59 and P138 were isolated from the same fields across two different dates (Table 2.7) In each case, the pair of isolates identified fell into Clade 1a and 1b respectively in the CoxII alignment. Furthermore, six *P. sylvaticum* isolates were collected from one field in Nottinghamshire (cv. Laguna). These isolates fell across the three sub-clades of *P. sylvaticum* clade in CoxII alignment (Clade 4, Figure 2.9).

NADH dehydrogenase subunit I

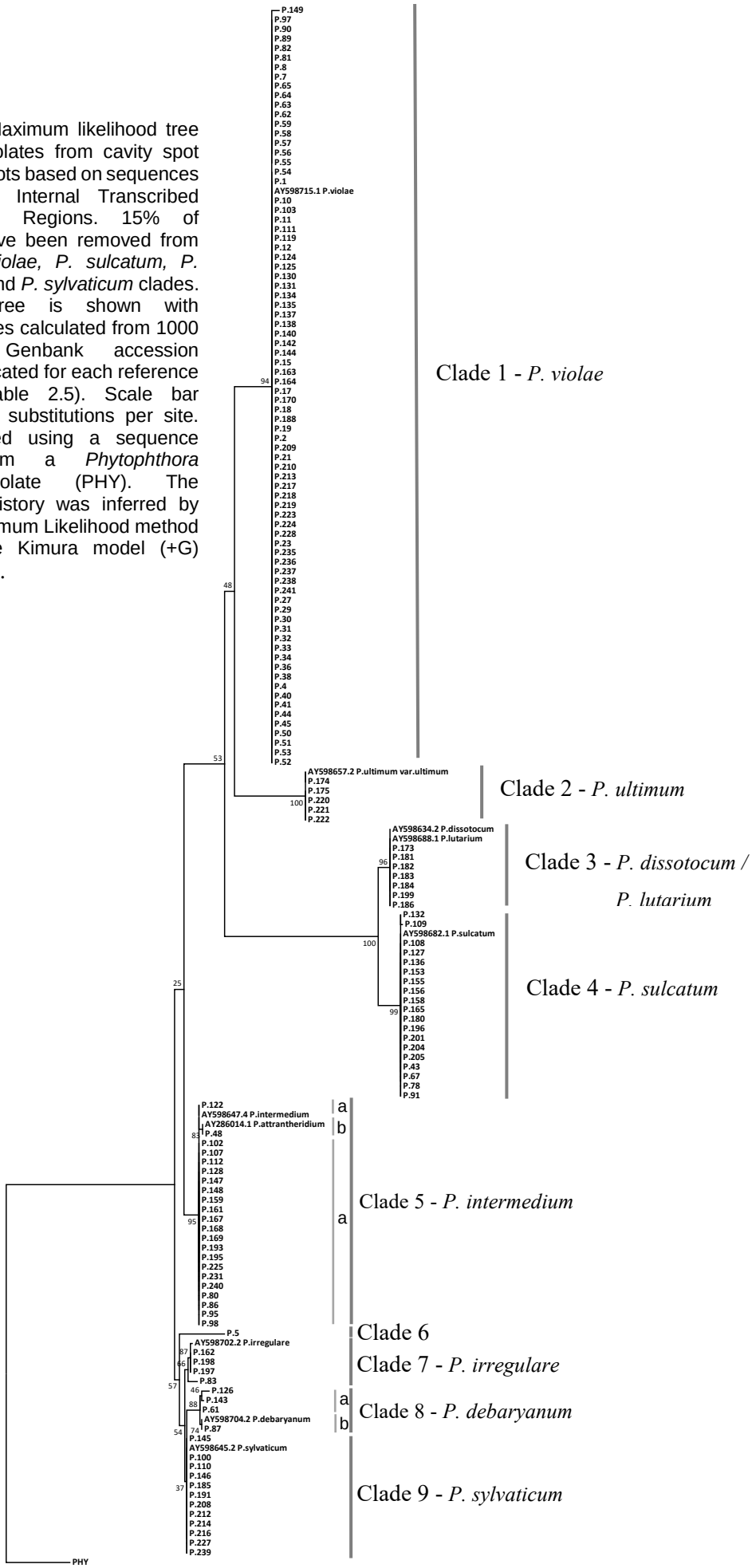
Clade 1 contained the majority of *P. violae* isolates, with one *P. violae* (P149) having a unique sequence type that did not fall within the main *P. violae* clade (this was also the case for the ITS and CoxII phylogenies). The remaining *P. violae* isolates fell into a separate clade (Clade 3) which contained all of the isolates that grouped into CoxII Clade 1b, apart from isolate P90 and P111 which did not fall into this clade. Clade 2 contained all *P. sulcatum* isolates with no intra-specific separation. Clade 4 consisted of all *P. sylvaticum* isolates, which grouped into two main sub-clades, with the majority of isolates falling into sub-clade a with a large amount of intra-specific genetic variation. As for the CoxII alignment, sub-clade b contained a small group of *P. sylvaticum* isolates from the same single field in Nottinghamshire. Clade 6 contained all *P. intermedium* isolates, with two sub-clades. As before, isolate P5 represented a unique sequence type in its own clade (Clade 5), whilst the rest of the *P. intermedium* isolates were distributed within sub-clades a and b in Clade 6, which were from a variety of locations and isolated from a range of carrot cvs.

Concatenated alignment

The concatenated alignment for ITS, CoxII and NADH sequences from *P. violae, P. intermedium* and *P. sulcatum* revealed four main clades (Figure 2.11). Clade 1 contained the *P. violae* isolates. Isolates P149, P188 and P11 formed sub-clade c, which was somewhat genetically distant from the majority of the *P. violae* isolates as previously described for the individual gene sequence alignments, although with a bootstrap value of 32. Most of the remaining *P. violae* isolates formed two sub-clades (a and b) within Clade 1 (Figure 2.11). Clade b contained the majority of the *P. violae* isolates from a range of locations while Clade a contained isolates previously slightly separated from the main clade in analysis of CoxII and NADH (Figure 2.9: CoxII: Clade 1b, Figure 2.10: NADH: Clade 3a). Isolates P89, P90 and P111, although within the main *P. violae* group,

were more genetically distinct from the majority of *P. violae* isolates (Figure 2.11). Clade 2 contained all *P. sulcatum* isolates which were separated into two main sub-clades each of which contained isolates from a range of different locations and carrot cvs. Sub-clade a revealed multiple sequence variation, whereas sub-clade b contained a small number of isolates with no within-clade variation (Figure 2.11). Clade 4 contained the majority of *P. intermedium* isolates split into two main sub-clades. Sub-clade a contained isolate with high-bootstrap support while isolate P148 formed a separate sub clade (a_1) as described for the individual gene phylogenies. *P. intermedium* isolate P5 formed its own clade (Clade 3; Figure 2.11), as previously described.

Figure 0.10 Maximum likelihood tree of *Pythium* isolates from cavity spot lesions on carrots based on sequences of the rDNA Internal Transcribed Spacer (ITS) Regions. 15% of sequences have been removed from each of *P. violae, P. sulcatum, P. intermedium* and *P. sylvaticum* clades. Consensus tree is shown with bootstrap values calculated from 1000 replications. Genbank accession number is indicated for each reference sequence (Table 2.5). Scale bar indicated 0.05 substitutions per site. Tree is rooted using a sequence obtained from a *Phytophthora cactorum* isolate (PHY). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura model (+G) (Kimura, 1980).



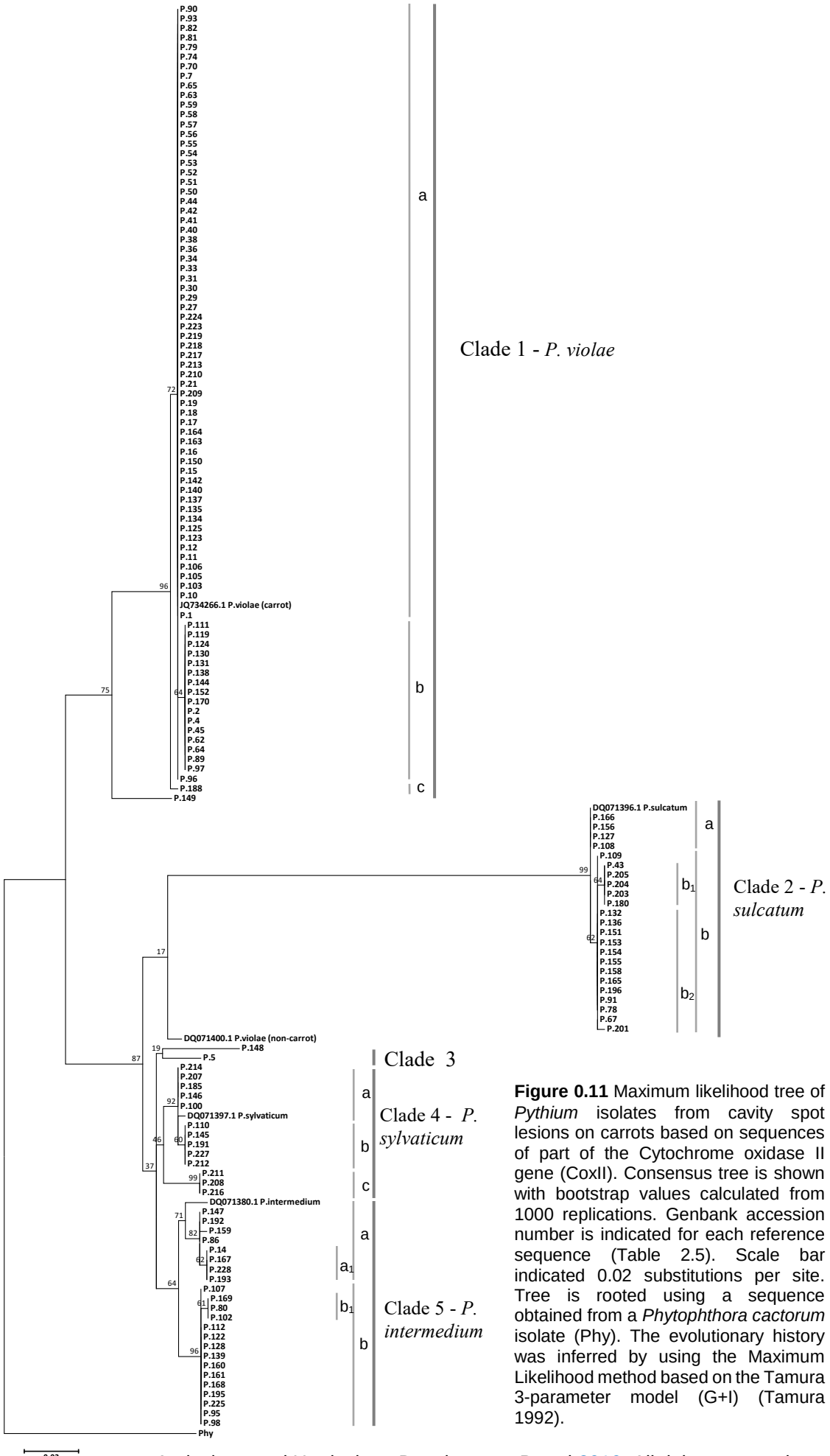


Figure 0.11 Maximum likelihood tree of *Pythium* isolates from cavity spot lesions on carrots based on sequences of part of the Cytochrome oxidase II gene (CoxII). Consensus tree is shown with bootstrap values calculated from 1000 replications. Genbank accession number is indicated for each reference sequence (Table 2.5). Scale bar indicated 0.02 substitutions per site. Tree is rooted using a sequence obtained from a *Phytophthora cactorum* isolate (Phy). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (G+I) (Tamura 1992).

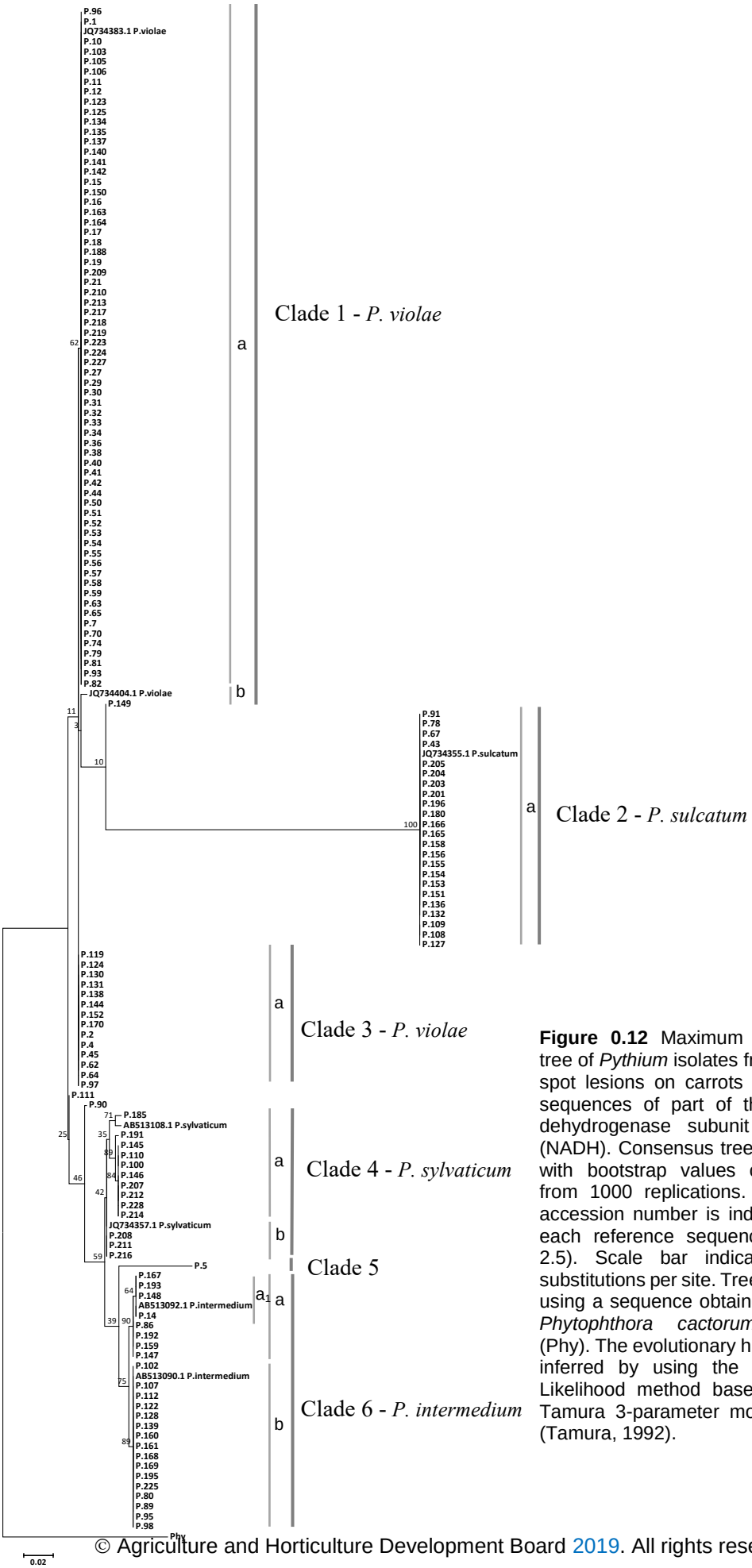


Figure 0.12 Maximum likelihood tree of *Pythium* isolates from cavity spot lesions on carrots based on sequences of part of the NADH dehydrogenase subunit 1 gene (NADH). Consensus tree is shown with bootstrap values calculated from 1000 replications. Genbank accession number is indicated for each reference sequence (Table 2.5). Scale bar indicated 0.02 substitutions per site. Tree is rooted using a sequence obtained from a *Phytophthora cactorum* isolate (Phy). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (G+I) (Tamura, 1992).

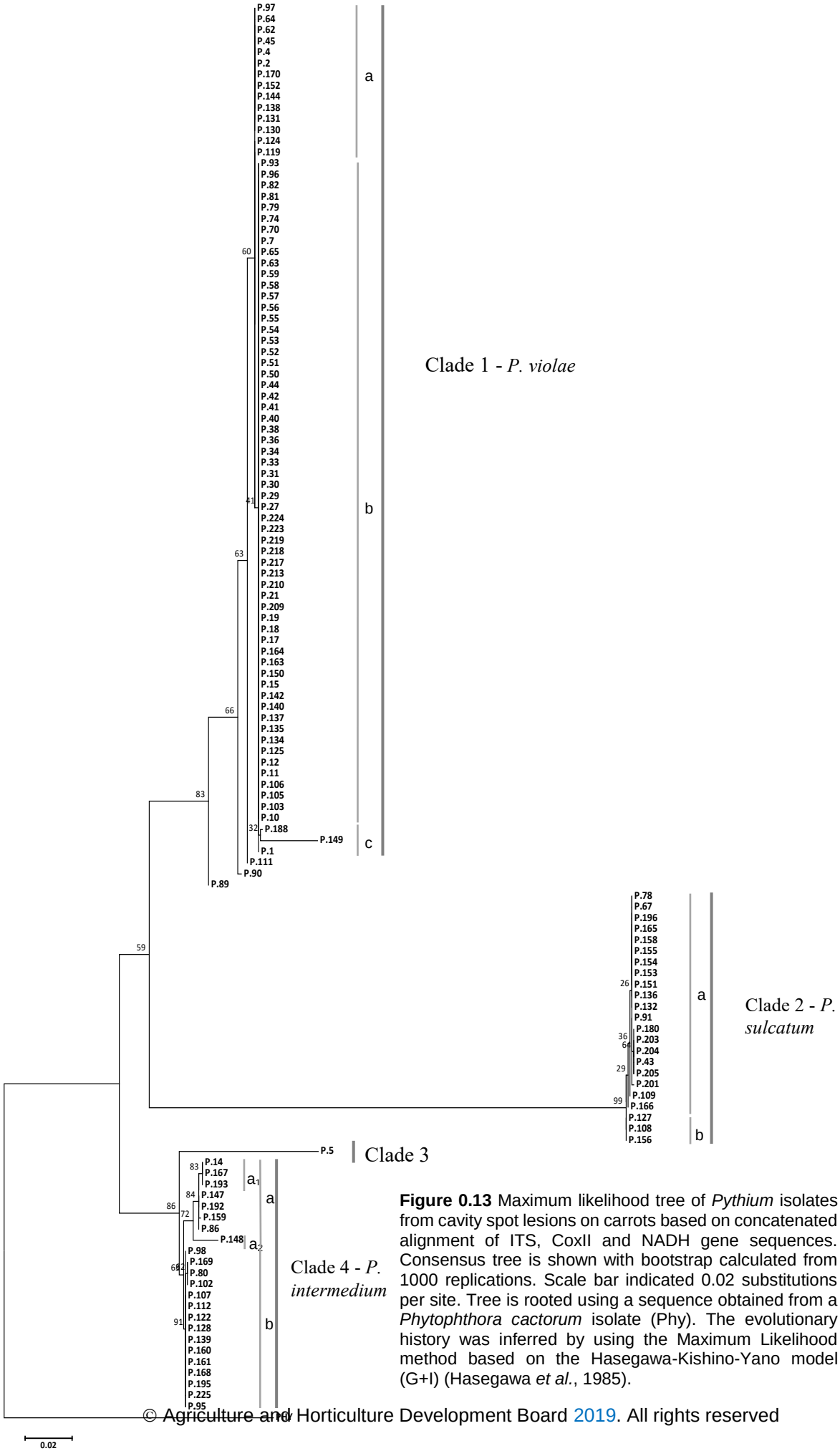


Figure 0.13 Maximum likelihood tree of *Pythium* isolates from cavity spot lesions on carrots based on concatenated alignment of ITS, CoxII and NADH gene sequences. Consensus tree is shown with bootstrap calculated from 1000 replications. Scale bar indicated 0.02 substitutions per site. Tree is rooted using a sequence obtained from a *Phytophthora cactorum* isolate (Phy). The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (G+I) (Hasegawa *et al.*, 1985).

Objective 3: To compare pathogenicity of selected *Pythium* species isolates based on inter- and intra-specific variation of housekeeping genes and geographic distribution

Introduction

Controlled laboratory experiments using freshly dug carrot roots has been used previously for pathogenicity testing. Groom and Perry (1985) were the first to induce 'cavity spot-like' lesions on carrot roots using a laboratory-based pathogenicity test. Agar plugs of mycelium (6 mm) from *P. violae* grown on CMA were placed on healthy roots and incubated at 20˚C for seven days, and lesions developed at the inoculation site within 48 hours. Furthermore, *P. violae* was re-isolated from lesions up to 11 days post inoculation, but not after this period. Since then, this approach has been used to determine the pathogenicity and virulence of a range of *Pythium* spp. with roots assessed by either measuring lesion area (El-Tarabily *et al.*, 1996) or a visual assessment scale (Hermansen *et al.*, 2007).

There is little published information on the susceptibility of different carrot varieties to different *Pythium* spp.. Work in Canada by Benard and Punja (1995) demonstrated that different varieties do possess varying amounts of resistance by using a 'plugs on roots' pathogenicity test, and identified a range of resistant, intermediate and susceptible varieties. Chantenay and Nairobi are both known to be susceptible to cavity spot (Gladders, 2014), however no experiments have been conducted looking at the relative susceptibility of different carrot cultivars and accessions to a range of *Pythium* isolates based on their genetic variation.

Materials and Methods

Inoculations were carried out to assess the ability of fourteen *Pythium* isolates representing three species (*P. violae* x6, *P. intermedium* x4, *P. sulcatum* x4) to produce cavity spot lesions on freshly harvested carrot roots.

Carrots were grown in Long Meadow West field at Wellesbourne (latitude 52˚12', longitude 1˚35'; Appendix B) in beds where a pre-sowing application of nitrogen (100 kg/ha) was applied, before the seed (cv. Nairobi) was drilled on 31/03/2017 (100 seeds/m, 4 rows at 35 cm). Five days post drilling, pre- and post-emergence weed control was applied (Linuron 1.35 L/ha and Stomp Aqua 2.9 L/ha), and the trial area fenced to protect from wildlife. Once seedlings had emerged (24/04/2017) the beds were covered with fleece for frost and carrot root fly protection (Tudor Environmental, UK). Throughout the growing season, plots were hand-weeded and irrigated as necessary. Roots were harvested at set intervals throughout the day to ensure time from harvest

to inoculation was kept to a minimum (< 3 hours) on 20th-21st November 2017 (replicate one) and 4th-5th December 2017 (replicate two), washed with tap water and kept sealed in plastic bags for immediate use. Roots were surface sterilised by submerging in 10% bleach for one minute and washing twice in SDW before inoculation with two 3 mm diameter agar plugs (cork borer) of *Pythium* mycelium from the edge of an actively growing colony of each isolate grown on CMA. Roots were incubated in the dark at 15˚C in a seedling propagator lined with damp sterile blotting paper (Figure 2.1).

A total of 24 roots per isolate were inoculated, with two agar plugs per isolate, with four replicate propagators containing six roots, giving 48 individual testing points. Control roots (uninoculated, CMA plug only) and a 'standard' *P. violae* isolate (P10) treatment had six replicate propagators with a total of 36 roots and 72 testing points. In total the 64 propagators were arranged in a randomised incomplete block design across six shelves with four replicates of all treatments, plus additional replicates of the control and standard isolate (P10). All roots were photographed every three days for nine days and lesion area measured using ImageJ (Rasband, 1997-2016). Two independent experiments were conducted.

Figure 0.14 Carrot roots (cv. Nairobi) inoculated with agar plugs of *Pythium.*

Statistical analysis was carried out with the support of Andrew Mead (Rothamsted Research) in Genstat® (18.1 edition, VSN International Ltd). Significant differences between isolate lesion area data were analysed using the Restricted Maximum Likelihood (REML) algorithm specifying a linear mixed model with a nested blocking structure (boxes within shelves; random model) and a nested treatment structure (isolates within species; fixed model). Interpretations of the analyses and comparison of treatment means were carried out by comparing REML treatment means using the standard error of the differences of the means (SED) at the 5% level.

VeGIN carrot accessions pathogenicity experiment

Root inoculations were carried out as described above to test the susceptibility of seven different accessions of carrot to a standard *P. violae* isolate (P10). These accessions were selected from a carrot diversity set (a resource developed in DEFRA-funded Vegetable Genetics Resource Network project (VeGIN) based on differences in cavity spot incidence in previous field experiments). The widely grown cultivar Nairobi was included alongside the seven accessions. Seed were collected and stored at Warwick Crop Centre UK Vegetable Gene Bank. Seed were from a range of donors including plant breeders, seed firms and collectors, and were acquired between 1983 and 1994 from a range of countries. Root accessions are anonymised (named A1-A7). All roots were grown in Sheep Pens West (Appendix B) at Wellesbourne where a pre-sowing application of nitrogen (100 kg/ha) was applied, before the seed was drilled on 03/05/17 (3x3 design across 3 beds). At drilling, pre- and post-emergence weed control was applied (Linuron 1.35 L/ha and Stomp Aqua 2.9 L/ha) and the trial area fenced to protect from wildlife. Once seedlings had emerged (22/05/17) the beds were hand-weeded and covered with fleece for frost and carrot root fly protection (Tudor Environmental, UK). On 18/08/17 carrots tops were mowed off and the fleece replaced. Throughout the growing season, plots were hand-weeded and irrigated as necessary. Roots were harvested in September 2017 in blocks of two or three accessions at three intervals throughout the day to ensure time from harvest to inoculation was kept to a minimum (< 3 hours). Experimental set-up was as described above (Figure 2.2).

Figure 0.15 Carrot roots arranged on shelves in controlled environment room, inoculated with plugs of *P. violae* isolates P10.

Results

Isolates from the three *Pythium* spp. tested (*P. violae, P. intermedium* and *P. sulcatum*) all produced cavity spot-like lesions on carrot roots over the two replicated pathogenicity experiments. *P. violae* and *P. sulcatum* were more aggressive and produced large, dark lesions within 72 hours which expanded during the next six days. However, lesions were smaller with less discolouration of the tissue following inoculation with *P. intermedium* isolates (Figure 2.13). Plugs of un-inoculated CMA applied to (control) carrot roots resulted in no lesions.

In the first experiment, there was significant variation in lesion size between *Pythium* spp. ($F_{3,40.2}$ = 33.57, p < 0.01; Table 2.8). *P. violae* isolates produced the largest lesion sizes (130.4-251.4 mm^2) followed by *P. sulcatum* (90.6-172.2 mm^2) and *P. intermedium* (61.4-101.2 mm^2; Figure 2.12a). When comparing across all individual isolates, there was no significant difference in lesion size ($F_{10,42.2}$ = 1.66, p = 0.123; Figure 2.12a). However, when comparing isolates within each species, there were no differences between isolates within the species (*P. violae* $F_{4,42.9}$ = 1.82, p = 0.143; *P. intermedium* $F_{3,43.0}$ = 0.68, p = 0.569; *P. sulcatum* $F_{3,43.0}$ = 2.43, p = 0.078; Figure 2.12a). Within *P. sulcatum*, P43 showed notably smaller lesion sizes than the other isolates, while P10 (standard isolate) produced the smallest lesion size of the *P. violae* isolates (Figure 2.12a).

In the second experiment, there was also significant variation in lesion size between *Pythium* spp. ($F_{3,39.7}$ = 18.54, p < 0.01; Table 2.8). In contrast to the first experiment, *P. sulcatum* had the largest lesion sizes (50.4-133.2 mm^2), followed by *P. violae* (23.72-113.52 mm^2); and *P. intermedium* (20.02-62.93 mm^2; Figure 2.12b). Also, in contrast to Experiment 1, there was a significant difference in lesion size when comparing across all individual isolates ($F_{10,41.3}$ = 4.32, p < 0.01, Figure 2.12b). Furthermore, there were significant differences in lesion size within both *P. violae* and *P. sulcatum* isolates ($F_{4,42.9}$ = 4.75, p = 0.003; $F_{3,41.6}$ = 6.31, p = 0.001 respectively; Figure 2.12b), but not within *P. intermedium* isolates ($F_{3,41.6}$ = 1.69, p = 0.183; Figure 2.12b). Within *P. violae*, isolate P10 and isolate P149 produced significantly smaller lesion sizes compared to the other *P. violae* isolates. Additionally, isolate *P. violae* P54 produced significantly larger lesions than all the other isolates. Within *P. sulcatum*, isolate P43 produced significantly smaller lesions than any of the other *P. sulcatum* isolates tested, whilst within *P. intermedium* isolates, isolate P107 produced significantly larger lesions that the other *P. intermedium* isolates tested.

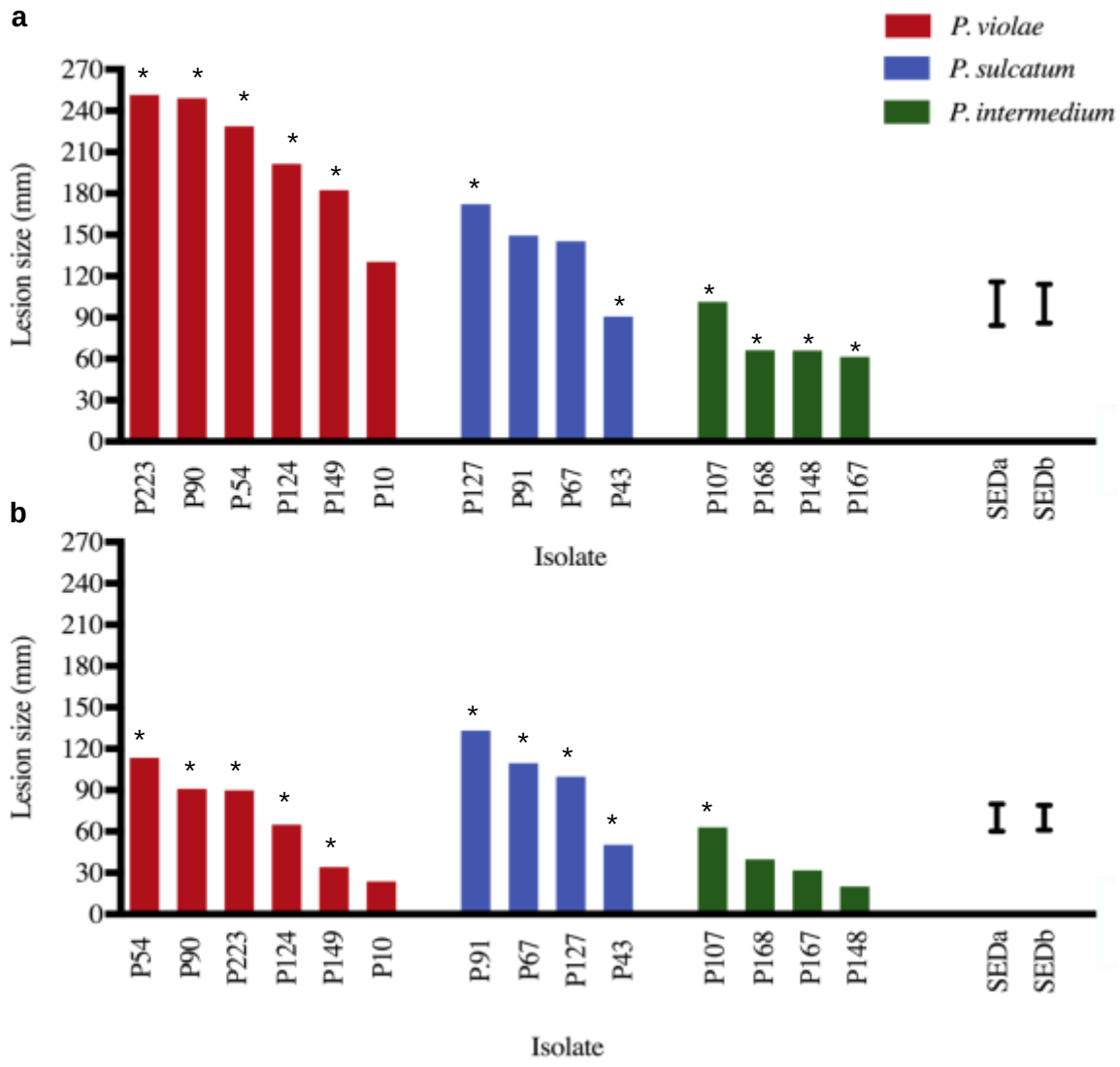


Figure 0.16 Pathogenicity of 14 *Pythium* isolates on carrot roots for Experiment 1 (a) and Experiment 2 (b); 6x *P. violae*, 4x *P. sulcatum*, 4x *P. intermedium*). Data are the mean lesion size produced on carrot roots nine days post inoculation at 15ºC. Error bar represents the standard error of the differences of the means (SED). SEDb represents the error for comparing the standard *P. violae* isolate (P10) to all other isolates. SEDa represents the error for comparing all other isolates between each other. * represents a significant difference at the 5% level from the standard *P. violae* isolate (P10).

There was no significant difference in the standard deviation of lesion size on carrots between species or across all isolates for either experiment. The variation in lesion size across carrots/boxes was similar for all species.

Table 0.9 The mean and standard error of lesion size across *Pythium* species inoculated on carrot roots. Three standard errors are provided for comparison with the standard isolate (a), for comparing either *P. intermedium*/*P. sulcatum* with *P. violae* (b), and for comparing between *P. intermedium*/*P. sulcatum.*

Experiment	Isolate	Mean	Standard error			
			a	**b**	**c**	
1	Standard (P10)	130.4	-			
	P. violae	226.6	20.89	-	-	
	P. intermedium	73.7	21.48	15.04	-	
	P. sulcatum	139.4	21.48	15.04	15.82	-
2	Standard (P10)	23.72	-			
	P. violae	78.65	12.86	-	-	
	P. intermedium	38.59	13.22	9.27	-	-
	P. sulcatum	98.23	13.22	9.27	9.78	

There was a significant effect of experiment on lesion size ($p < 0.001$). Lesion sizes in Experiment 2 were smaller overall than those in Experiment 1. There was also a significant experiment by species interaction in mean lesion size ($p < 0.001$). In Experiment 2, *P. sulcatum* produced the largest lesion sizes overall, unlike in Experiment 1 where *P. violae* produced the largest lesion sizes. Across experiments there was no significant difference in lesion size between isolates within species, i.e. the order of isolates (in terms of lesion size) did not significantly vary. The standard isolate P10 produced the smallest lesion sizes of the *P. violae* isolates, followed by P149 in both experiments. Isolate P43 produced the smallest lesion size of the *P. sulcatum* isolates, and P107 produced the largest lesion sizes of *P. intermedium* across both experiments

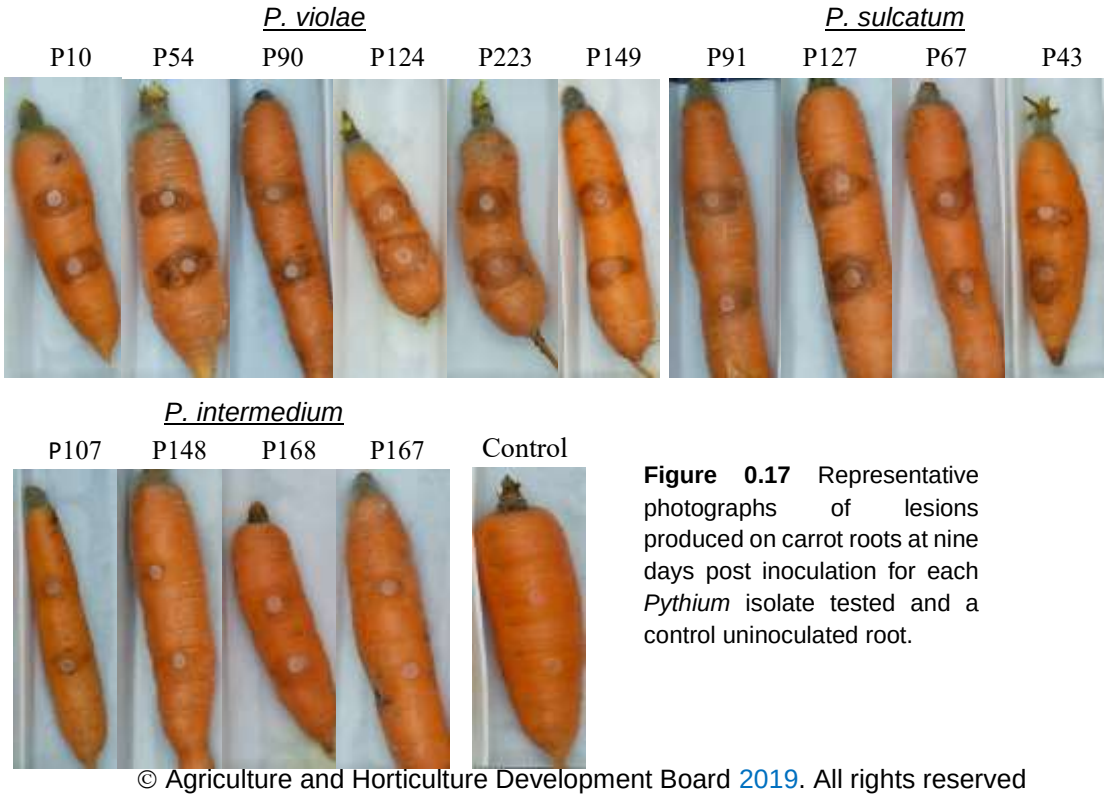


Figure 0.17 Representative photographs of lesions produced on carrot roots at nine days post inoculation for each *Pythium* isolate tested and a control uninoculated root.

VeGIN carrot accessions pathogenicity experiment

P. violae isolate P10 produced cavity spot lesions on all the carrot accessions tested, including cv. Nairobi (Figure 2.14). There was a large variation in average lesion size produced by isolate P10 across the roots, which on average ranged from 9.8-186.3 mm^2. Across all carrot accessions there was a significant difference in lesion size ($F_{7,21} = 10.10$, $p < 0.001$, Figure 2.15). Accessions A1 and A2 developed the smallest lesions, with most roots showing no lesion development, and those roots that did develop lesions were small, shallow and very light in colour (Figure 2.15). Accessions A7 and A8 developed large, dark coloured lesions which were deep and had defined edges. For cultivar Nairobi (A6), isolate P10 produced the third largest lesion size. These lesions were large and sunken, and although not as dark as accessions A7 and A8, still well defined and substantial (Figure 2.15).

The lesions size produced on cultivar Nairobi averaged at 97.3 mm, which falls within the range of lesion size than was produced by isolate P10 on Nairobi within the *Pythium* isolates pathogenicity test, where lesion size averaged as 130.4 mm^2 and 23.7 mm^2 in Experiment 1 and 2 respectively.

The standard deviation analysis showed that was a significant difference in standard deviation across accessions ($F_{7,21} = 5.31$, $p = 0.001$; Table 2.9) which ranged from 9.8 to 83.0, and in general, as average lesion size increased, the standard deviation of the lesion size increased, i.e., there was greater variance in lesion size when the mean lesion size was larger. However, this is not the case for accession A3, which produced the third smallest mean lesion size, but the second largest standard deviation (Table 2.9).

Table 0.10 The mean lesion size and standard deviation of lesion size across eight carrot varieties inoculated with *P. violae* isolate P10.

Accession number	Mean (mm^2)	Standard deviation
A1	9.8	12.1
A2	21.6	9.8
A3	64.6	79.8
A4	66.8	31.6
A5	83.4	39.5
A6	97.3	49.3
A7	149.4	75.2
A8	186.3	83.0

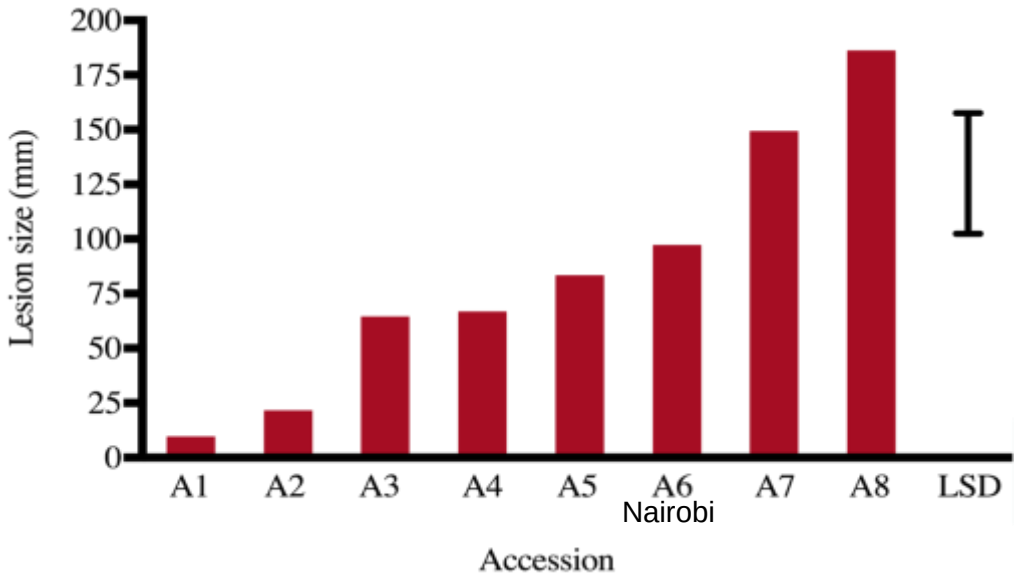


Figure 0.18 Lesion size produced by *Pythium violae* isolate P10 on eight carrot varieties. Data shown are the mean lesion size produced on whole carrot roots nine days post inoculation at 15˚C. Error bar represents the least significant difference (LSD) the 5% level.

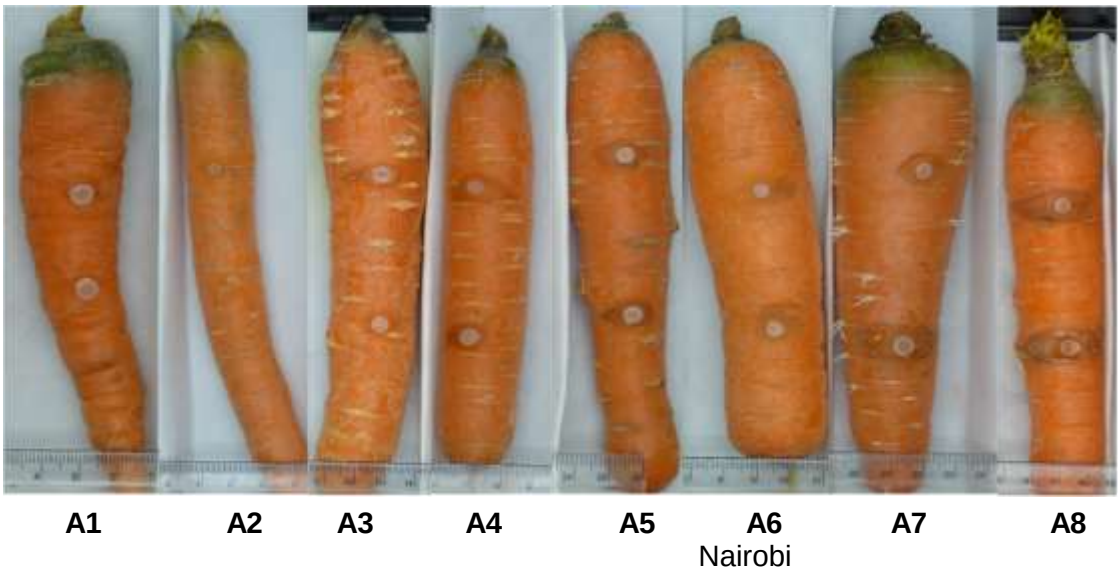


Figure 0.19 Representative photographs of lesions produced on carrot roots by *P. violae* P10 from different accessions at nine days post inoculation for each carrot accession tested.

Discussion

This survey of the *Pythium* species associated with cavity spot lesions in carrot production areas throughout England is the largest study of *Pythium* isolates associated with cavity spot in the UK. It revealed that several *Pythium* spp. can be isolated from roots with a variety of symptoms and the main causal agent is *P. violae*. Genetic analysis of housekeeping genes revealed a range of between- and within-species variation. Isolates representing different clades have shown variation in lesion size on carrot roots.

P. violae was consistently associated with cavity spot lesions in carrots sampled in all regions, and was more commonly isolated from 'younger' lesions and therefore should be considered the most important pathogen causing cavity spot in England. The lower optimum temperature for growth for *P. violae* (17-20˚C, compared to *P. ultimum*/*P. sulcatum* 25-30˚C; Hiltunen & White, 2002), may be one of the main reasons for its predominance in the UK compared to other regions of the world. However, previous studies have concluded that soil moisture may play a more important role than temperature in cavity spot disease development. Even before the cause of cavity spot had been identified, Guba *et al.* (1961) had correlated larger lesion sizes on carrots with high moisture content, and this has since been noted as a common phenomenon in poorly-drained soils and with high incidences of rainfall, particularly in late summer (Hiltunen & White, 2002). This conclusion is supported by Soroker *et al.* (1984) who were able to induce cavity spot lesions above 28˚C in controlled environment experiments using short periods of flooding. Samples of carrots with cavity spot in this study were generally collected between September and April/May, and the development of cavity spot in the UK over winter months (with cooler temperatures) for strawed crops was a common occurrence.

P. sulcatum was also associated with cavity spot lesions in this study, again in the majority of carrot samples from different regions but notably, none found in diseased roots from the East of England. However, a smaller number of isolates were obtained from this area (25) from fewer fields (15), and this may therefore be a reason for this observation rather than an absence of *P. sulcatum* in this region. *P. intermedium* was ubiquitous across all carrot areas, and was more often identified from 'older' lesions. *P. intermedium* is known to be fast-growing and a frequent inhabitant of soil and root zones (van der Plaats-Niterink, 1981). It is commonly found in a range of living and dead plant material, and has been isolated from ornamentals, arable and vegetable crops as well as tree species around the world (van der Plaats-Niterink, 1981). Although *P. intermedium* has been shown to cause cavity spot lesions (Guerin *et al.*, 1994; Suffert & Guibert, 2007) it is possible that the high occurrence of this species in this study is due to its fast growth and its ability to out-compete slower growing *Pythium* spp. such as *P. violae* and *P. sulcatum* (Kageyama, 2014). When lesions are first formed, it is more common to isolate a single species, generally either *P.*

sulcatum or *P. violae*, but as cavity spot lesions get older, often the faster growing *P. intermedium, P. irregulare* or *P. sylvaticum* are first to grow out, smothering growth of the slower growing species (Hiltunen & White, 2002). In this study it was more difficult to isolate *Pythium* from older, expanded lesions. Most of the isolations in this study were obtained between September-April, with a large number of carrot samples being isolated from post-strawing (January-April). The larger percentage of *P. intermedium* isolates identified may be due to the increasing age of the lesions, and the amount of *P. violae* present may have been underestimated. There is therefore a need to be cautious about assigning certain *Pythium* or other species as the initial cause of cavity spot, as the age and size of lesion (state of decay) needs to be taken into account.

In some fields, multiple different *Pythium* spp. were isolated and identified from cavity spot lesions. In the more intensively sampled fields where isolation was done from an increased number of cavity spot lesions, a greater number and wider range of *Pythium* spp. were recovered. This finding suggests that it may be common for multiple *Pythium* spp. to be found together, again highlighting the difficulty in knowing which *Pythium* spp. are the main cause of a lesion. Campion *et al.* (1997) also identified that multiple *Pythium* spp. may be isolated from a single region, field, carrot and even lesion, and that these may have differences in pathogenicity, symptom type, or colonisation potential of the carrot root.

The phylogenetic analyses in this study revealed that multiple isolates from the same field were in different sub-clades of the same *Pythium* spp.. This indicates that there may be local genotype differences and evolution/divergence of isolates within a field. The results of this study suggest some correlation between geographic origin and genetic sequence may be present, and indicates that genetic changes may be occurring within-field. Further studies involving a greater number of *Pythium* isolates is recommended to validate this.

In the phylogeny and pathogenicity experiments, carrot sampling and collection was extensive, but even with the use of selective media, isolation of *Pythium* spp. from cavity spot lesions proved challenging. Quite often, even though a large number of cavity spot lesions were plated onto agar, only one or two lesions would exhibit *Pythium*-like growth, and at times it was not possible to separate *Pythium* growth from other competing fungal species with a fast growth rate. This problem meant that obtaining a pure culture was sometimes difficult.

The pathogenicity test conducted with a range of carrot cultivars revealed that *P. violae* produced cavity spot lesions on a range of carrot varieties, but that lesion size varied significantly between cultivars. The lesions produced on cv. Nairobi, were the third largest of the eight tested varieties. A number of other cultivars had smaller lesions produced by the same *P. violae* isolate, confirming previous results showing cv. Nairobi is a relatively easily colonised by *P. violae*. Accessions A1 and A2 had very little to no lesions on roots, and seemed particularly promising lines for future breeding programmes. The variation in lesion size produced on cv. Nairobi was the fourth largest

of the eight varieties tested, indicating that either individual carrot roots show variation in susceptibility or that the ability of an individual isolate of *P. violae* to infect a carrot root varies. Further replications of this experiment with a larger number of roots would be needed to understand the variation in lesion size produced. Although there were no roots in this test which were uninoculated (i.e. there were no controls), in all other laboratory pathogenicity experiments conducted, no lesions were produced on control roots.

Few studies have published information relating to the susceptibility of different carrot cultivars to *P. violae*. White (1988) studied the susceptibility of carrot cultivars to *Pythium* spp., and found cv. Nandor was somewhat resistant to *P. violae* and *P. sulcatum*, but susceptible to *P. intermedium*, implying perhaps the mechanism of resistance is the not same process. Barbara (Warwick Crop Centre, unpublished) tested cavity spot resistance in two carrot varieties in replicated beds and found only 3/60 roots of cv. Volcano had cavity spot lesions compared to 38/80 for Nairobi. Results from this study support the observation that Nairobi is relatively susceptible to *P. violae*. Carrot samples from Norfolk (Table 2.5) included samples from a trial site which evaluated a range of commercial, common UK cultivars (anonymised). Roots of susceptible cultivars revealed greater colonisation from a range of *Pythium* spp. (*P. violae, P. intermedium* and *P. sulcatum*) than the resistant cultivar, from which only *P. intermedium* was isolated. In contrast, Nairobi was only heavily colonised by *P. violae*. Given the very high growth rate of Nairobi in UK commercial sites (due to the consistency of root growth and the robust root for packhouse processing), the high incidence of *P. violae* infection across UK fields may be attributed to Nairobi susceptibility.

In this study, the amount of intraspecific variation identified differed between *Pythium* spp. and different gene sequences. The phylogenetic analysis based on ITS sequences resulted in clear discrimination of different *Pythium* spp. ITS sequence analysis has been shown to be variable between, but largely conserved within, *Pythium* spp. (Wang & White, 1997). Martin (2000) found that sequence divergence among isolates within a single *Pythium* spp. generally varied only to a small extent, from 0-0.88% substitutions, and therefore use of ITS for *Pythium* identification to species level is generally robust. However, although ITS sequence analysis is now used universally for *Pythium* identification, not all species boundaries are resolved by the ITS region in isolation; i.e. closely related but separate species may have similar or identical ITS sequences (Levesque & de Cock, 2004; Robideau *et al.*, 2011). In the present study, a small number of isolates were not identified to species level using ITS sequence analysis; the *P. lutarium* and *P. dissoctotum* reference isolates from Levesque and de Cock (2004) both resided in the same clade as these unknown Pythium isolates, which were not distinguishable from each other.

Martin (2000) found a higher degree of nucleotide substitution occurred in a small number of *Pythium* spp. and hence multi-gene analysis may be needed to determine genetic differences between very closely related species. The results of the phylogenetic analysis of Cox II revealed

some intraspecific variation within the *Pythium* spp.. This gene is more variable than others associated with nuclear DNA as it is mitochondrially encoded (Villa *et al.*, 2006).

This work is the first study to determine the phylogenetic relationship between and within isolates of *P. violae*, *P. sulcatum* and *P. intermedium* based on the NADH dehydrogenase subunit one gene and identified sources of intraspecific variation not observed with the Cox II gene analysis, and may be useful when attempting to determine genetic lineage of the *P. violae* species. The NADH gene also revealed greater intra-specific variation within the *P. sylvaticum* isolates than the ITS or Cox II alignments, clearly separating out a single Dutch isolate from the remaining *P. sylvaticum* isolates. This suggests that NADH analysis may be better suited to determining how genetic variation may be linked with geographic origin of *Pythium* spp. isolates than previous attempts (Villa *et al.*, 2006); however a greater collection of isolates from a wider geographic range would need to be considered. The concatenated alignment showed the greatest amount of intraspecific variation of any of the gene analyses and was used to select isolates to take forward for pathogenicity testing. Isolates were selected based on their clade alignment and geographic location.

In this study, a range of *P. violae*, *P. sulcatum* and *P. intermedium* isolates were examined for their pathogenicity on carrot roots using an agar-plug inoculation method. This technique is useful for determining the pathogenicity and virulence of different isolates, however it does not always result in typical cavity spot lesions (Vivoda *et al.*, 1991), and carrot roots must be fresh out the ground (previous experiments found roots less than three hours old were most susceptible) to allow *Pythium* penetration, as the carrot skin starts to dry and 'seal' itself making penetration more difficult. This is the first examination of how representative *Pythium* isolates from different clades differ in their virulence on carrot. All isolates caused lesions on carrot roots, but these varied in size and colour, with *P. violae* and *P. sulcatum* isolates producing darker, deeper and larger lesions relative to *P. intermedium* isolates. *P. violae* and *P. sulcatum* are characterised as 'slow-growing' *Pythium* spp., whilst *P. intermedium* is 'fast-growing' and growth rate has previously been correlated with lesion size produced (Zamski & Petretz, 1995). Zamski and Petretz (1995) found slow-growing *Pythium* spp. (including *P. violae* and *P. sulcatum*) produced lesions on carrots, while fast-growing species (*P. aphanidermatum* and *P. paraecandum*) were not pathogenic. This is comparable with the results from this PhD study, where consistently *P. intermedium* produced smaller lesions than *P. violae* and *P. sulcatum*.

In contrast, El-Tarabily *et al.* (1996) conducted a laboratory pathogenicity test of 170 *Pythium* isolates from Australia on carrot and found *P. coloratum* (51 isolates) was more aggressive than *P. sulcatum* (120 isolates), where only 32 isolates produced lesions and these lesions were smaller. These results highlight the variation in virulence that can be seen both between and within a species, and indicate virulence may depend on the particular isolates used within a species as

well as the specific conditions in the pathogenicity experiment. Similar variation within and between species was seen in this study.

In this study, the pathogenicity experiments resulted in conflicting results between experiments and the virulence observed within *Pythium* species was diverse. Experiment 2 revealed significant differences in lesion size produced by isolates within *P. violae* and within *P. sulcatum*. However, a number of isolates produced consistent responses. For example within *P. violae*, isolates P10 and P149 produced significantly smaller lesion sizes than other *P. violae* isolates across both experiments. Furthermore, P149 consistently separated from the rest of the *P. violae* isolates in the phylogenetic analysis forming a unique out-group and therefore warrants further investigation with respect to genetic variation and pathogenicity. In Experiment 1, inoculation with *P. violae* resulted in the largest lesions while in Experiment 2, *P. sulcatum* produced the largest lesions. Notably, in Experiment 2, lesion sizes were significantly smaller than in Experiment 1, roughly half the size of those observed in Experiment 1. This decrease in size may be due to the date the experiment was conducted. These experiments used carrot roots grown in the field, but roots were harvested fresh for each experiment, three/four weeks apart. During this time, there was a change in the weather conditions with the temperature dropping considerably in this time. Carrot roots can suffer from frost damage at temperatures below 1.4˚C, and during cold periods, can 'harden' by increasing membrane stability to protect against freeze injury. If the carrots had entered an active 'hardening' phase, the penetration of the skin may have taken longer than the previous experiment, accounting for the smaller lesion size (Snyder & Paulo de Melo-Abreu, 2005).

P. sulcatum isolate P43 consistently produced the smallest lesion sizes of the *P. sulcatum* isolates across both experiments. The concatenated alignment revealed P43 to be in a separate clade from the remaining *P. sulcatum* isolates alongside four isolates obtained from The Netherlands, and was the only isolate obtained from the UK that fell within that clade. It would be of interest to examine the virulence of the Dutch isolates from this clade to see if this clade produces smaller lesions.

P. violae P10 was the standard isolate that was used in all experiments throughout this PhD, and was chosen based on previous initial pathogenicity experiments where it consistently produced a large lesion size on carrot. The reduced pathogenicity of this isolate (compared to other *P. violae* isolates) may be due to a change in growth habit that was noted just prior to this experiment. This habit was observed as sectoring, reduced growth rate and production of dense mycelium. Although P10 cultures used in these experiments exhibited 'normal' *P. violae* growth, subsequent sub-culturing revealed inhibited growth. It is known that sectoring can affect pathogenicity, so the reduced lesion size produced by this isolate may be due to this effect (Abdalla, 1975). Throughout the PhD, attempts were made to reduce the number of sub-cultures from all isolates, however,

given the extensive use of this isolate before and throughout this PhD, it is possible that the loss of virulence is associated with repeated use (Smith *et al.*, 2008; Songe *et al.*, 2014).

The present investigation identified a range of *Pythium* spp. currently associated with cavity spot in the UK, and established that there is intraspecific genetic variation within the *Pythium* spp. tested. Representative isolates from different clades varied in their virulence in *in-vitro* experiments.

Aim 2: Development of *P. violae* artificial inoculation systems for carrot

Objective 1: Define the growth media and conditions for *P. violae* mycelium/oospore inoculum production in a controlled environment.

Introduction

Management of cavity spot involves a variety of techniques including rotation, early harvesting and fungicides but none of these are effective alone. There is an urgent need to identify new active ingredients or approaches for control.To address this, research into the management and control of cavity spot has been undertaken in a number of AHDB Horticulture projects. However, low natural levels of disease have resulted in a failure so far to reliably identify any new active ingredients or approaches to control cavity spot.

Production of *P. violae* inoculum, and use in an artificial inoculation system, would allow now products to be tested reliably and consistently. There is currently no optimised protocol for production of *P. violae* inoculum. Furthermore, relating inoculum levels of soil-borne pathogens to subsequent disease severity can be very difficult, and has proved particularly challenging for *Pythium* spp. (Hiltunen & White, 2002).

Oospores are thought to be the primary survival structure of *P. violae*, and given the fall in temperature over winter, and the fragility of the mycelium (Hendrix & Campbell, 1973), it is likely that oospores initiate infection at the start of a carrot growing season. In this PhD, it was decided that use of a solid substrate to grow *Pythium* oospores would be most suitable, as this promotes oospore rather than mycelial growth. Furthermore, this method is more practical and realistic for use in the field, as well as having the advantage that the solid inoculum is easy to handle and mix, allowing the pathogen to be easily incorporated and evenly distributed into the growing media.

A solid medium comprised of horticultural sand (J. Arthur Bowers, UK) and an oat-based substrate was selected for oospore production. *P. violae* isolate P10 was derived from infected carrots (cv. Nairobi) collected in Holton, Lincolnshire in 2013. Isolate P4 was derived from infected carrots (cv. Match) collected in Wormegay, Norfolk in 2013. These isolates were selected because in preliminary experiments. Methods and results of inoculum production is detailed in AHDB report FV391a/b.

Objective 2: Test the efficacy of different concentrations of *P. violae* inoculum in inducing disease in carrot seedlings in a controlled environment.

Introduction

The inability to consistently produce and quantify inoculum, as well as the wide variation in infection rates, means there is currently no robust and reliable plant assay for *P. violae*. A standardised protocol for production of *P. violae* inoculum and infection of carrots needs to be developed. This will allow increased understanding of early *Pythium* infection, as well as testing of potential new products and approaches to cavity spot control. Production of solid inoculum generating *Pythium* oospores proved successful, and therefore this inoculum was used to determine the potential of this inoculum to cause cavity spot disease in carrot seedlings.

Materials and Methods

Seedling experiments: solid *P. violae* inoculum (Experiments 1-5)

Carrot seedling assays were designed to test the effect of different concentrations of *P. violae* solid inoculum on carrot seedling germination and seedling damping-off. Experiments and levels of inoculum were based on a preliminary test by C. Handy (unpublished, Warwick Crop Centre; data not shown). Flasks (500 ml or 1 L) containing the sand/oat medium were inoculated with either of the two *P. violae* isolates (P4 and P10) and incubated at 14˚C in the dark as described in FV391a/b. For the experiment, the contents of a number of flasks were emptied into sterile plastic bags and mixed by hand for 5 minutes. Oospore density was then determined for the inoculum for each isolate by agitating a 1 g sample from the flask in 10 ml of SDW for 10 minutes and oospores counted using a 1 ml counting cell (Sedgewick Rafter). A minimum of two replicate counts for each of four 1 g samples were counted under a light microscope.

This starting inoculum was diluted appropriately in sharp sand, (J. Arthur Bowers, UK) and mixed by hand to obtain a further batch of inoculum at a concentration of approx. 5000 oospores g^{-1}. Oospore density was checked by a further oospore count, after which a final dilution was carried out to obtain another batch of inoculum with a concentration of approx. 1000 oospores g^{-1}. Appropriate amounts of the 1000 and 5000 oospore g^{-1} inoculum were then diluted in sharp sand again to obtain the desired oospore concentration for each treatment. *P. violae* inoculum dilutions for isolates P4 and P10 (300 g) were placed into clear plastic containers (600 ml) to give final concentrations of 0-300 oospores g^{-1} (0-400 oospores/cm^3) and 20 ml SDW added. Twenty carrot

seeds (cv. Nairobi, Elsoms Seeds Ltd, UK) were then planted in each box at 1 cm depth and lightly covered with sand, the lids immediately closed and the boxes incubated in a controlled environment room at 14˚C under white fluorescent lighting (12 h/day). Boxes were weighed and watered every two weeks to bring them back to their original weight (Figure 3.2).

Five experiments were conducted over the course of 14 months (June 2015-August 2016) using different concentrations and ages of *P. violae* inoculum (Table 3.1). Untreated control treatments (no *P. violae* inoculum) were also included. Each replicate experiment was arranged in a randomized design as appropriate. Carrot seed germination and subsequent damping-off disease symptoms were assessed weekly for 10 weeks. The number of seedlings damped off as a percentage of those which germinated in each box was recorded.

Figure 0.20 Seedling experiment boxes in randomised design on shelving in controlled environment.

Table 0.11 Seedling Experiments 1-5: Expt. no., inoculum age, temperature, isolate used, no. of replicate boxes and oospore concentrations used to test the effect of different concentrations and ages of *P. violae* oospores produced in solid sand/oat medium on carrot seed germination and seedling damping-off between June 2015 and August 2016.

Experiment	Inoculum age (weeks)	Temp (ºC)	Isolate	Replicate boxes	Oospore concentration (oospores g^{-1})
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	6	0, 5, 10, 25, 50, 75, 100, 200
4	12	14	P10, P4	6	0, 10, 25, 50, 75, 100, 200, 300
5	10, 32, 64	14	P10	4	0, 25, 50, 100, 200, 300

Seedling experiments: liquid *P. violae* inoculum (Experiment 6)

A further seedling experiment (Experiment 6, Table 3.2) was carried out to evaluate the effect of *P. violae* isolate P10 oospores produced in both a solid and liquid medium, on carrot seed germination and damping-off in autoclaved and non-autoclaved sand. The liquid inoculum was applied either at sowing or to seedlings three weeks after sowing. V8B was selected as the liquid growth medium as it has been reported to support growth of *P. violae* as well other oomycete species (Sutherland & Cohen, 1983; Pettitt *et al.*, 2002). V8 juice (1 L) was stirred with 20 g $CaCO_3$ for 45 minutes and the mixture centrifuged at 9000 rpm for 30 minutes at 20˚C. For experiments, a 10% (v/v) V8B was prepared by combining 100 ml of the clarified supernatant with 900 ml SDW and autoclaving at 121˚C for 15 minutes. 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 (3 mm^2) from the actively growing edge of *P. violae* isolate P10 (grown on CMA) were used to inoculate 20 ml aliquots of V8B+C in flat cell culture flasks (50 ml; VWR, UK) with vented lids. After incubation at 15˚C for seven weeks in the dark, mycelial mats were removed, washed twice in 20 ml SDW, blotted dry on sterile filter paper and weighed. To extract oospores, mycelial mats from three culture flasks were transferred to a sterile bulbous flask (MSE, UK), and homogenised (MSE Homogeniser) on a low setting for 8 minutes in 45 ml SDW. The process was 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. A 200 µl sample was removed, vortexed for 20 seconds and *P. violae* oospore concentration determined using a haemocytomer (Fuchs-Rosenthal). Oospore inoculum was diluted as appropriate in SDW to achieve concentrations of 1 x 10^3, 1 x 10^4 and 1 x10^5 oospores ml^{-1} and 10 ml added to either autoclaved or non-autoclaved sharp sand (300 g, Westland, UK) contained in clear plastic boxes (600 ml) to give final concentrations of 3, 30 and 300 oospores g^{-1}. Carrot seed (cv. Nairobi, Elsoms Seeds Ltd, UK) was then 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 oospore suspension around the seedlings.

P. violae oospores produced in solid inoculum were prepared as previously described and used to amend sand to the same final concentrations as the liquid inoculum before sowing (Table 3.2). As the solid inoculum was mixed directly into the growing medium, for this treatment oospores could not be applied post germination.

For each treatment, there were four replicate boxes which were placed in a randomised block design over four shelves in a controlled environment room at 15˚C. Untreated control treatments

(no *P. violae* inoculum) were also included. 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 0.12 Seedling Experiment 6: Inoculum type, sand type, inoculation time and oospore concentrations used to test the effect of different concentrations and inoculation time of *P. violae* oospore inoculum produced in V8 liquid and sand/oat solid medium on carrot seed germination and seedling damping-off.

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

Results

Seedling experiments: solid *P. violae* inoculum (Experiments 1-5)

Overall, inoculation with solid *P. violae* inoculum resulted in considerable variation in damping-off symptoms in carrot seedlings. Across the five different experiments with solid inoculum, no clear relationship was observed between *P. violae* oospore concentration, percentage seed germination or post-emergence disease levels. Adjustments to treatments made over the course these experiments attempted to test factors that might affect damping-off development such as temperature, inoculum level and inoculum age, but no clear results were obtained. Over all experiments, if damping-off occurred, it began at around four w.p.s (1-2 weeks post germination).

In Experiment 1, very little damping-off due to *P. violae* was observed. All oospore concentrations for isolate P4 and all concentrations other than the highest inoculum level (200 oospores g^{-1}) for isolate P10 resulted in less than 10% seedling mortality. Inoculation with 200 oospores g^{-1} of isolate P10 resulted in a mean of 69% seedling mortality (results not shown). Experiment 2 was

set up using *P. violae* isolate P10 to establish if a lower temperature of 10˚C would be more conducive to disease development (compared to the standard temperature of 14˚C). Here, a high level of damping-off was observed at both temperatures for the four highest inoculum concentrations (50, 75, 100 and 200 oospores g^{-1}), with 95, 53, 67 and 95% seedling mortality for 10˚C and 94, 79, 82 and 89% mortality for 14˚C respectively (results not shown). There was no clear difference in seedling mortality between 10˚C and 14˚C.

Given the increase in damping-off in Experiment 2, a repeat of Experiment 1 was set up for both *P. violae* isolates P4 and P10 with a higher number of replicates at the original temperature of 14˚C (Experiment 3). This experiment did result in some damping-off, however mortality was at a lower level than observed in Experiment 2 (< 50% for isolate P10 and <15% for isolate P4 at the highest inoculum level of 200 oospores g^{-1}; Figure 3.8). Despite low levels of damping-off, inspection of the data suggested a dose-response effect, particularly with isolate P10, where the 5 oospores g^{-1} concentration resulted in only 2% seedling mortality, whilst the 200 oospores g^{-1} concentration resulted in 46% seedling mortality (Figure 3.8).

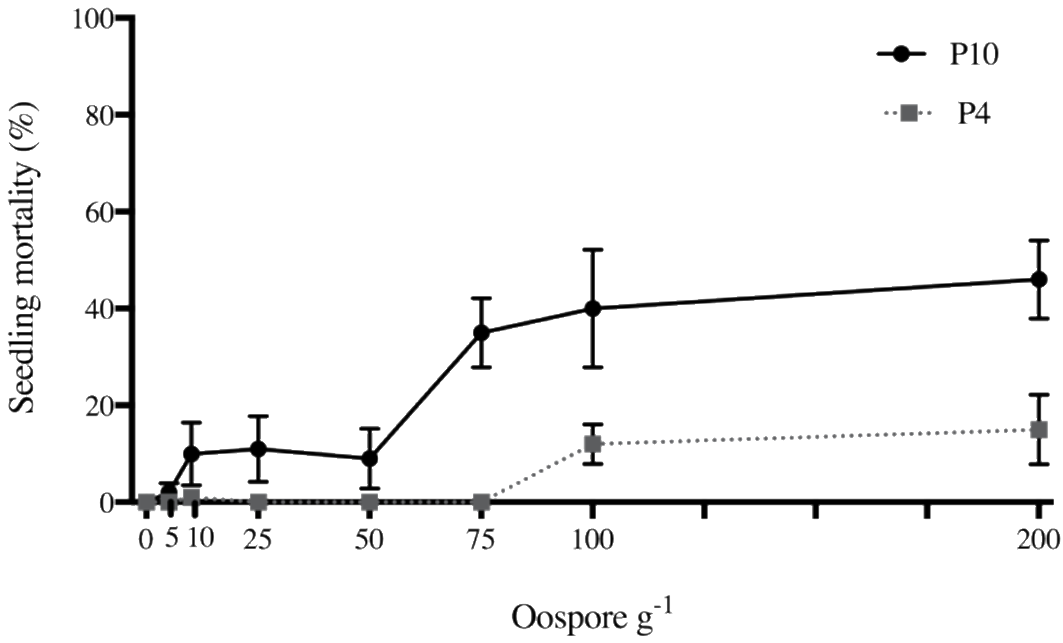


Figure 0.21 Seedling Experiment 3: effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on carrot seedling mortality at 10 w.p.s for isolates P10 (black solid line) and P4 (grey dashed line).

A further experiment was then prepared (Experiment 4), that included a higher inoculum *P. violae* concentration of 300 oospores g^{-1}. However, this experiment resulted in less than 1% damping-off for all oospore concentrations for both isolates P4 and P10 (results not shown).

From the four previous experiments, as the experiment that had produced the highest percentage mortality (Experiment 2) also used the oldest inoculum, it was hypothesised that the age of the *P. violae* inoculum, and hence the maturity of the oospores, may play a significant role in the ability to infect carrot seedlings. Hence Experiment 5 tested three different ages of *P. violae* isolate P10 inoculum on disease development. However, this experiment was terminated early (five w.p.s) due to an unknown factor causing mortality of all seedlings in all treatments, including the uninoculated controls. 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).

Seedling experiments: liquid *P. violae* inoculum (Experiment 6)

The *P. violae* oospore inoculum produced in liquid V8B did not result in any seedling mortality either at sowing or to three week-old seedlings when applied into autoclaved sand. However, when *P. violae* liquid oospore inoculum was applied to non-autoclaved sand, seedling mortality ranged from 27.6-54.2%, with no apparent effect of inoculum concentration. However, seedlings also died in uninoculated control treatments, with percentage mortality ranging between 44.1-51.4%, suggesting that seedling mortality was not due to *P. violae* infection. Similarly, the *P. violae* solid oospore inoculum showed high levels of seedling mortality (60.5-75%) including the uninoculated control treatments.

Objective 3: Test the efficacy of different concentrations of *P. violae* inoculum and a metalaxyl seed treatment on cavity spot symptom development for pot-grown carrots in the glasshouse and in field grown carrots

Introduction

One solution to the problem of variable levels of cavity spot in the field, and the inability to predict sites with high disease pressure, is to artificially inoculate carrots with *P. violae.* This can either be done in pots (in the glasshouse) or in the field to ensure consistent high levels of infection so that the efficacy of new potential control treatments can be reliably discerned and analysed. Ensuring a reliable and consistent level of infection requires development of a dependable source of inoculum, an understanding of infection levels needed to produce disease, and a set of defined environmental conditions known to reliably maximise disease development.

Five mature plant experiments, and two field experiments were carried out, all are detailed in AHDB project FV391a/b.

Discussion

This work aimed to produce *P. violae* inoculum and establish disease in a field-realistic and quantifiable way; i.e. via oospore infection, rather than mycelial infection. Therefore, the method of inoculum production needed to allow development of a large number of oospores with very little concomitant mycelial growth. Production of *P. violae* oospores in this study in the solid sand/oat media was successful and resulted in a large number of oospores: up to 40,000 oospores g^{-1}. Discussion of inouculum production is detailed in FV391a/b.

Experiments under controlled conditions with carrot seedlings aimed to quantify the effect of different *P. violae* oospore concentrations produced in a solid sand/oat substrate on the incidence of damping-off symptoms. Here, it was hypothesised that there would be a dose-response effect such that increased seedling mortality would occur with greater oospore concentrations. However, results across these experiments were highly variable, with damping-off ranging from < 1% to > 90% seedling mortality for the same inoculum concentrations over different experiments. These experiments have therefore proved inconclusive. *Pythium* spp. such as *P. ultimum* are often associated with damping-off, including in carrot (Al-Hamdani *et al.*, 1983), but this symptom has not been commonly associated with *P. violae*. Pettitt *et al.* (2002) was the first to report that *P. violae* could cause carrot seedling mortality, and observed 100% pre-emergence damping-off

when carrot seed was sown in sand amended with two-week old sand/oat solid inoculum as used in this study. However, the number of oospores used in the experiment was not enumerated and subsequently Pettitt (personal communication) stated the importance of establishing a quantifiable link between *P. violae* oospores and disease.

The amount of *P. violae* liquid inoculum required for infection of carrots is also not well established. Pettitt *et al.* (2002) produced *P. violae* oospores in V8B for five weeks, and used a 1 ml suspension of the oospores in water at 3 x 10^6 oospores ml^{-1} to inoculate carrot seedlings sown in sand contained in small modules. This resulted in up to 31% of roots becoming colonised with *P. violae* as measured by plating onto agar media, but no mortality (damping-off) was reported. In this PhD study, seven-week-old *P. violae* inoculum consisting of oospores produced in V8 broth was used in the final seedling experiment. This culture age was used to i) reduce the quantity of viable mycelium and ii) maximise the number of mature oospores (which can be accurately quantified) for infection. In addition, previous experiments indicated that in V8 culture, *P. violae* oospore numbers increase over time up to 10 weeks (Clarkson *et al.*, 2016). As carrot seedlings died across all treatments, including the uninoculated control, in both the liquid inoculum and solid inoculum applied to non-autoclaved sand, it is likely that another pathogen (most likely in the sand), was responsible. Attempts to identify contaminants with plating of seedling roots was unsuccessful, although numerous bacterial species were present. This potential contamination, along with the variable results with the solid sand/oat based inoculum, highlights the difficulties in establishing a robust seedling inoculation system for *P. violae*.

Despite the wide variation in results from the artificial inoculation with carrot seedlings, this study established that seedling mortality occurred mainly at levels > 75 oospores g^{-1}, and the greatest increase in mortality was between 50-75 oospores g^{-1}. Therefore, for the mature plant experiments, a maximum inoculum level of 75 oospores g^{-1} was selected with the expectation that this rate would cause a small amount of damping-off, but allow the majority of plants to survive.

This is the first report of successful artificial inoculation of mature carrot plants grown both in pots in the glasshouse and under field conditions using a *P. violae* solid substrate sand/oat inoculum. In glasshouse experiments, amending a compost/sand growing medium with different levels of this inoculum resulted in some seedling mortality, reduced seedling size, an initial decrease in growth of carrot foliage and the formation of stunted carrot roots with typical cavity spot lesions as well as brown and hairy tap roots. In field experiments, the macrocosms yielded a large number of mature carrot roots, with all inoculated treatments resulting in characteristic cavity spot lesions.

A full discussion of all field and pot experiments is detailed in AHDB final project reports for FV391a/b.

Aim 3: Development of quantitative methods for detection of *P. violae*

Objective 1: Develop a capture method for extracting *P. violae* oospores from soil

Introduction

Detection and isolation of *P. violae* both from soil and carrots has proven challenging (Hiltunen & White, 2002), but the development of diagnostic methods enabling the detection of *Pythium* spp. causing cavity spot in soil could provide essential tools for developing fundamental knowledge of the pathogen. An understanding of the dynamics of *P. violae*, including early infection events, and of how oospore numbers relate to cavity spot development, as well a means of monitoring of *P. violae* survival in both carrot and non-carrot cropped fields, would provide essential information to help reduce economic losses for growers (Klemsdal *et al.*, 2008). Ultimately, if accurate detection and quantification could be achieved, field soil could be analysed to determine the level of *Pythium* spp. causing cavity spot, and potentially provide a risk assessment of cavity spot development for growers (Klemsdal *et al.*, 2008).

There are a number of challenges when using molecular approaches for DNA extraction and PCR, especially for *Pythium* oospore detection in soil. Firstly, soil DNA extraction kits only allow very small amounts of soil to be analysed and can result in the extraction of other organic soil components alongside the target DNA, which may interfere with the PCR process (Tebbe & Vahjen, 1993; Kageyama *et al.*, 2003; Wang & Chang, 2003). In addition, reliable detection of *P. violae* in such a small amount of soil is likely to be variable and may not be representative of the disease potential across an entire field, as the pathogen is known to have a very heterogeneous distribution (Hiltunen & White, 2002). In one study with *P. myriotylum*, Wang and Chang (2003) found that reliable detection was made difficult because of the low density of oospores in the soil. Methods to separate *Pythium* oospores from soil would therefore be beneficial (Wang & Chang, 2003).

Previous attempts to monitor oospores of *Pythium* spp. in soil have also encountered difficulties in estimating densities due to inefficient extraction from soil and the challenges associated with developing reliable sampling protocols (Pavon *et al.*, 2007).

Materials and Methods

Production *of P. violae* oospores

For initial testing approaches to capture *P. violae* oospores, the sand/oat based solid substrate *P. violae* (isolate P10) inoculum was used as previously described in Chapter 3, Section 3.2.1. Inoculum from two 500 ml flasks (500 g) was combined and thoroughly mixed under sterile conditions, divided into approx. 50 g portions, sealed in 50 ml falcon tubes and stored at 18˚C. Samples (50 g) containing *P. violae* oospores for use in experiments were thoroughly mixed, and further subsamples taken for oospore capture and enumeration as described below. To obtain an initial quantification of the number of oospores in each sample, two 1 g portions were manually shaken in 10 ml SDW, vortexed for 1 minute to disperse oospores, and 1 ml pipetted into a 1 ml counting chamber (Sedgewick-Rafter). The total number of *P. violae* oospores were then counted under the microscope at x100 magnification. Three replicate counts for each 1 g sample were carried out.

Development of a *P. violae* oospore capture method

P. violae oospores were captured from the sand/oat inoculum using sucrose flotation. Inoculum samples (10 g) were dispensed into 50 ml sterile falcon tubes and 45 ml saturated sucrose solution (75%) added. Tubes were manually shaken for 2 minutes and centrifuged (Sorvall, UK) at 2190 x g for 5 minutes at 20˚C. The supernatant was successively filtered through 250 μm and 80 μm filters (Cadisch Precision Meshes Ltd., Hertfordshire) using a bottle-top filter unit (Nalgene) to remove large particles, after which the filtrate was passed through a 5 μm filter and washed with 5 ml SDW to capture *P. violae* oospores. The filter was removed from the bottle-top unit, placed in a 30 ml universal tube, agitated for 5 minutes in 5 ml 1% Tween-20 (Merck, USA) and finally rinsed with 1 ml SDW after which the oospores in the resulting suspension were enumerated in a 1 ml counting chamber under light microscopy at x100 magnification. This method was subsequently modified over time, to improve the rate of oospore capture and changes tested included rinsing the filtrate through the filters with 5-25 ml SDW, removing either the 250 µm or 80 µm filter, and agitating 5 μm filters in more concentrated Tween-20 solutions. The method was further refined based on work by Wang and Chang (2003) by introducing a pre-extraction step whereby the oospore samples from the sand/oat solid substrate inoculum were first suspended in SDW and sonicated before adding to the sucrose.

The final protocol used was as follows: *P. violae* sand/oat inoculum samples (10 g) were suspended in 55 ml SDW in a 100 ml conical flask, vortexed for 1 minute, sonicated for 5 minutes (MSE Soniprep 150, UK) after which 600 µl of 1% Tween-20 was added and the suspension

stirred for further 5 minutes. The suspension was added to a saturated sucrose solution (73 ml, 75%) in a 250 ml centrifuge bottle and the conical flask rinsed out with a further 15 ml SDW which was also added to the sucrose. The bottle was inverted several times before centrifugation at 2190 x g for 5 minutes at room temperature (18-20˚C) after which the supernatant was filtered through a 5 µm nylon filter using a bottle-top filter unit to capture the oospores.

To understand the effect of successive sucrose extractions of *P. violae* oospores, samples were retained following a single extraction and further extractions carried out to capture any remaining oospores. This was done by adding SDW (40 ml) to the pellet and vortexing for 1 minute to resuspend the whole sample. Saturated sucrose solution (53 ml) was then added, the bottle inverted to mix, centrifugation again carried out at 2190 x g for 5 minutes and the resulting supernatant filtered through a second 5 µm filter, as previously described. This process 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. To remove oospores, each of the five 5 µm filters resulting from these successive extractions was shaken in a flask shaker (Stuart Scientific Co. Ltd, SF1, UK) for 5 minutes with 5 ml 1% Tween-20, 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 carried out to ensure that all oospores were washed from the filter. The three 5 ml washes from each of the five filters were retained separately, and the number of oospores counted using a 1 ml counting chamber. Three replicate counts were conducted for each filter wash.

Testing efficacy of the *P. violae* oospore capture method

The efficacy of the final refined oospore capture method (Section 4.2.1.2) was tested using five replicate samples of the *P. violae* oospore sand/oat inoculum. The method and quantification were carried out as described above, with each sample extracted five times in saturated sucrose, and for each extraction three washes of the filter were carried out. In addition, to test capture rate when the starting concentration of oospores in the sample was lower, three different starting dilutions of 1 in 10, 1 in 100 and 1 in 1000 (w/w) were made with sand. Again, each sample was extracted five times and for each extraction, three washes of the filter were carried out (as described in Section 4.2.1.2) with oospores enumerated for each wash. Capture efficacy at different stages in the process was calculated for each sucrose extraction/wash as a percentage of a reference count made for each of the five samples using the counting chamber (as described in Section 4.2.1.1). Also, the total number of oospores extracted was calculated by addition of the number of oospores captured for each sucrose extraction.

The efficacy of the oospore capture approach was also tested for soil by adding known concentrations of *P. violae* oospores to a light sandy loam field soil (Wick Series; Sheep Pens West, Wellesbourne, UK). Soil was first passed through a 4 mm sieve then air dried at room temperature (18-20˚C) on a laboratory bench for 48-72 hours. The dried soil was then thoroughly mixed by hand and passed through a 2 mm sieve, before being hand-mixed again and stored in sealed bags at room temperature (18-20˚C) for use the following day. *P. violae* oospores captured from previous samples were used to produce suspensions in SDW of $1x10^2$, $5x10^2$, $1x10^3$, $5x10^3$, $1x10^4$ oospores ml^{-1} with the number of oospores determined in a haemocytometer (Modified Fuchs-Rosenthal, Hawksley, UK). Oospore suspensions were vortexed, and 1 ml aliquots added to replicate 10 g soil samples to obtain final concentrations of 10, 50, 100, 500, 1000 oospores g^{-1}. An uninfested control treatment (no oospores added) was also included. In total therefore, there were five replicate 10 g soil samples for each of the six oospore concentrations which contained a total of 0, 100, 500, 1000, 5000 and 10,000 oospores. For these soil samples, the oospore capture method was adapted to reduce the amount of soil residue trapped on the 5 µm filter. Following washing and centrifugation, during filtration, the final 40 ml (approx.) of supernatant was retained, transferred to a 50 ml falcon tube and centrifuged again at 2190 x g for 2 minutes at 20˚C, which allowed any remaining soil particles to be pelleted. The remaining supernatant was passed through the same 5 µm filter. For further sucrose extractions, the pellet from the 50 ml falcon tube was resuspended in 40 ml SDW and the suspension added to the 250 ml centrifuge bottle before vortexing. The method was repeated, as above, a total of three times. Oospores captured from the filters (as described above) were counted using a 1 ml counting chamber.

Results

Development of a *P. violae* oospore capture method from soil

P. violae oospores were successfully captured from both the *P. violae* sand/oat inoculum and the soil samples spiked with *P. violae* oospores using saturated sucrose extraction and filtration.

Using variations of the initial oospore capture approach (no sonication) for the sand/oat inoculum, less than 50% of the *P. violae* oospores were recovered after two or three re-extractions of the same sample (Table 4.5) compared to the reference count made using a counting chamber. Oospores extracted using both the 250 µm and 80 µm nylon filters to remove larger particles resulted in 25% and 35% capture respectively (Sample 1a and b, Table 4.5), whilst removing use of the 80 µm nylon filter resulted in over 40% capture. (Sample 2a and b, Table 4.5). All variations of the initial oospore capture approach (no sonication) for the sand/oat inoculum showed that the number of *P. violae* oospores released from the filters following washing decreased with

sequential washes (data not shown). The percentage of oospores being captured from the 2nd and 3rd sucrose extractions of the same samples generally decreased compared to the first sucrose extraction (Table 4.5).

Table 0.13 Percentage oospore capture (compared with reference count) from five *P. violae* sand/oat inoculum samples[1].

Sucrose extraction		1	2	3	Total capture (%)
Sample no.	**1a**	14.6	10.2	-	**24.7**
	1b	26.0	9.2	-	**35.2**
	2a	22.2	24.8	-	**46.9**
	2b	20.6	20.0	-	**40.6**
	3	20.5	15.2	9.8	**45.7**

[1] Oospores from Samples 1a and 1b were captured from the initial oospore capture method, using both the 250 µm and 80 µm pre-filters. Samples 2 (a,b) and 3 were captured from the initial oospore capture method without use of the 80 µm pre-filter. 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 and combined to give the total number of oospores captured for each sucrose extraction. The total percentage of oospores captured from each sample over the two/three sucrose extractions is shown in the final column.

Compared to the preliminary results obtained with the initial oospore capture method, the final optimised oospore capture method which involved sonication (Section 4.2.1.2) resulted in higher percentage capture of *P. violae* oospores. The mean number of oospores in the reference count (obtained by shaking 1 g of the sample in 10mls SDW) for the five replicate samples was 24,904 oospores g^{-1}, compared to a mean of 36,250 oospores g^{-1} for the optimised oospore capture method. Therefore, overall oospore capture increased by 45.6% compared to the reference count (Table 4.6).

As for the initial method, the percentage of oospores captured generally declined with subsequent sucrose extractions from the same sample. Approximately 50% of the oospores were captured after the first sucrose extraction, and combined, the first three sucrose extractions from the same sample recovered the majority of oospores from the soil sample with between 84 and 95% of oospores captured (Figure 4.1a). The final two sucrose extractions only captured an additional 11% of oospores (Table 4.6). As with the initial method, following oospore capture using the optimised method, the majority of *P. violae* oospores were washed off after the first of the three filter washes, with the 2nd and 3rd wash showing a considerable decrease in oospores (Figure 4.1b). The first filter wash on average released 88% of the oospores captured (Table 4.6).

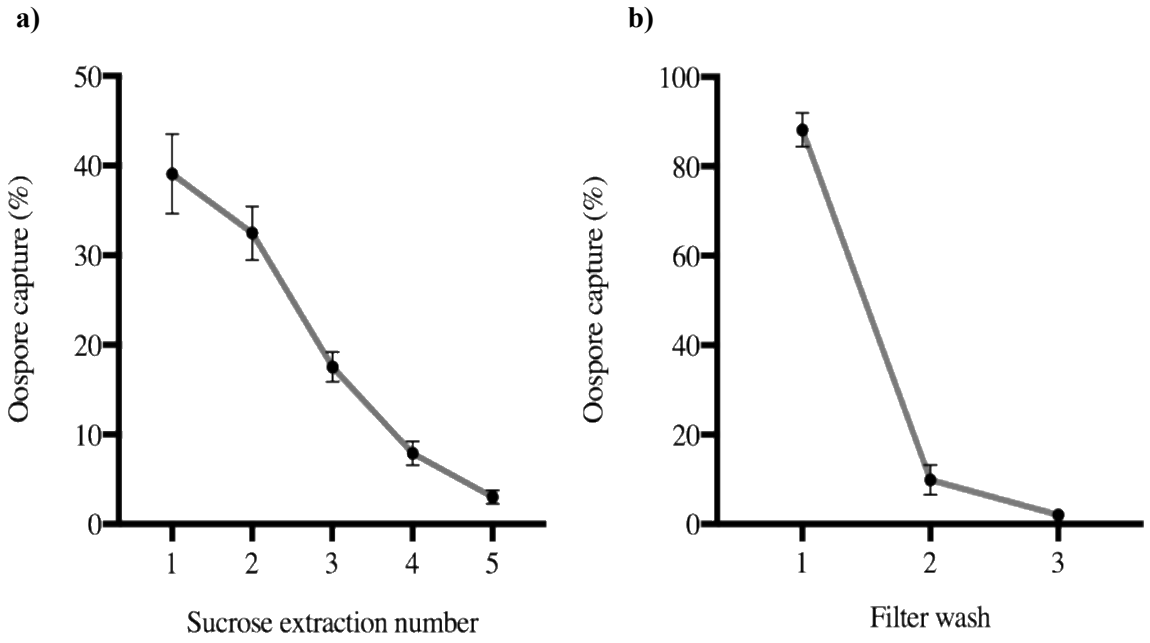


Figure 0.22 Mean percentage *P. violae* oospore capture from five extractions from the same sand sample (a); and mean percentage capture for each filter wash as a percentage of oospores captured in total in the sucrose extraction (mean of the five sucrose extractions) (b). Data represents mean ± SEM; N=5.

Table 0.14 Number of oospores captured from five *P. violae* sand/oat inoculum samples.

Sucrose extraction[1]		**1**	**2**	**3**	**4**	**5**	**Total capture**
Sample no.	**1**	14118.7	11712.8	7054.5	4773.9	1559.4	**39219.3**
	2	13583.7	11412.4	6713.0	3193.0	1592.0	**36494.0**
	3	11430.5	16181.3	6576.5	2034.0	631.5	**36853.8**
	4	17061.7	8095.8	3514.9	1414.8	201.8	**30289.0**
	5	13394.4	11724.5	8412.9	3218.2	1642.7	**38392.6**

[1]Oospores from each sample were captured from five sucrose extractions of the same sample and each filter was washed three times and combined to give the total number of oospores captured for each sucrose extraction. The total number of oospores captured from each sample over the five sucrose extractions is shown in the final column.

When the final optimised method for capture of *P. violae* oospores was used to quantify oospores in different dilutions of the sand/oat inoculum (36-3625 oospores g^{-1}), fewer oospores as a percentage of the reference count were recovered (Table 4.7) compared to the original samples which contained a much greater number of oospores (mean 36,250 oospores g^{-1}, Table 4.6). Capture as a percentage of the starting number of oospores decreased as the concentration of oospores in the sample decreased, with 72%, 61% and 53% capture for 1:10 (3625 oospores g^{-1}), 1:100 (362 oospores g^{-1}) and 1:1000 (36 oospores g^{-1}) dilutions respectively (Table 4.7). Again, the percentage of oospores captured decreased with successive sucrose extractions.

Table 0.15 Percentage of oospores captured from three *P. violae* sand/oat dilution inoculum samples compared to starting count of 36250 oospores g^{-1}.

Sucrose extraction[1]		**1**	**2**	**3**	**4**	**5**	**Total capture % (oospores g^{-1})**
Sample (oospores g^{-1})	**3625**	35.0	19.9	9.1	6.4	1.9	**72.3**
	365.2	25.5	16.0	8.8	4.3	6.6	**61.2**
	36.52	23.3	12.9	7.7	7.7	1.5	**53.1**

[1]Oospores from each sample were captured from five sucrose extractions of the same sand sample and each filter was washed three times and combined to give the total number of spores captured for each sucrose extraction. The total percentage of oospores captured from each sample over the five sucrose extractions is shown in the final column.

When the final optimised method for capture of *P. violae* oospores was used to quantify oospores in the spiked soil samples, a similar pattern to the sand/oat inoculum samples was observed with respect to the sucrose extractions, with the percentage of oospores captured decreasing with the 2nd and 3rd sucrose extraction (Figure 4.2). The total number of oospores captured increased as the concentration of oospores in the spiked samples increased. In the 10 oospores g^{-1} sample, a mean of 59 oospores were captured, and with the 1000 oospore g^{-1}, a mean of 1258 oospores were captured. However, in contrast to the results with the sand/oat inoculum dilution samples, the percentage of oospores captured decreased as the concentration of oospores in the original sample increased (Figure 4.2). Nearly 60% of oospores were captured from the 10 oospores g^{-1} sample, whilst only 11% and 12% of oospores were captured from the 500 and 1000 oospores g^{-1} samples respectively (Figure 4.2). This equates to only 1258 oospores being captured from the 1000 oospore g^{-1} concentration, which actually contained a total of 10,000 oospores (Figure 4.2).

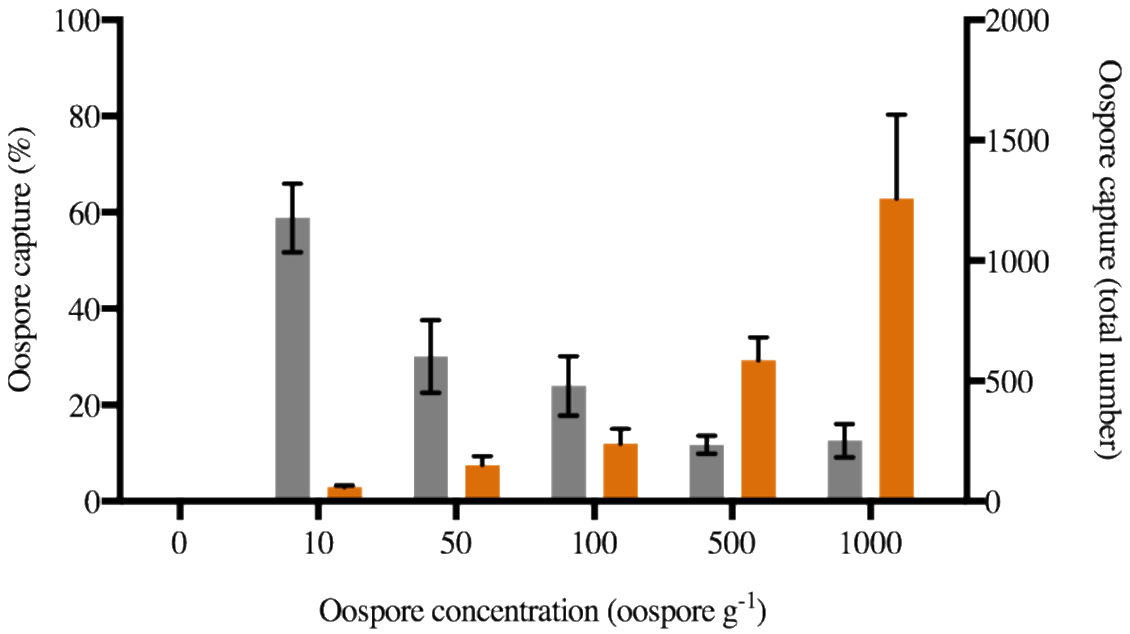


Figure 0.23 The percentage of *P. violae* oospores captured (grey bars, left y axis) and total number of *P. violae* oospores captured (orange bars, right y axis) from soil spiked with *P. violae* oospores at a range of concentrations. Data represents mean ± SEM.

Objective 2: Develop a DNA extraction method for *P. violae* oospores

Introduction

Traditional methods of enumerating and identifying *Pythium* spp. to study dynamics have included dilution plating onto selective media and morphological identification (Schroeder *et al.*, 2006). A range of selective media have been developed and assessed (Ali-Shtayeh *et al.*, 1986), but all contain a mix of antibiotics and/or fungicides to selectively inhibit bacteria and fungi without inhibiting *Pythium* growth (Schroder *et al*., 2006). However, isolation and conventional identification are time-consuming and prone to error, and misidentification of *Pythium* spp. can be common even by experienced scientists (Wang & White, 1997; Schroeder *et al.*, 2006; Pavon *et al.*, 2007). Furthermore, the very low germination rate and dormancy of *P. violae* oospores, as well as the slow growth of *P. violae* mycelium means that conventional soil dilution plating is unsuitable for isolating and quantifying *P. violae* as it can be outgrown by other *Pythium* spp. (Wang & Chang, 2003; Schroeder *et al.*, 2006; Li *et al.*, 2010). The development of serological techniques in the 1990's allowed more sensitive detection of plant pathogens in diseased plants (Lyons & White, 1992). These techniques rely on specific polyclonal or monoclonal antibodies to detect their respective antigens in the test sample (Goulter & Randles, 1997). The presence or absence of this antibody can then be detected via the use of an enzyme that results in a substrate colour change. An enzyme-linked immunosorbent assay (ELISA) is highly sensitive, does not require identification expertise, and is also useful for detecting organisms in mixed populations (Lyons & White, 1992). ELISA assays have been developed for detection of *P. violae* and *P. sulcatum* in cavity spot lesions (Lyons & White, 1992) but were only successful in identifying *P. violae* from cavity spot lesions where *P. violae* had already been detected via plating (Fang & Ramasamy, 2015). Furthermore, the antibodies cross-reacted with *P. sylvaticum* and *P. intermedium*, both of which are associated with cavity spot (Lyons & White, 1992). Using DNA-based molecular detection techniques is a promising alternative approach for identification and quantification of *Pythium* spp. in plant material and also for detecting oospores in soil (Wang & Chang, 2003).

Molecular techniques based on DNA amplification through PCR can achieve much more sensitive and rapid detection of *Pythium* spp. and other oomycetes even when the pathogen cannot be isolated, or the sample is asymptomatic (Kageyama *et al.*, 2003; Bilodeau *et al.*, 2007). In one study with *P. myriotylum*, Wang and Chang (2003) found that reliable detection was made difficult not only because of the low density of oospores in the soil, but also because of incomplete lysis due to the thick oospore walls (up to 2 µm). *P. violae* oospore walls are up to 3 µm thick (van der

Plaats-Niterink, 1981) so ensuring adequate lysis is an important step that may have hindered detection previously (Kageyama *et al.*, 2003).

Materials and Methods

Lysis testing

The efficacy of different lysis approaches was tested for *P. violae* oospores extracted using the initial oospore capture method described above (Section 4.2.1.2) before the addition of a sonication step. Two experiments were conducted to optimise oospore lysis: the first examined the effect of different mechanical lysis techniques, and the second examined the effect of different bead types. *P. violae* oospore suspensions (200 µl) containing approximately 20,000 oospores ml^{-1} were prepared and placed inside a 2 ml screw-cap tube (STARLAB, Germany). For Experiment 1, the efficacy of three mechanical lysis approaches for breaking up oospores was tested; the first approach (Tests 2-5, Table 4.1) used a Fast Prep® machine, the second approach used manual grinding, and the third used vortexing, with conditions as described in Table 4.1.

Table 0.16 Lysis conditions tested for Experiment 1 and 2

Test number	Experiment 1: mechanical lysis	Experiment 2: bead testing
1	Control (no lysis step)	Control (no lysis step)
2	Fast Prep (40 s, 5.5) with garnet matrix	Large glass beads (2.5-3.5 mm) and silica beads (0.1 mm)
3	Fast Prep (30 s, 5.5) with garnet matrix, followed by 10 min incubation at 65°C	Small glass beads and silica beads (0.1 mm)
4	Fast Prep (30 s, 5.5) with garnet matrix x2	Silica beads (0.1 mm) and molecular sand
5	Fast Prep (30 s, 5.5) with garnet matrix x3	Ceramic beating bead (6.35 mm) and silica beads (0.1 mm)
6	Molecular sand and micro-pestle (30 s manual grinding)	
7	Lysis buffer (from DNA extraction kit) and horizontal vortex (5 min, full speed)	

After each process, the tube contents were checked using light microscopy under 100x magnification for the presence of whole oospores. Three replicate counts of oospores were performed using a haemocytometer (Modified Fuchs-Rosenthal) and a final mean oospore count for each lysis condition calculated. The garnet lysing matrix provided for use with the Fast Prep® machine (Lysing matrix A, MP Bio, Germany) and used in Experiment 1 is costly, and therefore a less expensive alternative was sought. For Experiment 2, the most effective mechanical lysis

method from Experiment 1 was chosen and the effect of different bead types and sizes tested (Table 4.1). The number of intact oospores was calculated as described above for Experiment 1.

DNA extraction

DNA extraction was tested using extracted *P. violae* oospores (isolate P10; as washed from 5 µl filters) using a DNeasy Plant Mini Kit (Qiagen, UK). Initially, a 200 µl oospore suspension at a concentration of approx. 20,000 oospores ml^{-1} in SDW was used for DNA extraction. Subsequently, in order to define the sensitivity of the DNA extraction process for detection, different total amounts ranging from 50-5000 oospores were prepared in 200 µl SDW and transferred into 2 ml screw-cap tubes. To further test PCR sensitivity, lower oospore concentrations of 1-50 oospores were prepared in 200 µl SDW. Oospores were counted manually using a pipette under a light microscope (x100 magnification) and transferred into 2 mltubes.

DNA extraction was carried out in accordance with the manufacturer's instructions but subsequently amendments were made, such that the final protocol consisted of the following modifications: lysis as optimised involved shaking oospore suspensions three times in a Fast Prep® machine at speed setting 5.5 for 30 seconds using a ceramic bead (6.35 mm) and silica mix, with 0.005 g polyvinylpolypyrrolidone (PVPP; Sigma-Aldrich, UK) and 100 µl extra lysing buffer (500 µl total) added prior to shaking. For each component added throughout the DNeasy protocol, 1.5x the volume was added to take into account the extra liquid from the oospores in the starting material. DNA was eluted in 30 µl of elution buffer and incubated for 5 minutes at 60˚C. This final step was repeated using the eluate and DNA was stored at -20˚C. Six replicate DNA extractions were performed on the oospore dilution series (1-5000 oospores) and PCR performed using PviolF/R (primer pair 1, Table 4.2) designed by Klemsdal *et al.* (2008). PCRs were set up using REDTaq^{VR} ReadyMix^{VR} (Sigma-Aldrich, Gillingham, Dorset, UK) in 20 µl volumes containing 1 µl of primers (10 µM), 10 µl of RedTaq (Sigma) and 1 µl of DNA. Thermocycling conditions are described in Table 4.2. PCR products (4 µl) were run on a 1.2% agarose electrophoresis gel to assess amplification.

Results

Development of a DNA extraction protocol for *P. violae* oospores

Lysis testing

The different lysis methods tested all resulted in a reduction of visible whole *P. violae* oospores compared to a control treatment with no lysis. For Experiment 1, the Fast Prep® conditions

consistently resulted in better lysis than either vortexing or manual grinding (Table 4.8). When the contents of the Fast Prep® lysed tubes were observed under the microscope, a mean of between 0.5 and 7 oospores were left intact across the varying treatments compared to between

Table 0.17 The mean oospore count for lysis conditions tested for Experiment 1 and 2.

Test number	**Experiment 1: mechanical lysis**	**Mean oospore count**
1	Control (no lysis step)	37
2	Fast Prep (40 seconds, 5.5) with garnet matrix	5
3	Fast Prep (30 seconds, 5.5) with garnet matrix, followed by 10 minute incubation at 65˚C	7
4	Fast Prep (30 seconds, 5.5) with garnet matrix x2	3
5	Fast Prep (30 seconds, 5.5) with garnet matrix x3	0.5
6	Molecular sand and micro-pestle (30 seconds manual grinding)	8
7	Lysis buffer (from DNA extraction kit) and horizontal vortex (5 minutes, full speed)	15.5
	Experiment 2: bead testing	**Mean oospore count**
1	Control (no lysis step)	31
2	Large glass beads (2.5-3.5mm) and silica beads (0.1 mm)	8
3	Small glass beads and silica beads (0.1 mm)	8.5
4	Silica beads (0.1 mm) and molecular sand	Liquid cloudy: oospores not visible
5	Ceramic beating bead (6.35 mm) and silica beads (0.1 mm)	0

8 and 15.5 intact oospores for the vortex and manual grinding conditions (Table 4.8). Furthermore, the number of whole oospores still visible after lysis decreased with increasing number of successive shakes on the Fast Prep® machine. After three Fast Prep® treatments, less than one oospore was left intact over three replicate counts (Table 4.8). The addition of a heated incubation step after one Fast Prep® shake did not increase lysis (Table 4.8). Following Experiment 1, the treatment which resulted in fewest intact oospores was three Fast Prep® machine treatments at 5.5 for 30 seconds, and hence this treatment was used in Experiment 2 which examined different beads.

Both small and large glass beads, as well as the silica beads tested, resulted in less lysis of *P. violae* oospores than the garnet matrix, with a mean of 8 and 8.5 oospores still remaining intact (Table 4.8). The use of silica beads and molecular sand resulted in the mixture being too cloudy to view the oospores. Both of these bead types consist of very fine particles, meaning the length of time taken for the particles to settle was too long. The use of a large ceramic beating bead alongside the silica beads left no whole oospores visible after three treatments with the Fast Prep® machine (Table 4.8). The large ceramic beating beads were also re-useable and provided complete lysis of all oospores. This bead matrix, alongside three treatments on the Fast Prep® machine, was therefore used for all subsequent DNA extractions.

DNA extraction

Effective DNA extraction from *P. violae* oospores was measured by successful PCR amplification with primer pair PviolF/R (primer pair 1, Table 4.2) designed by Klemsdal *et al.* (2008). Initially, 200 µl samples of approx. 20,000 oospores were used for DNA extraction, and amendments made until clear and consistent amplification in PCR was achieved (results not shown). Following this testing, the oospore dilution series (Section 4.2.2.2) was used to test sensitivity. The amplification of DNA from oospores using this primer pair resulted in a strong PCR product of the expected fragment size (Figure 4.3). The six replicate DNA extractions and subsequent PCR reactions confirmed consistent results, with similar detection seen across the six replicates. Band brightness decreased with decreasing numbers of oospores, but amplification was possible from 5000 oospores down to 1 oospore. However, detection of DNA from below 10 oospores was variable, with very weak or sometimes no amplification (Figure 4.3).

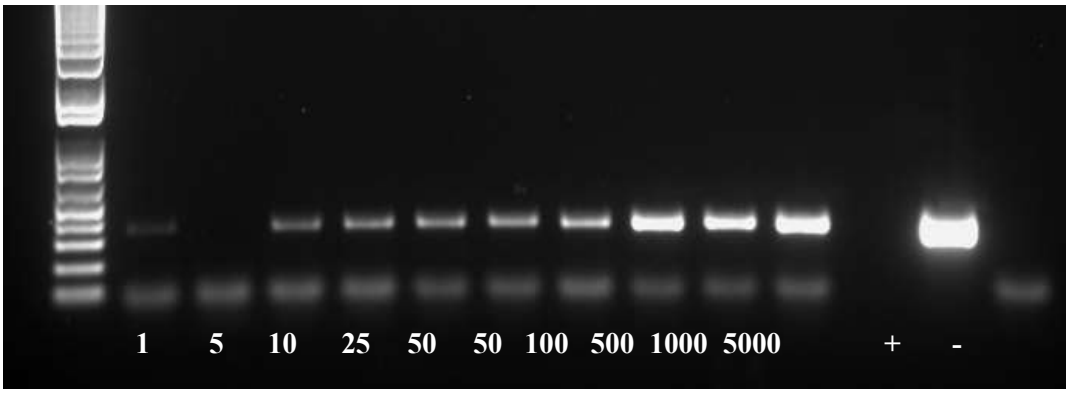


Figure 0.24 Representative electrophoresis gel showing sensitivity of *P. violae* DNA detection following optimised DNA extraction protocol using primer pair 1 (Klemsdal et al., 2008). Gel shows PCR amplicons following amplification of DNA extracted from 1-5000 *P. violae* oospores; + = positive control (*P. violae* DNA at 10ng μl^{-1}), - = negative control (water). A 1 Kb ladder was used as the molecular marker.

Objective 3: Develop a robust, accurate and sensitive qPCR assay for *P. violae*.

Introduction

Many of the molecular methods designed for detection of *Pythium* spp. have not been quantitative (Schroeder et al., 2006). Quantitative PCR (qPCR) has been widely used in plant pathology for DNA quantification and diagnosis of plant pathogens (Schaad & Frederick, 2002; Fang & Ramasamy, 2015). qPCR quantifies DNA using a florescent dye or a probe to detect the product, allowing the initial concentration of target DNA to be calculated (Schroeder et al., 2006; Kageyama, 2014). SYBR Green based assays are often used due to their low cost, and a simple requirement to design two specific primers to amplify DNA. Taqman assays, although more expensive, may allow greater specificity and sensitivity (Tajadini et al., 2014) as they involve the design of an additional probe to the target DNA.

Previous methods to detect *Pythium* spp. causing cavity spot based on polyclonal antibodies and ELISA assays (Lyons & White, 1992; White *et al.*, 1994; Kageyama *et al.*, 2002) have not been specific enough or provided adequately sensitive detection prior to cavity spot development (Klemsdal *et al.*, 2008). In 2008, specific PCR primers targeting the ITS regions of the rDNA for five *Pythium* spp. associated with cavity spot in Norway, including *P. violae*, were developed (Klemsdal *et al.*, 2008). This process allowed sensitive detection of *P. intermedium* and *P. vipa*, but the sensitivity of *P. violae* detection was poor, with no detection below 50 pg μl^{-1} of DNA (Klemsdal *et al.*, 2008). ITS regions are particularly useful for distinguishing *Pythium* spp. as they are conserved within a species but variable between species (Matsumoto *et al.*, 1999; Levesque & de Cock, 2004; Schroeder *et al.*, 2006). Furthermore, ITS regions have a high copy number, allowing a greater sensitivity of detection to be achieved (Kageyama *et al.*, 1997; Vincelli & Tisserat, 2008; Li *et al.*, 2010).

The Barbara qPCR test used the same primers as those designed by Klemsdal *et al.* (2008) for conventional PCR, and testing by Andrew Taylor (Warwick Crop Centre, unpublished, data not shown) revealed that the amplicon at 352 bp was too long. This resulted in the formation of secondary structures and inadequate selectivity and sensitivity, meaning a new alternative qPCR assay is required.

Materials and Methods

Development of *P. violae* specific primers for quantitative PCR

A number of primer sets were tested for *P. violae* specificity against different *Pythium* spp. (Table 2.6) comprising *P. violae* (P2, P17), *P. ultimum* (P174), *P. lutarium* (P173), *P. irregulare* (P26), *P. sylvaticum* (P24), *P. intermedium* (P5) and *P. sulcatum* (P178). DNA was extracted from cultures of each *Pythium* spp., and diluted to 10 ng μl^{-1} for use in PCR reactions. Primer pairs 2-7 were designed manually based on several different target gene sequences which were aligned for a range of *Pythium* spp. using the ClustalW package (Thompson *et al.*, 1994) in MEGA v7 (Kumar *et al.*, 2016). Primers were designed around regions of dissimilarity and were tested for primer dimers (Oligo-analysis tool, Eurofins Genomics) and folding/secondary structure formation (M-fold Web Server, University St Albany). Primers with predicted self/pair dimers and those predicted to result in significant secondary structures were excluded. Primers 2-6 were designed to be used with qPCR SYBR-Green assays, primer pair 7 was designed for use with a qPCR Taqman assay.

Primers targeting ITS regions of the rDNA

Primer pair 1

The first pair tested (primer pair 1, Table 4.2) was specifically designed for *P. violae* by Klemsdal *et al*. (2008). PCRs were set up using REDTaq^{VR} ReadyMix^{VR} (Sigma-Aldrich, Gillingham, Dorset, UK) in 20 µl volumes containing 1 µl of primers (10 µM), 10 µl of RedTaq (Sigma) and 1 µl of DNA. Thermocycling conditions are described in Table 4.2 but different numbers of PCR cycles were tested (25, 30, 35, and 40 cycles). PCR products (4 µl) were run on 1.2% agarose electrophoresis gel to assess amplification.

Primer pairs 2 and 3

Primer pairs 2 and 3, which also targeted the ITS regions (Table 4.2), were designed as described above and tested using conventional PCR. Annealing temperatures, times and cycle numbers were adjusted to improve specificity.

Table 0.18 Primer pair number, target DNA, product size, thermocycling conditions, primer name, sequences and origin of primers used in this study and for the development of a *P. violae* quantitative PCR. WCC: Warwick Crop Centre

Primer pair no.	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 no.	Primer name	Primer sequence	Reference
1	ITS1	352	93 2 mins	93 1 min	61 1 min	72 1 min	72 10 mins	40	PviolF / PviolR	ATGTGTGTGTGCGGGACT CCACTCCCCAAAGAGAGAAGT	(Klemsdal *et al.*, 2008)
2	ITS1	81	94 5 mins	94 1 min	60 1 min	72 30 secs	72 10 mins	35	PviolNEWF / PviolNEWR	TGGTGTTTTCGGACGCTGCGC T TCCCGCACACACACATTGCTG	A. Taylor, unpublished, WCC
3	ITS1	76	93 2 mins	93 1 min	62 1 min	72 1 min	72 10 mins	35	PVIOL KH FOR / PVIOL KH REV	GGTGTTTTCGGACGCTGCG AGTCCCGCACACACACAATT	K. Hales, unpublished, WCC
4	OCM1	126	94 5 mins	94 45 secs	55 30 secs	72 30 secs	72 5 mins	30	PviolOCM1 918F / PviolOCM1 918R	GACCATCCCTATCCCCTA ACCGTCGTCCCACCGTG	K. Hales, unpublished, WCC
5	OCM1	100	94 2 mins	94 1 min	62 30 secs	72 30 secs	72 5 mins	30	PviolOCM1 1128F / PviolOCM1 1128R	CGGTGTTGGGGACAGTGACC CCGGCAAGCCAGTGACGGTA	K. Hales, unpublished, WCC
6	Cellulose Synthase	199	94 5 mins	94 1 min	65 10 secs	72 30 secs	72 5 mins	30	CSViol FOR / CSViol REV	CGAATGCGCGTGTACTGACAT CGAGCAGCAAGAGCGGTCCCA	A. Taylor, unpublished, WCC
7	ITS1	81	95 3 mins	95 5 secs	67 30 secs	-	40 30 secs	45	AT_FOR1 / AT_REV1 (Probe)	TGGTGTTTCGGACGCTGCGCT G TCCCGCACACACACATTGCTG (6FAM)CGGAGGAGGAACGAAG GTTGGTCTTGT(TAMRA)	A. Taylor, unpublished, WCC

Taqman qPCR targeting ITS regions of the rDNA

Due to the lack of specificity or sensitivity of primer pairs 1-6 for *P. violae* detection, further primers (primer pair 7; Table 4.2), targeting the ITS regions were designed and tested against DNA from the different *Pythium* spp. with a probe for use in a Taqman qPCR assay. Primer pair 7 was initially tested with conventional PCR at a range of annealing temperatures/cycle numbers to increase specificity.

Following robust amplification with conventional PCR, the Taqman qPCR was tested as follows. A 10-fold dilution series of *P. violae* DNA (isolate P10) was made from 1 ng μl^{-1} to 0.1 fg μl^{-1}. Reactions were set up in triplicate using a qPCRBIO Probe Mix Lo-ROX (PCR Biosystems) and a total reaction volume of 10 µl was made up as in Table 4.3 with primers diluted to 4 µM. The samples were loaded into a 384-microwell plate and the qPCR reaction run on a LightCycler® 480 Real-Time PCR system (Roche). Thermocycling conditions consisted of initial denaturation at 95˚C for 3 minutes followed by 45 cycles of denaturation at 95˚C for 5 seconds with annealing and extension at 67˚C for 30 seconds. DNA quantification occurred during the 45 cycles of denaturation, annealing and extension, and values extrapolated using a standard curve method. Initially, different primer concentrations were tested and 0.2 µM was found to be optimum (data not shown). The same primer pair was tested using a Taqman probe: 5' (6FAM)CGGAGGAGGAACGAAGGTTGGTCTTGT(TAMRA)-3'. Taqman qPCR was also tested against *P. lutarium, P. ultimum, P. attrantheridium, P. irregulare, P. cryptoirregulare, P. intermedium, P. debaryanum, P. sulcatum* and *P. sylvaticum* with all these non-target DNA samples diluted to 10 ng μl^{-1}.

Table 0.19 Reagents for qPCR Taqman reaction with primer pair 7

Reagent (concentration)	**µl/10 µl reaction**
	Conc. A (0.2 µM)
Probe mix (x2)	5
Forward primer (10 µM)	0.5
Reverse Primer (10 µM)	0.5
Probe (5 µM)	1
DNA	1
Water	2

The Taqman qPCR assay was also tested for sensitivity. Six independent replications of a dilution series of oospores ranging from 1-5000 oospores were extracted via the optimised DNA extraction method as described in Section 4.2.2.2, and the DNA used with the Taqman qPCR

assay. The reaction was set up as described above and all thermocycling conditions were the same.

The Taqman qPCR assay was also tested using DNA from soil samples from AHDB project FV405 (Clarkson, 2014), as described above (Section 4.2.3.2). As part of this study, with the aid of Andrew Taylor (Warwick Crop Centre) the correlation between quantity of DNA detected with primer pair 7 using qPCR, and the band brightness score from the conventional PCR primers (Klemsdal *et al.*, 2008), was calculated in GraphPad Prism (Pearson correlation; Version 7.0c). The correlation between DNA quantity and number of carrots with cavity spot (Clarkson, 2014) was also calculated.

Assessing *P. violae* DNA concentrations in soil using oospore capture and Taqman qPCR

Following the development and optimisation of *P. violae* oospore capture, DNA extraction and Taqman qPCR, the utility of this combined approach was evaluated firstly using soil samples spiked with oospores and secondly using field soil samples collected at different time points in a carrot field where severe cavity spot developed.

Quantifying *P. violae* oospores in spiked soil

Samples of soil (100 g) taken from a field with no history of carrot production (Wick Series, Wellesbourne), air dried (48-72 hours) at room temperature (18-20˚C), then mixed and passed through a 4 mm sieve. *P. violae* oospore suspensions were prepared as described in Section 4.2.2.2, and added to soil samples in 10 ml of SDW to achieve final concentrations of 10, 100 and 1000 oospores g^{-1}. Each sample was thoroughly mixed and left to air dry for 48-72 hours at room temperature (18-20˚C). The dried soil was passed through a 2 mm sieve and samples mixed again before dividing into 10 g portions and storing at -20˚C. Two replicate 10 g samples of soil for each oospore concentration, as well as two non-spiked soil samples were processed through the optimised oospore capture method described in Section 4.2.1. Following capture of oospores on the 5 µm filter and washing, oospore suspensions were stored at -20˚C until used. After thawing, oospores were concentrated into 200 µl SDW, DNA extraction was performed, as outlined in Section 4.2.2.2, and DNA stored at -20˚C. For comparison, DNA from three 0.5 g samples of the spiked soil for each oospore concentration and a non-spiked control were extracted using a soil DNA extraction kit (GeneAll Exgene soil SV mini, Korea) in accordance with the manufacturer's instructions and diluted 1 in 5 before storing at -20˚C. The optimised lysis approach was not used with this soil extraction kit, as the standard lysis protocol was followed. The Taqman-based qPCR assay with primer pair 7 (as developed in Section 4.2.3) was used to quantify *P. violae* DNA.

Results

Primers targeting ITS regions of the rDNA

Primer pair 1

The ITS region of the rDNA of *P. violae* isolate (P2) was successfully amplified by PCR using primer pair 1 for all numbers of cycles tested. However, at 30 cycles, amplification was also seen for DNA from *P. intermedium* and at 35 and 40 cycles for DNA from both *P. sylvaticum* and *P. irregulare* (Figure 4.4). This primer pair therefore was not considered fully specific for *P. violae*.

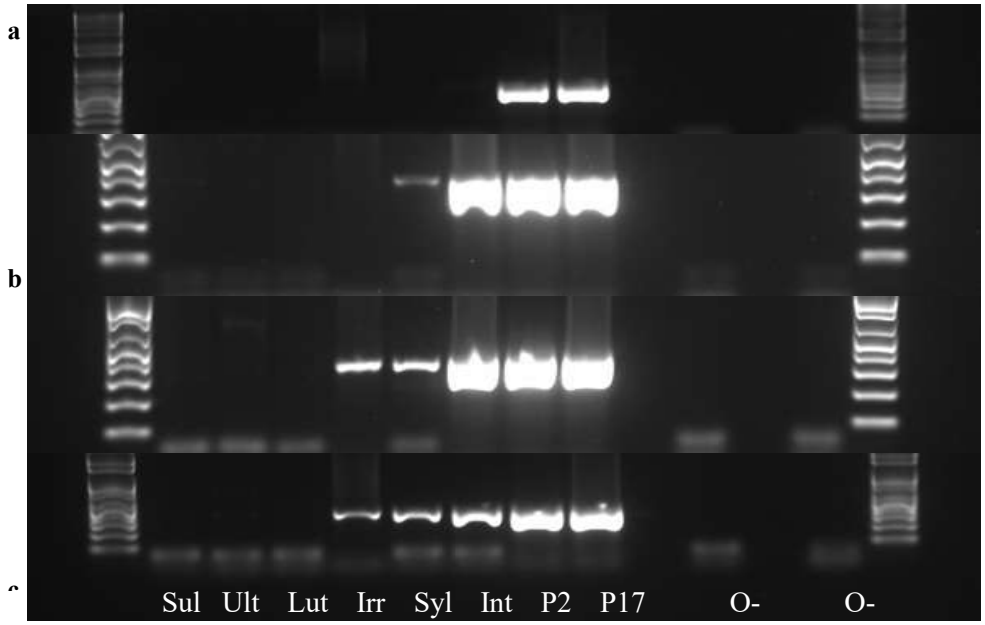


Figure 0.25 Electrophoresis gel showing amplification of *Pythium* spp. DNA following DNA extraction using primer pair 1 (Klemsdal *et al.*, 2008). Testing was carried out at 25 (a), 30 (b), 35 (c) and 40 (d) cycles. Sul = *P. sulcatum*, Ult = *P. ultimum,* Lut = *P. lutarium,* Irr = *P. irregulare,* Syl = *P. sylvaticum, Int* = *P. intermedium*, P2 and P17 = *P. violae,* O- = negative DNA control (*Fusarium* DNA at 10 ng μl^{-1}), O- - = negative control (water). A 1 Kb ladder was used as the molecular marker.

Primer pairs 2 and 3

Primer pairs 2 and 3 were designed based on the ITS region of the rDNA gene for *P. violae*. *P. violae* isolates (P2 and P17) were successfully amplified by PCR using both primer pair 2 and primer pair 3 (Table 4.9). However, the PCR reaction also resulted in amplification of DNA from non-target *Pythium* spp. (Table 4.9). After various adjustments, it was not possible to reduce amplification of *P. irregulare*, *P. sylvaticum* or *P. intermedium* whilst maintaining good amplification of *P. violae* (Table 4.9).

Table 0.20 PCR amplification of DNA from a range of *Pythium* spp. isolates when tested against seven primer pairs developed for specificity to *P. violae*. Black squares indicate a bright band (strong amplification), grey squares indicate a faint band (weak amplification) and white squares indicate no band (no amplification). Grey diamonds indicate that species was not used as part of testing. The *Pythium* spp. tested were: *P. violae* (Viol; x2 isolates), *P. sulcatum* (Sul), *P. ultimum* (Ult), *P. lutarium* (Lut), *P. irregulare* (Irr), *P. sylvaticum* (Syl). Primer pair 7 was also tested against *P. attrantheridium* (Att), *P. cryptoirregulare* (Cry) and *P. debaryanum* (Deb).

Primer pair no.	**Primer code**	***Pythium* species**										
		P. Vio		***P.sul***	***P.ult***	***P.lut***	***P.irr***	***P.int***	***P.syl***	***P.att***	***P.cry***	***P.deb***
		P2	**P17**									
1	PviolF / PviolR	■	■				▒	▓	▒	◇	◇	◇
2	PviolNEWF / PviolNEWR	■	■				▒	▒	▒	◇	◇	◇
3	PVIOL KH FOR / PVIOL KH REV	■	■	▓	▒		▓	▓	▒	◇	◇	◇
4	PviolOCM1 918F / PviolOCM1 918R	■	■					▒		◇	◇	◇
5	PviolOCM1 1128F / PviolOCM1 1128R	■	■				▒	▒		◇	◇	◇
6	CSViol FOR / CSViol REV	■	■					▒		◇	◇	◇
7	AT_ITS FOR1 / AT_ITS REV1	■	■				▒	▒			▒	

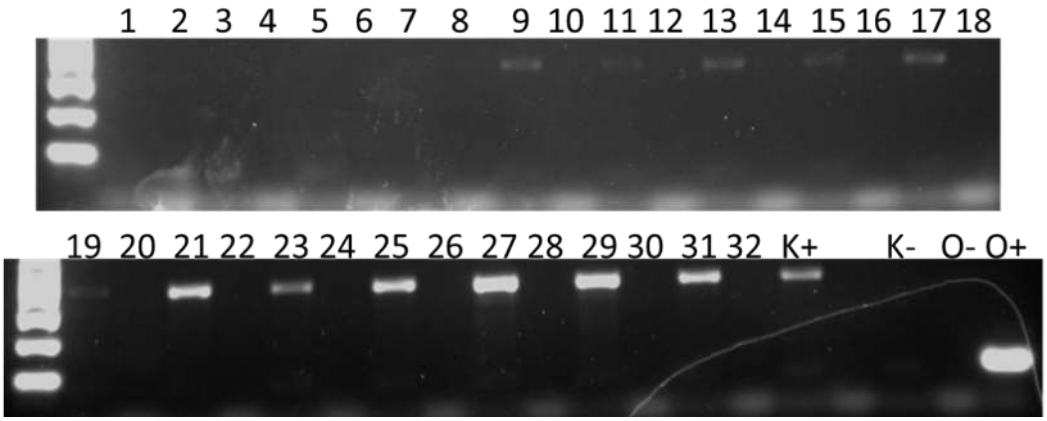


Figure 0.26 Comparison of primer pair 5 (OCM1 1128F/R) and primer pair 1 (PviolF/R) for detection of *P. violae* in DNA extracted from soil samples collected from fields with cavity spot affected carrots. PCR products from DNA extraction of each soil sample were run in adjacent wells numbered 1-32, e.g. 1 & 2 are the same soil sample, tested with each primer). Odd numbers are PCR products amplified by primer pair 1 and even numbers by primer pair 5. K+ = positive *P. violae* DNA control; K- = negative control (water) for primer pair 1; O+ = positive *P. violae* DNA control; O- = negative control (water) for primer pair 5. A 1 Kb ladder was used as the molecular marker.

Taqman qPCR targeting ITS regions of the rDNA

All the previous assays described above (using primer pairs 2-6) were not specific or sensitive enough to detect *P. violae* at low concentrations found in soil samples. The assays had been designed to be potentially used as qPCR SYBR green assays, so the potential of a Taqman probe-based assay (based on the ITS region) was also investigated.

Primer pair 7, targeting the ITS region of the rDNA gene, was designed, as well as a probe for the product. Initially, primer pair 7 was tested with conventional PCR, and found to be very specific to *P. violae*, with strong amplification of DNA extracted from the two *P. violae* isolates tested (Table 4.9). Following further testing using DNA from a wider range of *Pythium* spp., no amplification was observed for isolates of *P. sulcatum*, *P. ultimum*, *P. lutarium*, *P. sylvaticum*, *P. attrantheridium* or *P. debaryanum*. However, some amplification occurred for three other *Pythium* spp. tested (*P. intermedium*, *P. irregulare* and *P. cryptoirregulare*) but this amplificaiton was weak (Table 4.9).

When primer pair 7 was combined with the probe in a Taqman qPCR assay, accurate detection of *P. violae* DNA down to 1 fg μl^{-1} (1 x 10^{-6} ng μl^{-1}) was observed with an efficiency of 99% and error of 0.0078 (Figure 4.6). The Taqman assay was found to be highly specific to *P. violae* with minimal detection of the other *Pythium* spp. tested (Figure 4.7). The greatest level of amplification for another *Pythium* spp. was *P. irregulare*, but the 10 ng μl^{-1} sample of *P. irregulare* DNA was detected at an equivalent cycle number to the 1x10^{-5} ng μl^{-1} *P. violae* DNA sample. (Figure 4.7).

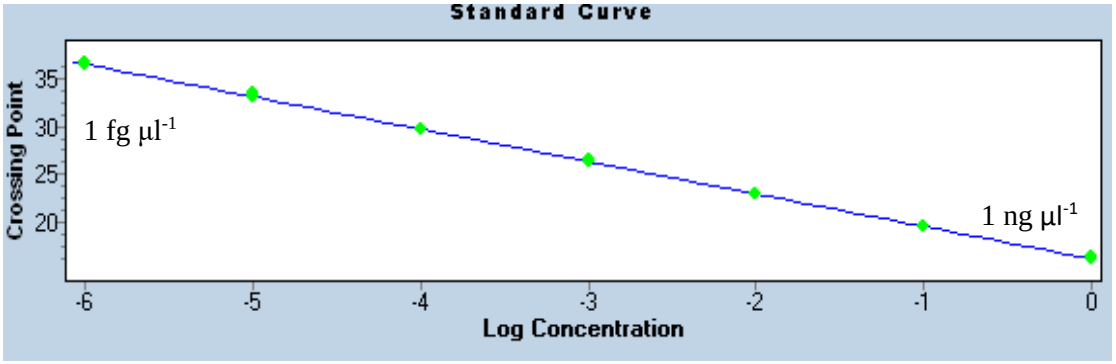


Figure 0.27 Standard curve calculated from serial dilutions of *P. violae* DNA from 1 ng µl^{-1}-1 fg µl^{-1} following a qPCR using a Taqman assay. Ct values shown are the mean values for triplicate reactions.

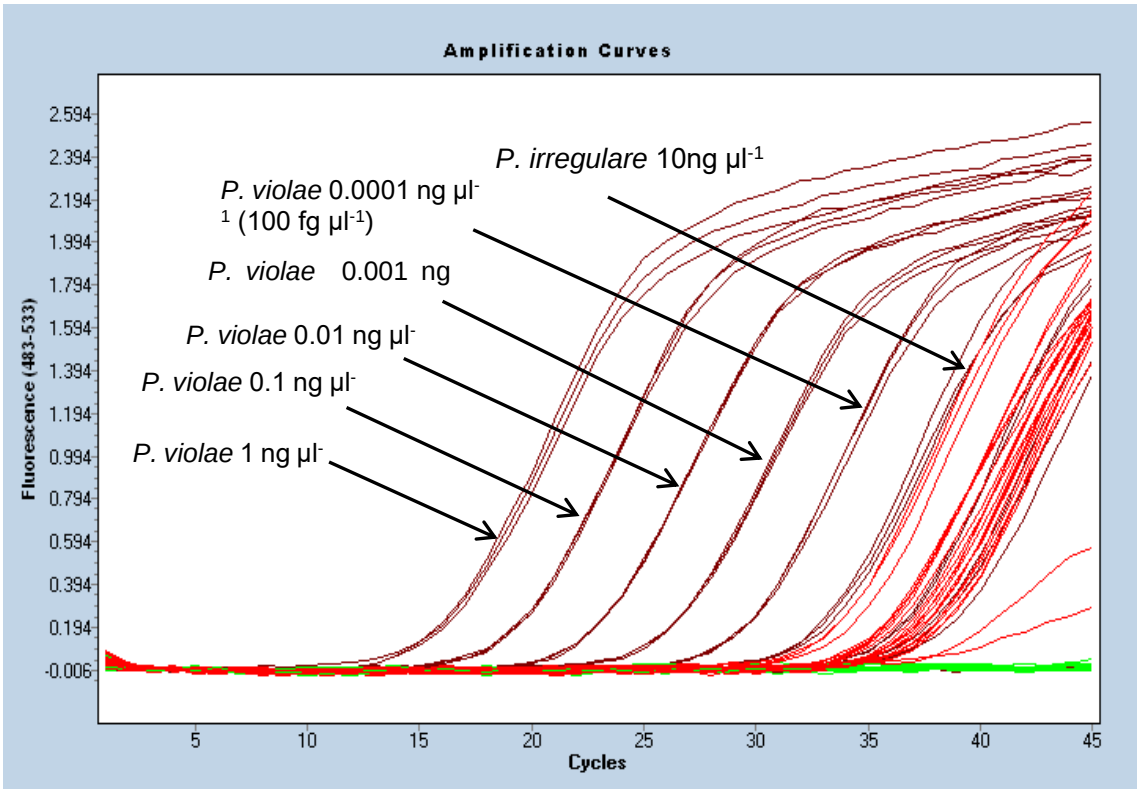


Figure 0.28 qPCR amplification curves for *P. violae* DNA at 1 ng ul^{-1}-10 fg µl^{-1} and other *Pythium* spp. (*P. sulcatum, P. ultimum, P. lutarium, P. irregulare, P. sylvaticum, P. intermedium*) DNA at 10 ng ul^{-1} concentration produced with Taqman assay.

When the Taqman assay was tested on DNA extracted from the *P. violae* oospore dilutions in SDW, amplification was observed over the full range from 1-5000 oospores (Figure 4.8). Across the six independent replicates, Ct numbers ranged from 28-40 cycles and overall, the variation in the *P. violae* DNA concentration was very small across the different oospore concentrations (Figure 4.8) with mean DNA concentrations ranging from 0.021-0.046 pg µl^{-1} for the 1-1000

oospore samples. Overall, for both the oospores that were counted out manually (1-50; grey line, Figure 4.8), and for the majority of the larger number of oospores which were part of a dilution series (50-5000 oospores, orange line, Figure 4.8), there was no consistent relationship between DNA concentration and oospore number. The DNA concentration detected also varied considerably between the six replicates. For example, for 1 oospore, the six independent replicate *P. violae* DNA extractions resulted in a detection range of 0.003-0.100 pg μl^{-1}, whilst the 1000 oospore replicates resulted in detection between 0.004-0.094 pg μl^{-1}, a very similar range to that of the 1 oospore DNA concentration. The only substantial increase in DNA concentration detected was with the 5000 oospore samples. Here, DNA concentration was still highly variable, ranging from 0.017-0.740 pg μl^{-1}, but was considerably higher than for the other oospore concentrations (Figure 4.8).

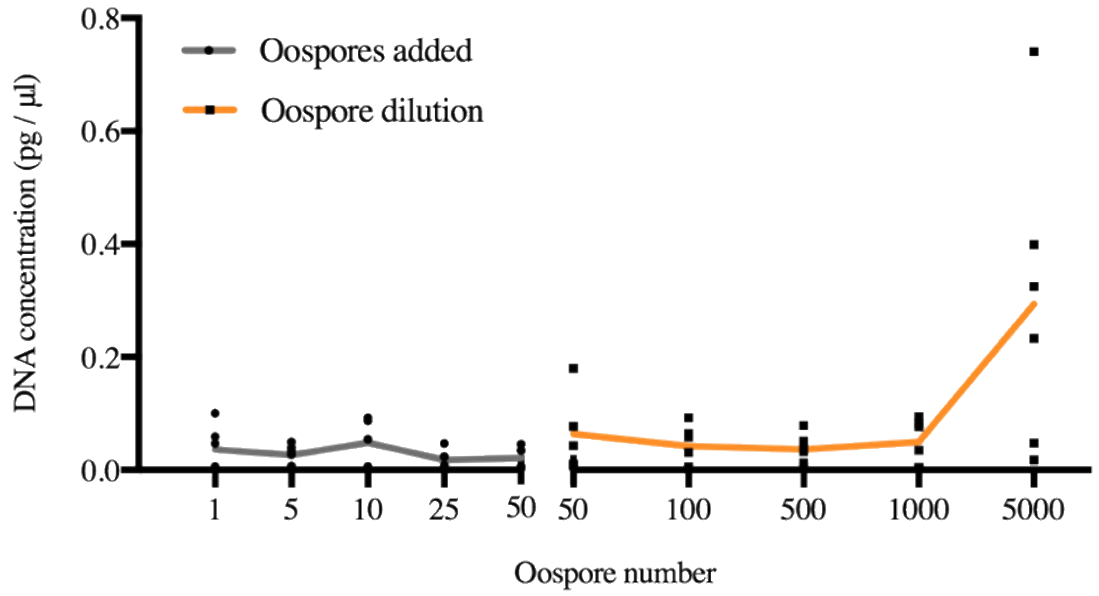


Figure 0.29 *P. violae* DNA concentration detected using the Taqman assay (primer pair 7) for different numbers of oospores. Oospores were individually counted out for samples containing between 1 and 50 oospores (grey line) while samples containing between 50 and 5000 oospores (orange line) were made from serial dilutions. All samples were in 200 µl SDW before DNA extraction was performed. Lines represents the mean of 6 independent replicate DNA extractions and dots represent the individual data points contributing to the mean.

The Taqman assay using primer pair 7 also effectively detected *P. violae* DNA in soil samples collected as part of AHDB project FV405 (Clarkson, 2014) (Figure 4.9a) with high sensitivity. The assay efficiency was 99.5% with an error of 0.0090. DNA concentration ranged from 0.0038-0.1740 pg μl^{-1} in soil samples that had previously shown *P. violae* detection with primer pair 1 (PviolF/R) (Klemsdal *et al.*, 2008) in conventional PCR. *P. violae* DNA was also detected in samples that had not shown amplification following PCR primer pair 1, although the DNA concentrations detected were very low (0.0004-0.0006 pg μl^{-1}; Figure 4.9). There was good

correlation between the conventional PCR with primer pair 1 (as measured using a 0-3 scale) and the Taqman assay (Figure 4.9a, $R^2 = 0.9336$, $p < 0.001$). Soil samples scored in conventional PCR as band brightness 3 resulted in detection of a mean of 0.1298 pg μl^{-1} *P. violae* DNA in the Taqman assay, compared with 0.0063 pg μl^{-1} for those scored at band brightness 1 (Appendix F). However, *P. violae* DNA concentration as measured by the Taqman assay did not correlate well with the incidence of cavity spot in the fields from which the soil samples were collected (Figure 4.9b, $R^2 = 0.007473$).

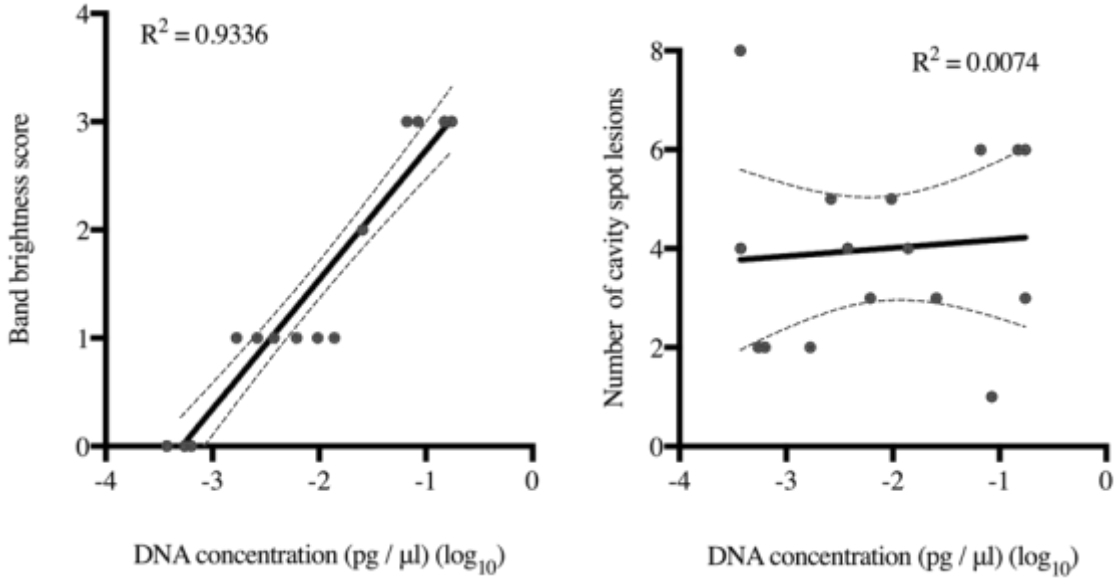


Figure 0.30 Relationship between *P. violae* DNA concentration in qPCR Taqman assay using primer pair 7 and band brightness score following conventional PCR using primer pair 1 (Klemsdal *et al.*, 2008) for soil samples collected from around cavity spot affected carrots (a) and cavity spot incidence recorded for carrots from which soil samples were taken (b).

Assessing *P. violae* DNA concentrations in soil using oospore capture and Taqman qPCR

Quantifying *P. violae* oospores in spiked soil

P. violae DNA from all the different concentrations of oospores in the spiked soil samples was successfully detected using the Taqman assay following DNA extraction with or without prior use of the oospore capture method (Figure 4.10). In the absence of prior oospore capture, and using 0.5 g soil in the GeneAll soil DNA extraction kit, an increase in *P. violae* DNA concentration was detected between the control (no oospores) and 10 oospores g^{-1} samples, but concentration decreased between 10 oospore g^{-1} and 100 oospore g^{-1} as well as between 100 oospore g^{-1} and 1000 oospore g^{-1} (Figure 4.10). Mean DNA detection in the 1000 oospore g^{-1} samples was 0.0052 pg μl^{-1}, approximately the same as detected in the control samples (0.0054 pg μl^{-1}). For the 10 g samples used with the oospore capture method and subsequent DNA extraction with the

DNAeasy extraction kit, the *P. violae* DNA concentration detected was greater, ranging from 0.0121-0.0364 pg μl^{-1} (Figure 4.10). Again, although there was not a consistent rise in DNA concentration in relation to oospore number, the 1000 oospore g^{-1} samples resulted in considerably higher DNA detection than the other concentrations, but this rise was not consistent between the two replications, as indicated by the large standard error of the mean (Figure 4.10).

The actual number of *P. violae* oospores in the spiked soil samples tested varied depending on whether the soil DNA extraction kit was used (where DNA was extracted from 0.5 g of soil), or if the oospore capture and the DNAeasy kit were employed (where 10 g soil samples were used). For the former method, the number of oospores in the spiked samples ranged from 5-50 oospores while for the latter this ranged from 100-10,000 oospores per sample (Figure 4.10). Despite these differences in numbers of oospores, *P. violae* DNA was detected at roughly the same level across both methods for the 100 oospore g^{-1} samples (approximately 0.016 pg μl^{-1}), despite there being 20 times more oospores present in the larger soil sample used for oospore capture.

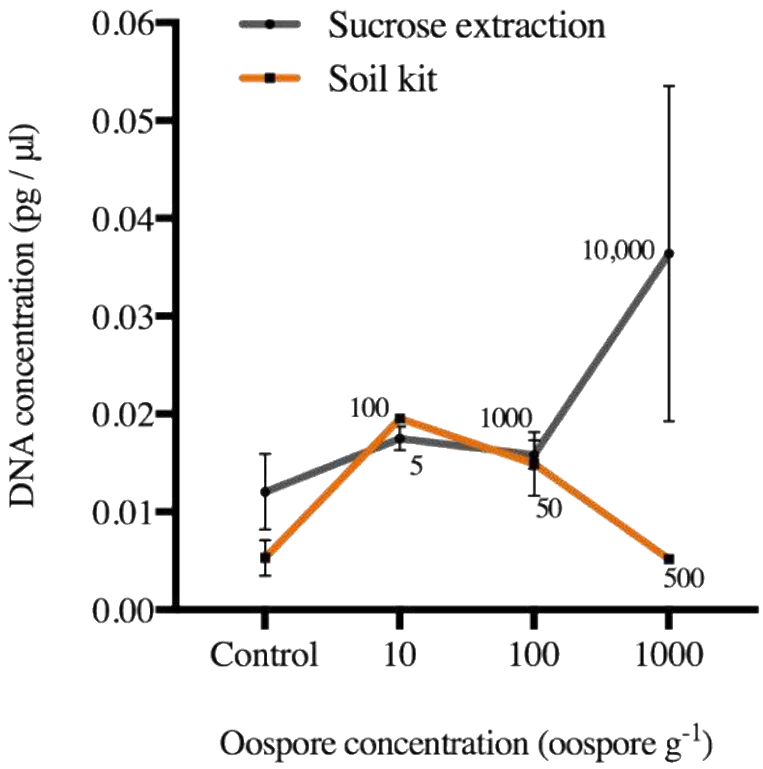


Figure 0.31 *P. violae* DNA concentration detected in soil samples spiked at a range of oospore concentrations. Orange line: oospores extracted using the GeneAll soil DNA extraction kit (0.5 g soil). Grey line: oospores extracted using oospore extraction method and DNAeasy kit (10 g soil). Numbers written above (grey line) and below (orange line) data points represent the actual number of oospores in each sample. Data represents mean DNA concentration (pg μl^{-1}) values ± SEM of three independent replicates for the soil DNA extraction kit, and two independent replicates for the oospore extraction method.

Objective 4: Monitor a commercial carrot field for cavity spot development and assess the dynamics of *P. violae* in soil

Introduction

Little is known about the dynamics of *P. violae* in soil, and only one study by Barbara (Warwick Crop Centre) has used a qPCR approach to address this problem (Anon., 2009). Here, results indicated that *P. violae* was undetectable in soil pre-planting, but reached a peak of detection in carrot crops 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* does not survive in the soil as it produces long-lived oospores (Hendrix & Campbell, 1973; Mitchell, 1978) and, hence, the failure to detect the pathogen pre-planting and post-harvest may be due to issues with sampling, the DNA extraction process, or the sensitivity of the PCR test. Quantifying the inoculum in field soil over the course of a growing season would help understand the dynamics of *P. violae* in the soil and help aid management decisions.

Materials and Methods

Cavity spot development in a commercial field site

A commercial carrot crop in North Yorkshire was identified where root/soil samples could be collected and analysed for presence of *P. violae* continually throughout the growing season. The field was previously cropped with carrots which had developed significant cavity spot. An area of the field comprising three beds (2 m wide, 100 m in length), each of which was divided into four to produce 12 plots of 20 m length (Appendix E), was used for sample collection. Soil samples were collected approx. once a month from January 2015 to January 2016 (Table 4.4). For the January-June 2015 samples, soil samples were taken from around the edge of the carrot bed as it was covered with plastic and each plot was further split into two, resulting in 24 subplots (dashed red line, Appendix E). Six soil samples were pooled for each of the 24 subplots to produce a sample of approximately 400 g. From June 2015 onwards, soil was taken from around the surface of harvested carrot roots. Here, 20 carrots were randomly sampled from across each of the 12 plots. Each root was carefully pulled up with soil still attached around the outside and placed in plastic bags before being taken to Warwick Crop Centre the same day.

On arrival, the carrots were laid out to dry overnight, after which soil from the outside of the carrot was rubbed off, collected and stored at -20˚C until used. The carrots were then washed and assessed for cavity spot incidence (number of carrots with cavity spot lesions) and cavity spot severity (the number of cavity spot lesions per carrot). The total number of cavity spot lesions per plot were subjected to statistical analysis using ANOVA using Genstat® (18.1 edition, VSN International Ltd).

Table 0.21 Description of sampling undertaken for each date at the commercial carrot field monitored from January 2015 to January 2016 in Yorkshire.

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

Quantifying *P. violae* oospores in field soil

Following final assessments of incidence and severity of cavity spot at the field site, the three plots with the highest incidence of cavity spot (Plot 1, 5 and 9, Appendix E) were selected for quantification of *P. violae*. Frozen soil samples were defrosted in the sealed plastic bags for 24 hours at room temperature (18-20˚C), after which the soil was mixed and passed through a 4 mm sieve, before being left to dry at room temperature (18-20˚C) for 48-72 hours. Dried soil was passed through a 2 mm sieve and samples mixed again. One 10 g sample from each of four time points (June, September, November and January) for each of the three plots was selected and processed through the oospore capture method. As with the spiked soil samples in Section 4.2.4.1, oospores captured were stored at -20˚C until DNA extraction. After thawing, oospores were concentrated into 200 µl SDW, DNA extraction performed as outlined in Section 4.2.2.2 and DNA diluted 1 in 5 prior to storage at -20˚C. For comparison, DNA from two 0.5 g samples of soil from each of the three plots and four time points was extracted using a soil DNA extraction kit

(GeneAll Exgene soil SV mini, Korea) in accordance with the manufacturer's instructions and diluted 1 in 5 before storing at -20˚C. The Taqman-based qPCR assay with primer pair 7 (as developed in Section 4.2.3) was used to quantify *P. violae* DNA.

Results

Cavity spot development in a commercial field site

During the 2015-2016 season, significant cavity spot disease developed in the commercial carrot field site monitored, as observed by typical dark, sunken, elliptical lesions (Figure 4.11). Cavity spot was first seen in the crop during August (10/08/2015, Figure 4.11a), although a total of only three lesions were recorded in carrots across all plots. These first lesions were shallow, 'clean' and relatively small. However, substantial cavity spot developed between the August and September sampling time points (29/09/2015), with the total number of lesions rising to 237 over the 12 plots (Appendix G). Across the 20 carrots sampled from each plot, a mean of 20 cavity spot lesions were recorded (Figure 4.12). Lesions at this time point were generally small to medium sized, with some lesions presenting a 'clean' appearance, whilst many lesions had a darker appearance, particularly around the edges (Figure 4.11b).

From September to January, across all plots, the total number of cavity spot lesions increased significantly ($F_{2,35}$ = 6.29, p = 0.007; Figure 4.12). At the November sampling time point (23/11/2015) the number of cavity spot lesions had increased from the September sampling, with the total number of cavity spot lesions observed across the 20 carrots from each of the 12 plots rising to 818 (Figure 4.12). A mean of 68 cavity spot lesions were recorded on the 20 carrots from each plot in the November sampling (Figure 4.12); however Plot 1 showed a considerably higher mean number of cavity spot lesions than any other plot (200 lesions; Table 4.10).

The total number of cavity spot lesions increased further by the final sampling in January (18/01/2016), rising to 1087 cavity spot lesions observed across the 12 plots (Appendix G). A mean of 91 cavity spot lesions were recorded across the 20 carrots per plot (Figure 4.12). Lesions were often very large, expanded and darkened, and roots were more affected by a variety of pathogens (Figure 4.11d). Eight of the 12 plots showed an increase in the total number of cavity spot lesions observed from the November sampling time point, however four of the plots showed a decrease in cavity spot incidence (Appendix G). The three plots with the highest cavity spot incidence over the September, November and January sampling time points were chosen for qPCR analysis (Plots 1, 5 and 9; Table 4.10).

Figure 0.32 Examples of carrots from the commercial carrot monitoring field in Yorkshire. Roots were collected on 10/08/15 (a), 29/09/15 (b), 23/11/15 (c) and 18/01/16 (d). Roots were dug from the carrot field, taken to Warwick Crop Centre, left to dry overnight, the surface soil rubbed off and collected, then the roots washed in tap water before being scored for cavity spot incidence.

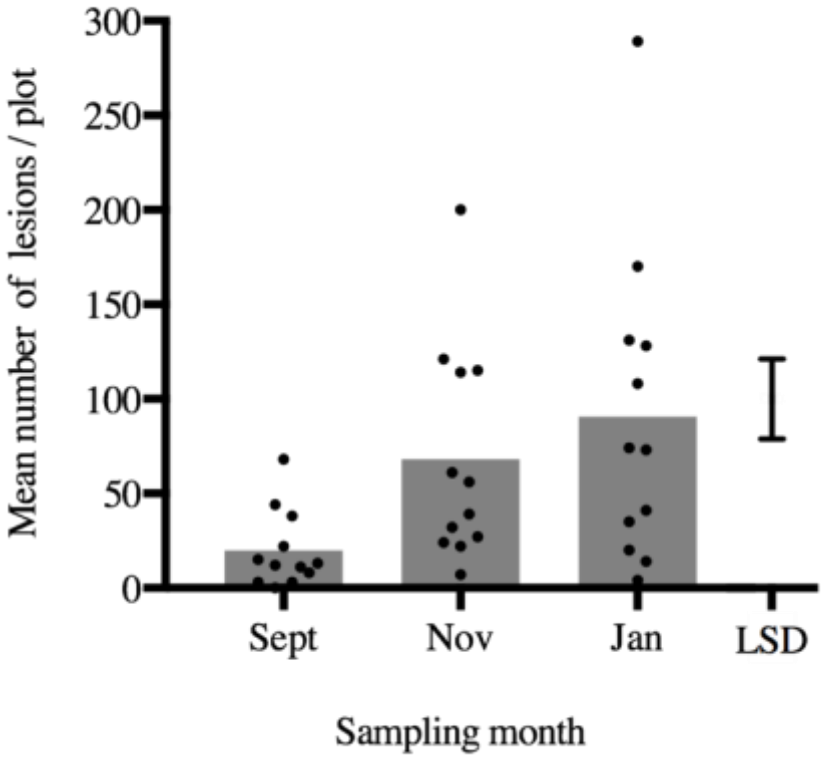


Figure 0.33 The mean total number of cavity spot lesions recorded for 20 carrots in each of 12 replicate plots from a commercial field site during the September, November and January sampling time points. Bars represent the mean number of lesions across all 12 plots, and dots represent the number of cavity spot lesions in each of the 12 plots. Error bar represents the least significant difference between treatments (LSD) at the 5% level.

Table 0.22 Total number of cavity spot lesions scored across 20 carrots taken from Plot 1, 5 and 9 from the commercial field site for the sampling dates from June 2015-January 2016.

Date	Total no. of cavity spot lesions		
	Plot 1	**Plot 5**	**Plot 9**
08/06/15	0	0	0
13/07/15	0	0	0
10/08/15	1	0	0
29/09/15	38	8	12
23/11/15	200	121	115
12/01/16	128	289	170

Quantifying *P. violae* oospores in field soil

For the soil samples collected from around carrots in the commercial carrot field site, *P. violae* DNA was detected in all months (June, September, November and January) both using the Taqman assay following DNA extraction with the oospore capture method, and with the soil DNA extraction kit without prior use of the oospore capture method (Figure 4.13).

Using the GeneAll soil DNA extraction kit, *P. violae* DNA detection was low in June, before any cavity spot lesions were observed on roots (Figure 4.13), and there was an overall trend of increasing *P. violae* DNA detection as the season progressed with lowest DNA concentration in the June samples, and highest in the January samples. However overall, there was not a large range in DNA concentration; across all four sampling time points *P. violae* DNA concentration ranged from 0.0042-0.0135 pg μl^{-1} (Figure 4.13).

Using 10 g samples of soil with the oospore capture method and subsequent DNA extraction with the DNAeasy extraction kit, *P. violae* DNA concentration detected was also very low in the June sampling time point (Figure 4.13). However, in contrast to use of the soil extraction kit, the *P. violae* DNA concentration detected using the oospore capture method was much higher in the September samples (Figure 4.13). This increase can mainly be attributed to Plot 9 where the DNA concentration (0.259 pg μl^{-1}) was considerably higher than the DNA concentration for Plot 1 (0.057 pg μl^{-1}). The Plot 5 sample for the September time point could not be calculated due to a large amount of contaminants. Interestingly, the overall trend of *P. violae* DNA concentration detected over the season was different when using the samples with the oospore capture method compared to the soil DNA extraction kit. The soil DNA extraction kit showed an overall trend of increasing *P. violae* DNA concentration over time. In contrast, the oospore capture method showed a rise in the DNA concentration for the September time point, followed by a decrease in DNA concentration for the November and January time points resulting in the DNA concentration for January falling to only slightly above the concentration for the June time point (Figure 4.13).

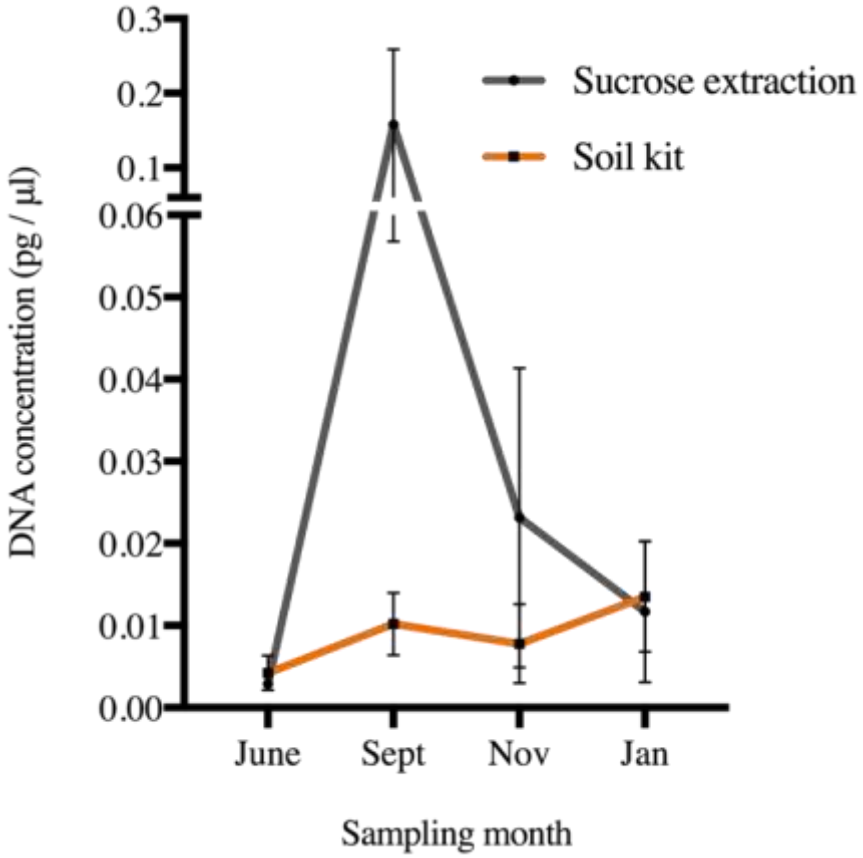


Figure 0.34 DNA concentration measured from extraction of DNA from *P. violae* oospores from soil samples taken from the commercial carrot field. Samples were from June, September, November and January time points. Orange line = oospores extracted via GeneAll soil DNA extraction kit without prior use of an oospore capture method (0.5 g soil). Grey line = oospores extracted via oospore capture method and subsequent DNA extraction with DNAeasy extraction kit (10 g soil). Data represents mean ± SEM of three independent replicates for the soil DNA extraction kit, and two independent replicates for the oospore capture method.

Discussion

This is the first study to report the use of an oospore capture method to separate oospores of *P. violae* from sand and soil samples. After optimisation, the final protocol resulted in *P. violae* oospores being efficiently separated from 10 g sand/oat inoculum samples and using this method achieved substantially greater capture of oospores than the previous method used for the reference counts (an increase in capture of 45.6% compared to the reference count). The oospore capture method was designed for use with larger soil samples (10 g) prior to DNA extraction, and then with a standard DNA extraction kit. This protocol improves on the use of very small soil samples (typically less than 1 g) in more expensive soil DNA extraction kits (Schena *et al.*, 2013). Many soil-borne pathogens are unevenly distributed, and therefore this approach should reduce the variation in detection often observed with soil-borne pathogens at small scales (Rodriguez-Molina *et al.*, 2000; Schena *et al.*, 2013).

Oospore capture was particularly efficient from sand/oat inoculum with a high *P. violae* oospore concentration, and although percentage capture decreased as oospore concentration decreased, over 50% capture of *P. violae* oospores was still achieved with the lowest oospore concentration tested using the sand/oat inoculum dilutions. Oospores were also captured from spiked soil samples, although this was less efficient, and unlike the sand/oat samples, efficacy decreased as oospore concentration increased. Previous work found a major factor hindering detection of soil organisms is that they can bind strongly to soil particles such as clay or organic matter (Kageyama *et al.*, 2003; Wang & Chang, 2003). The soil used in this PhD study was taken from Sheep Pens West field at the Wellesbourne site (Appendix B), a Wick series soil with approximately 80% sand, 10% clay and 10% silt (NVRS, 1974). Given that the only difference between using the assay with the sand/oat inoculum samples and soil samples was the change in substrate, the properties of this soil compared to sand (the clay and silt components) most likely explain the observed differences in oospore capture efficacy. It is difficult to explain the reduction in oospore capture efficacy in this study, but testing a range of soil types on oospore capture efficacy in the future would be beneficial to determine if different soil components (e.g. silt, clay etc.) affect recovery.

Previous studies have reported difficulties in lysis of oomycete oospores which have hindered subsequent PCR detection. For example, Wangsomboondee and Ristaino (2002) attempted numerous lysis methods to optimise detection of oospores of *Phytophthora infestans*, and found that neither direct PCR (no lysis) nor freeze-thawing of oospores followed by DNA extraction resulted in detection by PCR. Wang and Chang (2003) conducted similar lysis tests on oospores of *P. myriotylum*, and found disruption by vortexing with sand was most efficient, while simply grinding soil in liquid nitrogen was less successful. The testing of different lysis techniques, as

carried out in this work, is therefore an essential step for effective DNA extraction. The successive FastPrep® treatments alongside the slight modification of the DNA extraction protocol resulted in efficient lysis of *P. violae* oospores, and hence reliable detection by PCR.

DNA extraction and PCR of the *P. violae* oospore dilution series in SDW with the published primer pair 1 (Klemsdal *et al.*, 2008) was successful. The modified lysis and DNA extraction methods consistently amplified DNA extracted from 10 oospores (a detection limit of 1 oospore g^{-1}). Despite generally consistent detection of *P. violae* oospores in the dilution series, there was some variability in PCR amplification of DNA from samples containing between 1 and 10 oospores. Bead-beating lysis approaches as used here can shear DNA (Miller *et al.*, 1999; Robe *et al.*, 2003) which may explain the variability observed.

Following testing of *P. violae* PCR primers (primer pair 1) published by Klemsdal *et al.* (2008) using DNA from different *Pythium* spp., amplification was observed for *P. intermedium, P. irregulare* and *P. sylvaticum* after 30-40 cycles. The published conditions for specificity with *P. violae* requires 40 cycles, and hence amplification of the three other *Pythium* spp. for this cycle number was unexpected. Although specificity did increase when the number of cycles was reduced, this decreased cycling caused a concomitant decrease in sensitivity of detection for *P. violae*. Furthermore, these other *Pythium* spp. detected are also known to be associated with cavity spot disease (Lyons & White, 1992), and *P. intermedium* is capable of causing lesions on carrot roots (Chapter 2, Section 2.3.3). Therefore amplification from a soil DNA sample using primer pair 1 would fail to specifically identify the causal species. Furthermore, these primers are not suitable for use with qPCR. Nonetheless, these primers are still appropriate for detection of *Pythium* spp. associated with cavity spot where species identity is not important.

The development of new *P. violae* specific primers suitable for use with qPCR using the ITS regions of the rRNA gene initially proved difficult. There is little sequence variation between different *Pythium* spp. in the ITS region, and the small amplicon size required for qPCR leaves a very limited range in which to find sequence differences (Hiltunen & White, 2002; Levesque & de Cock, 2004). However, the OCM1 (Robideau *et al.*, 2014) and cellulose synthase (Blum *et al.*, 2012) genes provided much more sequence variation between *Pythium* spp. for primer design. Although primer pairs developed for both these gene targets resulted in good amplification of *P. violae* and very minimal detection of other *Pythium* spp., the comparison of these primers with primer pair 1 (Klemsdal *et al.*, 2008), which had previously tested positive for *P. violae* in DNA from commercial field soil samples, showed primer pair 5 and 6 failed to detect the DNA in the same samples. This could be because both the OCM1 gene and the cellulose synthase gene are single or low copy number genes (Zerillo *et al.*, 2013) which results in considerably lower sensitivity than when multi-copy gene targets such as the rRNA gene are used (Pastrik & Maiss, 2000; Schaad & Frederick, 2002; Kageyama, 2014).

Due to the problems with PCR sensitivity and specificity, a new Taqman qPCR assay was developed for *P. violae* again based on the ITS region of the rRNA gene. The Taqman approach has the advantage that the internal probe provides an extra source of specificity and can give substantial benefits when attempting to distinguish between two closely related species. The Taqman assay developed in this work resulted in specific and highly sensitive detection of *P. violae* DNA with a detection limit of less than 1 fg μl^{-1}. When the Taqman assay was tested with the *P. violae* oospore dilution in SDW, it was possible to detect the DNA from just one oospore, although results across the six replicates were highly variable. This variability may be partly due to the fact that the ITS region exists in multiple copies in genomes and there is likely to be variation in extraction efficiency between replicates (Klemsdal *et al.*, 2008; Li *et al.*, 2010). Given that a qPCR assay amplifies the template exponentially, any variations in the template will be magnified. Variations across all aspects of the DNA extraction, from lysis efficiency to DNA purity can introduce biases that substantially affect the outcome of qPCR assays (Krsek & Wellington, 1999).

The Taqman assay, however, did not result in a clear correlation between the number of oospores in the SDW oospore dilution series, and *P. violae* DNA concentration. Despite very low *P. violae* DNA concentrations detected in the oospore dilutions in SDW, good detection of the pathogen was achieved using the Taqman assay for the naturally infested field soil samples collected as part of AHDB project FV405 (Clarkson, 2014). The Taqman DNA concentrations correlated well with the results from conventional PCR conducted with primer pair 1 (Klemsdal *et al.*, 2008). However, there was no correlation between the Taqman DNA concentration and the number of cavity spot lesions recorded on the carrots from where the soil samples were collected. Similar work by Barbara as part of a DEFRA funded project (Anon., 2009) developed a *P. violae* qPCR assay using the primers designed by Klemsdal *et al.* (2008) to monitor pathogen dynamics and recorded cavity spot development in a carrot crop. Here, there was similarly no correlation between *P. violae* DNA concentration in soil collected from around carrot roots and disease severity (mean lesions/root). This was the case either early in the season (June/July) or late in the season (October/November), but some correlation was reported for samples collected in August and September (R^2 = 0.80). However, as stated above, the assay used primers which were not wholly suitable for use with qPCR. In this present study, the soil samples used were collected from carrot fields between October and November, and therefore may have been less likely to correlate with disease severity.

The Taqman qPCR was also tested using DNA from soil samples spiked with different numbers of *P. violae* oospores, using both a soil DNA extraction kit and the oospore capture method in combination with a DNAeasy DNA extraction kit. Although DNA was detected in all samples, the DNA concentrations detected were very low. This low concentration could be attributed to a

number of issues commonly related to DNA extraction from soil. Even though soil DNA extraction kits are designed to remove some PCR inhibitors, and the oospore capture method removed the majority of soil which could affect DNA extraction with the DNAeasy kit, both methods could still have been affected by soil residues which may have affected the subsequent Taqman qPCR assay.

The commercial carrot field site that was monitored for cavity spot over a period of 12 months from January 2015 to January 2016 showed a clear progression in symptoms of cavity spot incidence and severity. The corresponding soil samples would have been expected to show an increase in *P. violae* DNA detection from June through to January, and this was the case for the samples extracted using the soil DNA extraction kit, although all DNA concentrations were very low. However, when DNA from the same samples was extracted using the standard DNAeasy DNA extraction kit following the oospore capture method, a large increase in *P. violae* DNA concentration was detected in September, before decreasing over the November and January samples. This pattern of *P. violae* detection was similar to that described by Barbara (Anon., 2009) where the pathogen was detected more consistently during August and September possibly related to the disease levels on carrots. There is substantial evidence, including from this PhD, that indicates that *P. violae* is easy to isolate from cavity spot lesions when they first develop (August and September) but is largely absent from mature and expanded lesions later in the season (Hiltunen & White, 2002). Given that the majority of cavity spot developed between the August and September time points, it is likely that the large increase in DNA concentration detected in September was due to an increase in *P. violae* activity. Whether this activity involved increased oospore production, or increased mycelial growth (or a combination of both) is unclear.

Overall, the development of an oospore capture and improved molecular detection method for *P. violae* was somewhat successful, although a number of factors need to be addressed before this combined approach can be used routinely to quantify *P. violae* in soil and related to cavity spot disease progression. There was considerable and unexpected variation in detection of *P. violae* DNA in the commercial field site soil samples when using these methods, which was also the case with the standard soil DNA extraction kit. This variation may be due differences in the efficacy of each approach in extracting DNA from *P. violae* oospores or mycelium. The improved lysis protocol was not used with the soil DNA extraction kit, and no specific testing was done to test whether oospores were lysed sufficiently, and therefore the DNA extracted may have been predominantly from mycelium fragments rather than oospores (or a combination of both). By comparison, the oospore capture method was designed to primarily remove oospores from the soil, but may have also have trapped small mycelium fragments (Lees *et al.*, 2012), which may have contributed to the increase in *P. violae* DNA concentration observed in September.

The oospore capture approach followed by DNA extraction and Taqman qPCR failed to identify a relationship between DNA concentration and *P. violae* oospore number either in the dilution series in SDW or in the spiked soil samples and therefore this approach cannot reliably determine *P. violae* inoculum levels/oospore numbers in soil. However, results from the oospore dilution in SDW indicated that a large number of oospores (>5000) results in a substantial concentration of *P. violae* DNA detected. This suggests the soil sample collected from the commercial field site in September, which also had a high *P. violae* DNA concentration, likely had very high levels of *P. violae* inoculum. However, for the majority of the soil samples in which low levels of *P. violae* DNA was detected, it would be difficult to estimate levels of the pathogen.

The number of *P. violae* oospores present in carrot growing soils is also largely unknown. Previous research using a soil plating procedure suggested that *P. violae* oospore populations can range between 0 and 30 oospores g^{-1} of dry soil, and can rise to between 80 and 200 oospores g^{-1} of dry soil in heavily infested soils (Pettitt *et al.*, 2002). In this PhD project, soil samples from cavity spot infected carrot fields acquired in 2015 and quantified using the initial oospore capture method were found to contain 57 oospores g^{-1} of dry soil three months after harvest of a heavily infected carrot crop, and 280 oospores g^{-1} of dry soil from field soil collected from around cavity spot infected carrots. Given that the two extraction methods in this PhD project produced inconsistent results, it has not been possible to build understanding of *P. violae* dynamics in field soil via quantitative molecular methods, or reliably detect when *P. violae* levels increase in a cavity spot infected crop.

Despite the considerable challenges encountered, substantial progress has been made towards the capture, detection and accurate quantification of *P. violae* oospores in field soil. The oospore capture method developed was successful with the sand/oat samples, and to some extent with the soil samples and is a significant first step toward increasing the soil sample size for PCR quantification of *P. violae* from soil. However, the capture method is very time-consuming, as at least three sucrose extractions were required to capture the majority of the oospores in one sample. Furthermore, there is a clear need for further work to identify the issues surrounding the capture of oospores from a soil substrate rather than sand. The lysis and DNA extraction method development was also successful and combined with the Taqman qPCR assay has resulted in a highly sensitive and specific assay for *P. violae* DNA. However, when used with field samples, the highly variable results and differing trends in detection and quantification of *P. violae* DNA highlight the need for further work in order to be able to successfully and reliably detect *P. violae* oospores in soil to inform disease risk or to monitor pathogen dynamics.

Conclusions

Cavity spot disease, caused principally by the oomycete *Pythium violae*, is a major problem for carrot growers worldwide, affecting over 10% of the crops grown and causing millions of pounds of losses each year. Current control measures include cultural practices such as selecting fields with good drainage and no previous carrot crop history, and a heavy reliance on the fungicide metalaxyl. However, the effectiveness of these approaches is threatened by the enhanced degradation of metalaxyl, the decreasing availability of land not previously cropped with carrots and the issues surrounding identifying new effective crop protection products. An increased understanding of *P. violae* as a pathogen, and development of effective molecular detection tools could help provide new routes to alternative control measures.

Although there has been previous research on cavity spot and *P. violae*, this has been limited and there is still a lack of understanding of the biology and epidemiology of the pathogen and a failure to develop methods to reliably induce cavity spot disease artificially in either a pot or field setting. Research has also been further hampered by a lack of effective molecular tools to detect and quantify *P. violae* in the soil.

The aims of this thesis, as outlined in Chapter 1, were to:

1. Identify the current *Pythium* species associated with cavity spot in the UK and investigate the phylogeny and pathogenicity of a range of isolates.

2. Develop an artificial inoculation system for *P. violae* to reliably induce cavity spot disease in carrots.

3. Develop molecular tools to effectively capture, detect and quantify *P. violae* from field soil.

Pythium: advancing understanding of phylogeny and pathogenicity

During this PhD project, the largest survey of *Pythium* spp. associated with cavity spot in England to date was conducted, and provided clear evidence that *P. violae* is the main causal agent of cavity spot in England. This study was also the first phylogenetic study of *Pythium* spp. associated with cavity spot, which was helpful in selecting diverse isolates to test for pathogenicity. Moreover, a small number of *P. violae* isolates from The Netherlands clustered separately from UK isolates suggesting that a similar phylogenetic approach may provide a

means of determining differences in population structure of *P. violae* isolates from different geographic locations.

The *in vitro* pathogenicity experiments provided useful insight into the variation in lesion size that could be produced by different *Pythium* spp., as well as different isolates within a species. Slower growing *P. violae* and *P. sulcatum* isolates consistently produced larger and deeper lesions than faster growing *P. intermedium* isolates. This work may potentially be beneficial in identifying the size and type of lesion that is produced in the field depending on the dominant *Pythium* spp.. Given the variation in virulence both between *Pythium* spp. and within *P. violae* isolates, a promising next step would be to conduct genome or transcriptome-based studies to understand the basis for this observation.

Breeding carrots for resistance to cavity spot is a more sustainable approach to control and a preliminary *in vitro* experiment with a small collection of carrot lines tested in this project showed a wide range of differences in susceptibility to *P. violae*. However, further work is required to determine if results of this type of root assay are comparable with tests using whole plants in the glasshouse or field. A rapid and reproducible screening method for carrot using artificial inoculation with *P. violae* is an essential first step in identifying new sources of resistance for breeding.

Artificial inoculation of cavity spot: successes, improvements and future directions

Previous research attempting to identify new control measures for cavity spot disease has been severely impeded due to a lack of natural infection in field experiments (Gladders, 2014). There has been little research conducted in developing artificial inoculation systems for carrot, and most have used mycelium-based inoculum or methods which were difficult to quantify accurately (White, 1986; Pettitt *et al.*, 2002; El-Tarabily *et al.*, 2004; Suffert & Guibert, 2007). Furthermore, the primary *P. violae* inoculum in the field is thought to be oospores. Therefore, this project investigated artificial inoculation systems for *P. violae* using oospores, and this thesis is the first report of some success using this approach.

Although glasshouse experiments did result in the formation of cavity spot lesions as expected, there was also a striking effect on carrot growth with consistent stunting of roots and browning of tap roots. In addition, field experiments resulted in carrot roots over two years with consistently high levels of cavity spot disease, which has not been reported previously. This finding is a significant step in being able to identify new control measures.

The most significant problem encountered across the artificial inoculation experiments was the lack of a dose response effect of increasing disease severity with increasing *P. violae* oospore concentration. The potential reasons for this are complex (see Chapter 3, Section 3.4), but oospore germination is likely a major issue that was not solved within this project. Based on the

results of these experiments, better understanding of oospore germination, alongside the ability to induce germination experimentally, would potentially provide the missing link that is currently hindering reliable and consistent controlled artificial inoculation. The factors affecting germination of *P. violae* oospores are unknown, and future research into finding the optimal conditions and media for supporting and triggering *P. violae* germination *in vitro* would be valuable.

Another area of research less well studied is the influence of the soil microbial community on cavity spot disease development. It is well known that the composition of soil microbes can be linked to conducive or suppressive soils that influence disease development (Weller *et al.*, 2002; Agtmaal, 2015; Latz *et al.*, 2016). Given that *P. violae* is such a poor competitor (Hendrix & Campbell, 1973), its ability to cause high disease levels without any consistent or specific environmental triggers (that have so far been identified) is puzzling (T. Pettitt, personal communication). If *Pythium* spp. can be components of disease complexes, other pathogens may affect the ability of *Pythium* to cause disease. High-throughput sequencing methods using multiple primer pairs for fungi, bacteria and oomycetes would allow the whole community of microorganisms to be sequenced and determine which species form complexes both within cavities, and the surrounding soil. This could not be investigated as part of this PhD project due to time constraints. However, initial sample collection and storage has been achieved, therefore this work could be conducted in the future to further develop understanding of *Pythium* ecology.

Advancements in *P. violae* detection and quantification

Studies preceding this PhD project have not successfully monitored *P. violae* dynamics throughout a carrot growing season. Detection has often been inconsistent, and there have been concerns over the lack of reliability in terms of specificity, sensitivity and sampling (Barbara, 2010b). This project aimed to develop a reliable research tool that would enable *P. violae* to be accurately quantified in soil, to allow pathogen dynamics to be studied.

The oospore capture method developed was successful in retrieving a high percentage of oospores from sand inoculum samples; however the capture of oospores from spiked soil was more complex and less efficient. Nonetheless, this methodology has considerable potential benefits for use in the future as it is allows much larger soil samples to be tested, which overcomes some of the problems associated with the heterogenous distribution of *P. violae* across fields (Hiltunen & White, 2002).

The Taqman qPCR developed in this PhD project is the first description of such a highly sensitive and specific assay for quantification of *P. violae* DNA and is a significant improvement in sensitivity compared to previous molecular detection tools.

The combination of efficient oospore capture with development of the specific and sensitive Taqman qPCR assay for *P. violae* represents a major advance in *P. violae* detection and

quantification, which will be a valuable tool for further research. Use of a sensitive detection and quantification tool would aid understanding of *P. violae* dynamics, as well as help identify fields with *P. violae* inoculum prior to sowing or strawing. Furthermore, it is still unclear as to when *P. violae* infects the carrot roots, and understanding of early infection events would help with the development and application of future control measures such as fungicides.

Knowledge and Technology Transfer

- Abstract for University of Warwick School of Life Sciences Postgraduate Symposium, March 2015.
- Poster presented at British Plant Pathology Society annual conference, September 2015. Awarded John Colhoun Poster Prize
- Poster presented at 2015 AHDB Horticulture studentship conference, September 2015. Awarded Best Poster Prize.
- 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)
- Poster: AHDB Crops Studentship Conference, Stratford Upon Avon, 16th/17th November 2016 (prize won for best poster).
- 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 (prize won for best poster).
- Presentation: British Society for Plant Pathology (BSPP) Presidential Meeting 2017, Nottingham, 11th-13th September 2017 (awarded PH Gregory Oral Presentation award).
- Presentation: University of Warwick School of Life Sciences Postgraduate Symposium, University of Warwick, 21st March 2018 (prize awarded for best presentation)
- Presentation: British Carrot Growers Association / AHDB R+D Meeting, 21st November 2018
- Presentation: CERTIS Carrot and Parsnip Event, Peterborough, 16th January 2019

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Appendices

Appendix A: Whole genome sequencing

Based on results of previous pathogenicity testing, two isolates of *P. violae* (P10 and P4) were selected for whole genome sequencing. These were a strongly pathogenic isolate (P10) and a less pathogenic isolate (P4). DNA extraction was performed on freeze-dried mycelium and library preparation and sequencing carried out by H. Bates using an Illumina MiSeq machine at NIAB EMR. All subsequent work and analysis was carried out with the assistance of Dr A. Armitage (NIAB EMR). 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 (> 500 bp and > 10X coverage).

Table A.1 Assembly statistics for isolates used in whole genome sequencing

Isolate	Assembled genome size (Mb)	Number of contigs	N_{50} (kb)	Largest scaffold (Mb)	G+C content (%)
P10	50.6	6216	38.2	6.68133	52.41
P4	51.7	6050	37.4	6.68139	52.43

Appendix B: Warwick Crop Centre Site Map

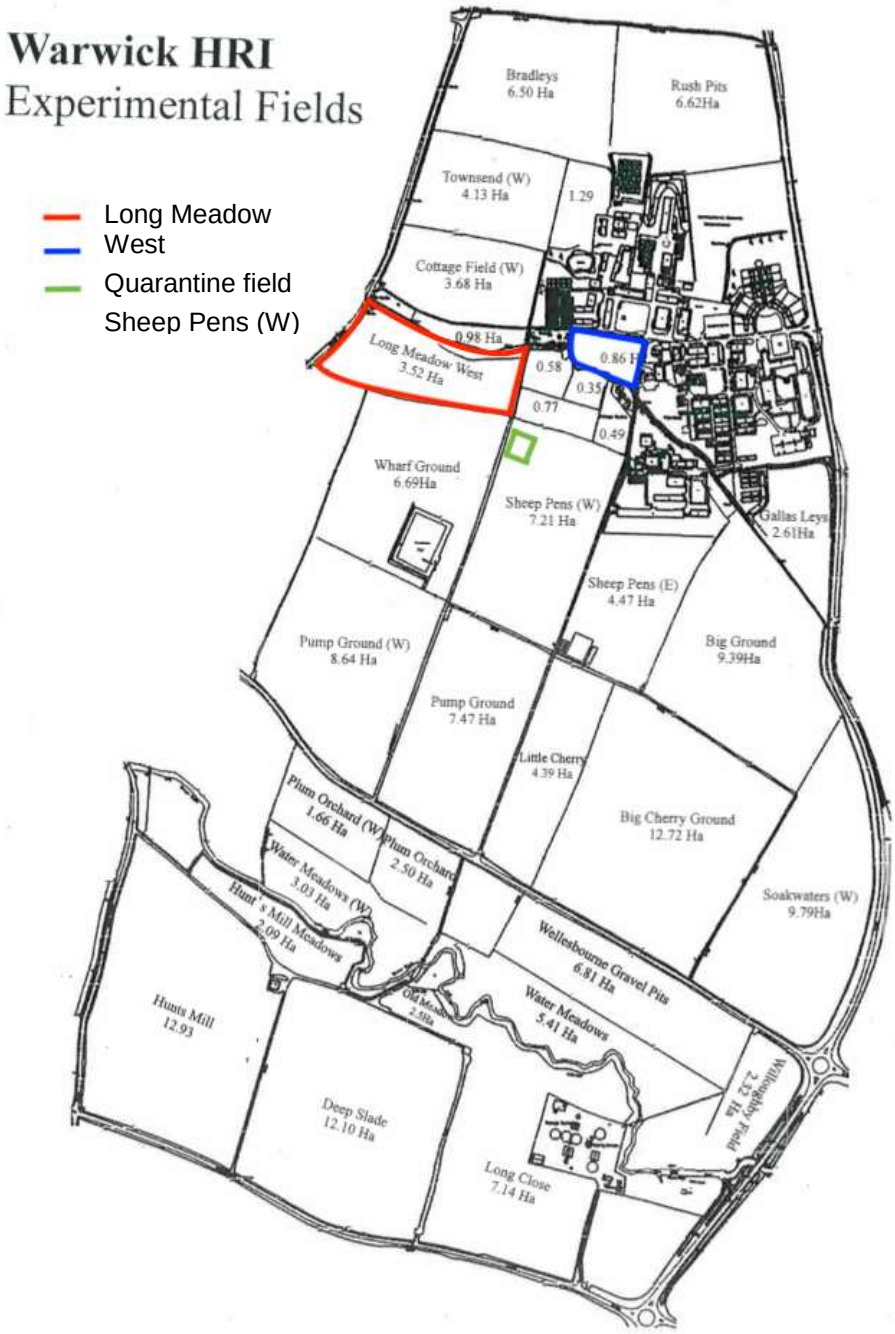

Figure B.1 Map of Warwick Crop Centre, Wellesbourne Campus (Horticultural Services, 2007). Long Meadow West Highlighted in red, quarantine field highlighted in blue, Sheep Pens (W) highlighted in green.

Appendix C: Deformed roots in mature plant artificial inoculation experiments

Experiment 1 and 2

The percentage of deformed roots (classified as any misshapen appearance) was measured and in Experiment 1 there was there was a main effect of concentration on the percentage of roots deformed ($F_{5, 85} = 2.63$, $p = 0.029$). There was no apparent dose-response effect observed. Very few roots showed any signs of deformation in Experiment 2, and there was no evidence of an effect of concentration.

Figure C.1 Examples of deformed roots from mature pot experiment 1.

Experiment 3

The percentage of deformed (classified as any misshapen appearance other than a forked tap-root) and forked (characterised by the formation of multiple tap-roots) roots were measured in this experiment. With both variables, there was no evidence of an effect of seed treatment or oospore concentration by seed interaction, but there was a main effect of treatment (Deformed: $F_{4, 99} = 14.57$, $p < 0.001$; Forked: $F_{4, 99} = 3.77$, $p = 0.007$). The number of deformed and forked roots increased as the oospore concentration increased: with both variables both the 50 and 100 oospore g^{-1} concentrations showed significantly greater number of roots with deformities/forking than the uninoculated control (results not shown).

Figure C.2 Examples of deformed and forked roots from mature pot experiment 3. 100 and 50 refer to oospores g-1, M/U refers to metalaxyl-treated and untreated seed respectively.

Appendix D: Field macrocosms carrot yield: total root weight, mean root weight and total number of roots.

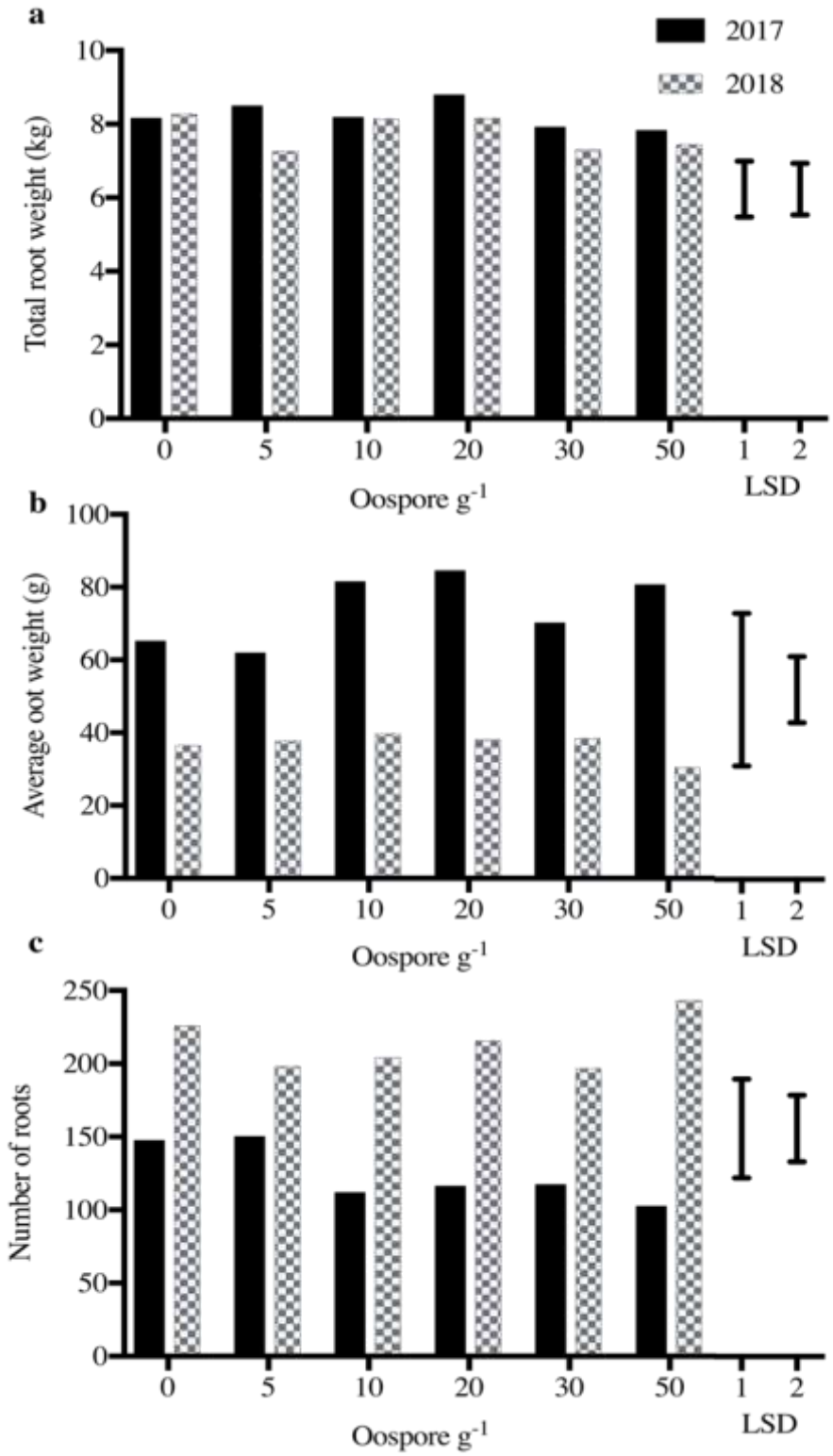

Figure D.1 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on carrot weight and number in field macrocosms: total root weight per macrocosm (a) and mean root weight per carrot (b) and total number of roots per macrocosm (c) for 2017 (black solid bars) and 2018 (grey hashed bars) harvest. Error bars represent the LSD at the 5% level

Appendix E. Commercial carrot field monitoring site

3x beds, 100 metres in length. 10m buffer zone at end of each bed to remove any ‘edge effects’. Each bed split into 4 plots and 8 subplots.

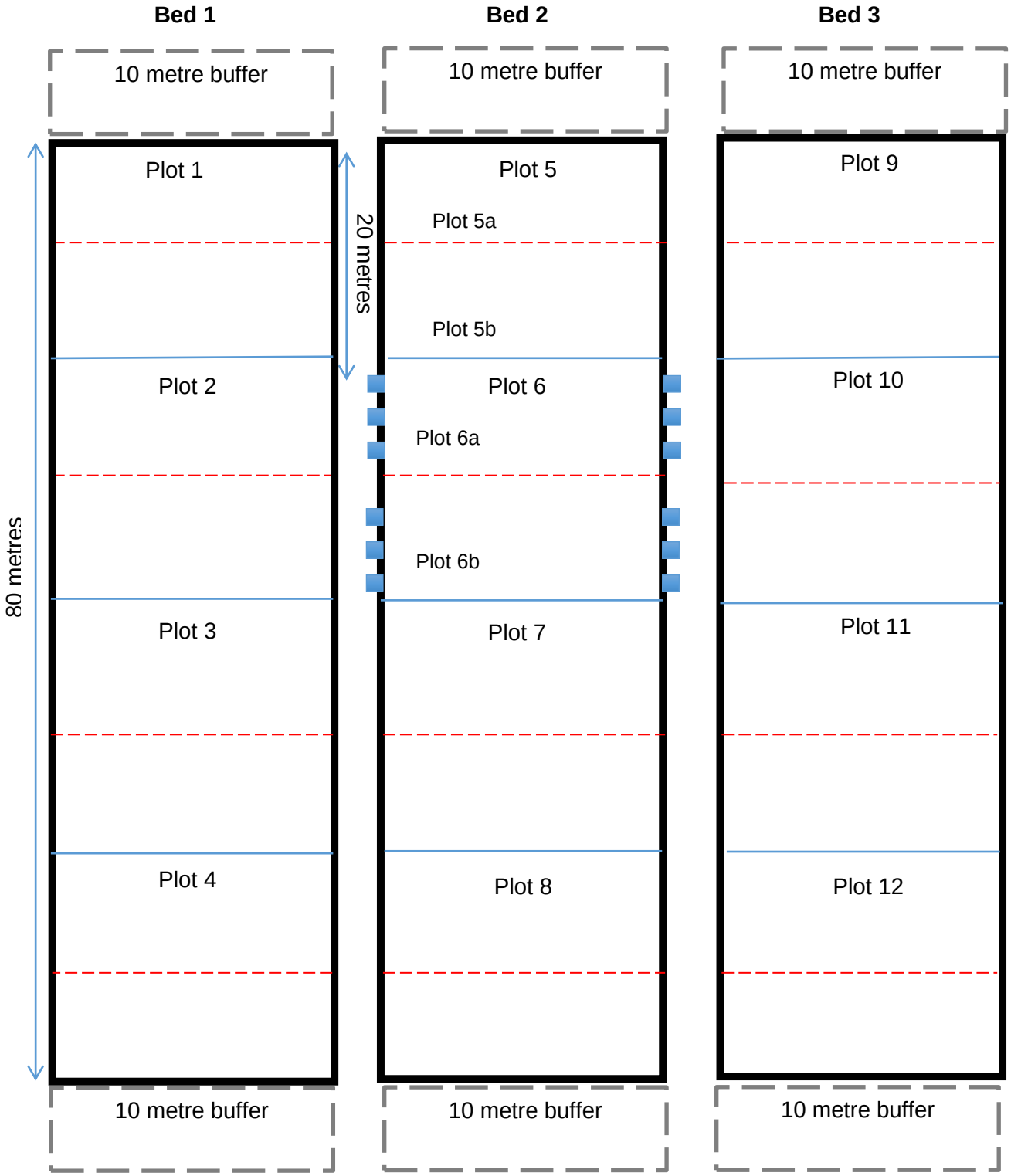

Figure E.1 Commercial carrot monitoring field site trial plan. Solid black lines indicate the three beds, blue lines within each bed indicate the 12 plots, orange dashed lines indicate the 24 sub-plots. Blue squares in plot 6 indicate how soil was samples prior to the June sampling time points (when carrots were covered with fleece).

Appendix F: Quantification of DNA samples from PviolF/R (Klemsdal et al, 2008) and newly developed Taqman qPCR assay

Table F.1 Quantification of DNA samples from non-target species (top section) and soil (bottom section) using a scale of band brightness for PviolF/R primers (Klemsdal et al, 2008) and a newly developed Taqman assay (AT_ITS) for *P. violae.* For conventional PCR with PviolF/R primers, DNA quantification was assessed with band brightness and scored on a scale from 0 to 3, where 0 showed no band (no amplification) and 3 showed a bright band (good amplification). For the Taqman qPCR, DNA quantities were calculated using a standard curve method. SEM=standard error of mean.

Sample	Details	Band Brightness (PviolF/R)	DNA detected (AT_ITS) (pg)	SEM
P173	*P. lutarium*		0.0000	0.00
P174	*P. ultimum*		0.0004	0.18
P48	*P. attrantheridium*		0.0001	0.13
P86	*P. intermedium*		0.0006	0.05
P87	*P. debaryanum*		0.0003	0.17
P91	*P. sulcatum*		0.0002	0.08
P100	*P. sylvaticum*		0.0001	0.06
P162	*P. irregulare*		0.0051	0.76
P175	*P. ultimum*		0.0019	0.25
P24	*P. sylvaticum*		0.0013	0.15
P25	*P. intermedium*		0.0002	0.18
P197	*P. irregulare*		0.0006	0.22
780	soil sample	0	0.0006	0.35
781	soil sample	0	0.0006	0.08
758	soil sample	0	0.0004	0.22
783	soil sample	0	0.0004	0.21

765	soil sample	1	0.0061	0.65
774	soil sample	1	0.0017	0.42
775	soil sample	1	0.0097	1.34
760	soil sample	1	0.0038	0.23
764	soil sample	1	0.0139	1.05
757	soil sample	1	0.0026	0.28
761	soil sample	2	0.0256	1.23
770	soil sample	3	0.1740	4.58
779	soil sample	3	0.0848	2.29
776	soil sample	3	0.1743	2.60
784	soil sample	3	0.1493	1.33
777	soil sample	3	0.0668	6.41

Appendix G. Commercial field carrot monitoring site cavity spot lesions data

Table G.1 Total number of cavity spot lesions for each of the 12 plots sampled in commercial carrot field site for the September, November and January time points. Mean indicates the mean number of lesions observed on the 20 carrots from each plot for all three time points (60 carrots total). Total (plot) indicates the total number of lesions observed on the 20 carrots from each plot for all three time points (60 carrots total)/ Total (time point) indicates the total number of cavity spot lesions observed on the 20 carrots from each plot for each sampling time point (120 carrots total).

Plot	September	November	January	Mean	Total (plot)
1	38	200	128	122	**366**
2	13	27	108	49.3	**148**
3	15	7	41	21	**63**
4	11	56	14	27	**81**
5	8	121	289	139.3	**418**
6	22	114	131	89	**267**
7	0	39	20	19.7	**59**
8	3	22	35	20	**60**
9	12	115	170	99	**297**
10	3	61	73	45.7	**137**
11	44	32	4	26.7	**80**
12	68	24	74	55.3	**166**
Total (time point)	**19.8**	**68.2**	**90.6**	-	-

