Developing research tools to investigate the biology and epidemiology of *Pythium violae* causing cavity spot on carrot to enable disease management

By

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work in this thesis has been undertaken by the author except in the case outlined below: The mature plant experiments carried out in Chapter 3 (Development of *P. violae* artificial inoculation systems for carrot) were carried out jointly with Nicole Pereira as part of AHDB projects FV391a and FV391b.

Summary

Cavity spot is a major disease of carrots worldwide, resulting in high-value losses each year in Britain. Cavity spot is caused by the oomycete *Pythium*, which attacks the mature carrot root causing sunken, elliptical lesions making the crop unmarketable. Disease management is challenging due to variable fungicide efficacy and difficulty implementing long rotations. New control approaches have been hampered by a lack of fundamental knowledge concerning *Pythium violae* epidemiology, basic tools for research including diagnostics, and no reproducible inoculation procedure. The work in this thesis aimed to address this problem by building greater understanding of *Pythium* biology/genetics, and developing molecular detection tools and artificial inoculation methods.

To determine the *Pythium* species associated with cavity spot, 150 isolates were collected from diseased carrots. Following DNA extraction and sequencing, the main species associated with cavity spot disease in the UK was *P. violae*. Further characterisation with housekeeping genes separated *Pythium* isolates into multiple clades within species. Pathogenicity experiments using representative isolates of *P. violae*, *P. sulcatum* and *P. intermedium* from different clades identified a range of virulence within and between species.

A reproducible source of *P. violae* oospore inoculum was developed and used to inoculate carrot seedlings, and pot/field-grown carrots. This resulted in abnormal carrot growth and development of typical cavity spot lesions. Results were variable in seedling and pot experiments, but commercially significant cavity spot was achieved for two consecutive years in the field.

An oospore capture method and qPCR assay were developed to improve capture, detection and quantification of *P. violae* in soil. The oospore capture was successful in detecting *P. violae* oospores from sand/oat samples. The qPCR assay was highly sensitive and specific to *P. violae*. Capture and detection from soil proved challenging with highly variable results. A commercial field site was monitored to establish *P. violae* dynamics, but results were inconsistent.

Abbreviations

χ^2	Chi-squared	*p*	Probability
A.D	*Anno domini*	*P. att*	*P. attrantheridium*
AHDB	Agriculture and Horticulture Development Board	*P. col*	*P. coloratum*
ANOVA	Analysis of Variance	*P. cry*	*P. cryptoirregulare*
Approx.	Approximately	*P. deb*	*P. debaryanum*
B.C	Before Christ	*P. int*	*P. intermedium*
BLAST	Basic Local Alignment Search Tool	*P. irr*	*P. irregulare*
bp	Base pair	*P. sul*	*P. sulcatum*
C	Celsius	*P. syl*	*P. sylvaticum*
CA	California	*P. ult*	*P. ultimum*
CFU	Colony forming unit	*P. vio*	*P. violae*
cm	Centimetre	PCR	Polymerase chain reaction
CMA	Corn Meal Agar	PDB	Potato Dextrose Broth
CoxII	Cytochrome oxidase subunit II	pg	Picogram
Ct	Cycle quantification	pH	Power of hydrogen
cv(s)	Cultivar(s)	Ppm	Parts per million
DEFRA	Department for Environment, Food and Rural Affairs	PVVP	Polyvinylpolypyrrolidone
DMSO	Dimethyl sulfoxide	qPCR	quantitative PCR
DNA	Deoxyribonucleic acid	R	Reverse
ELIZA	Enzyme-linked immunosorbent assay	rDNA	Ribosomal DNA
EU	European Union	REML	Restricted Maximum Likelihood
Expt.	Experiment	RNA	Ribonucleic acid
F	Forward	rpm	Revolutions per minute
F:	F-statistic	rRNA	Ribosomal RNA
f. sp.	*Formae speciales*	SDW	Sterile Distilled Water
g	gram	SED	Standard error of the differences of the mean
GA	Georgia	SEM	Standard error of the mean
h	Hour	spp.	Species
ha	Hectare	SYBR	Synergy Brands
HCl	Hydrochloric acid	UK	United Kingdom
ITS	Internal transcribed spacer region	USA	United Stated of America
Kb	Kilobase	UV	Ultraviolet
kg	Kilograms	v/v	Volume by volume
L	Litre	V8B	Vegetable Juice Broth
Log	Logarithm	VB8+C	VB8+cholesterol
LSD	Least significant difference	VBA	Vegetable Broth Agar
M	Metre	w.p.s	Weeks post sowing
max.	Maximum	w/w	Weight by weight
mg	Milligram	WCC	Warwick Crop Centre
mm	Millilitre	WI	Wisconsin
NADH	NADH dehydrogenase Subunit 1	x g	Relative centrifugal force
ng	Nanogram	µl	Microlitre
no.	Number	µm	Micrometre
OCM1	*Ochromonas mastigoneme*-like protein	µM	Micromole
OH	Ohio		

1 General Introduction

1.1 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 (R. Hobson, Hobson Farming Ltd., UK, personal communication). 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).

The domesticated carrot is thought to have originated in Afghanistan and Central Asia around 2500 B.C. First uses are believed to have been medicinal, using the carrot seed (Simon, 2000). The first report of cultivation is around 900 A.D, where a larger root was selected for (Simon, 2000). Carrot roots at those times were yellow or purple, the selection for an orange root occurred in Europe around the early 17th century (Simon, 2000). Carrots have a wide range of significant health benefits. Carrots contain high levels of the phytochemicals carotenoids, and as the carrot has been cultivated these levels have increased (Silva Dias, 2014). Carotenoids are the yellow, orange and red coloured phytochemicals found in yellow/orange cultivars, and are converted into vitamin A in the body, which aids the growth of healthy bones, teeth and eyes (Silva Dias, 2014; BCGA, 2018). The most widely used orange carrot is high in both α- and β-carotene, and is therefore a rich source of provitamin A (Silva Dias, 2014). Carrots also have anticarcinogen benefits. The carotenoids act as antioxidants which have been shown to

decrease the risk of some cancers (Zaini *et al.*, 2011). Carrots also contain bioactive polyacetylenes such as falcarinol, which are thought to stimulate cancer-fighting mechanisms in the body (Silva Dias, 2014). Carrots are rich in dietary fibre, and a good source of the trace mineral molybdenum, not found in many vegetables. They are also a good source of potassium, magnesium and manganese (Silva Dias, 2014).

The time taken for carrots to reach maturity depends on the cultivar used and the growing conditions during the season. Typically, a characteristic root will form 1-2 months after sowing, and will swell and turn orange as it matures (3-6 months post germination) (CABI, 2017). Land for growing carrots must be free-draining, hence very sandy soils are preferred as heavy, clay soils can induce malformations in the root (CABI, 2017). Soil must also be de-stoned for roots to grow straight (I. Holmes, Strawsons Ltd., UK, personal communication). Carrots are a temperate crop: temperatures over 25˚C will slow growth and may leave roots with poor colour (Simon, 2000; CABI, 2017). Irrigation is vital to achieve good quality, smooth roots as well as a high yield. 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).

1.2 Cavity spot disease of carrot

1.2.1 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).

1.2.2 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).

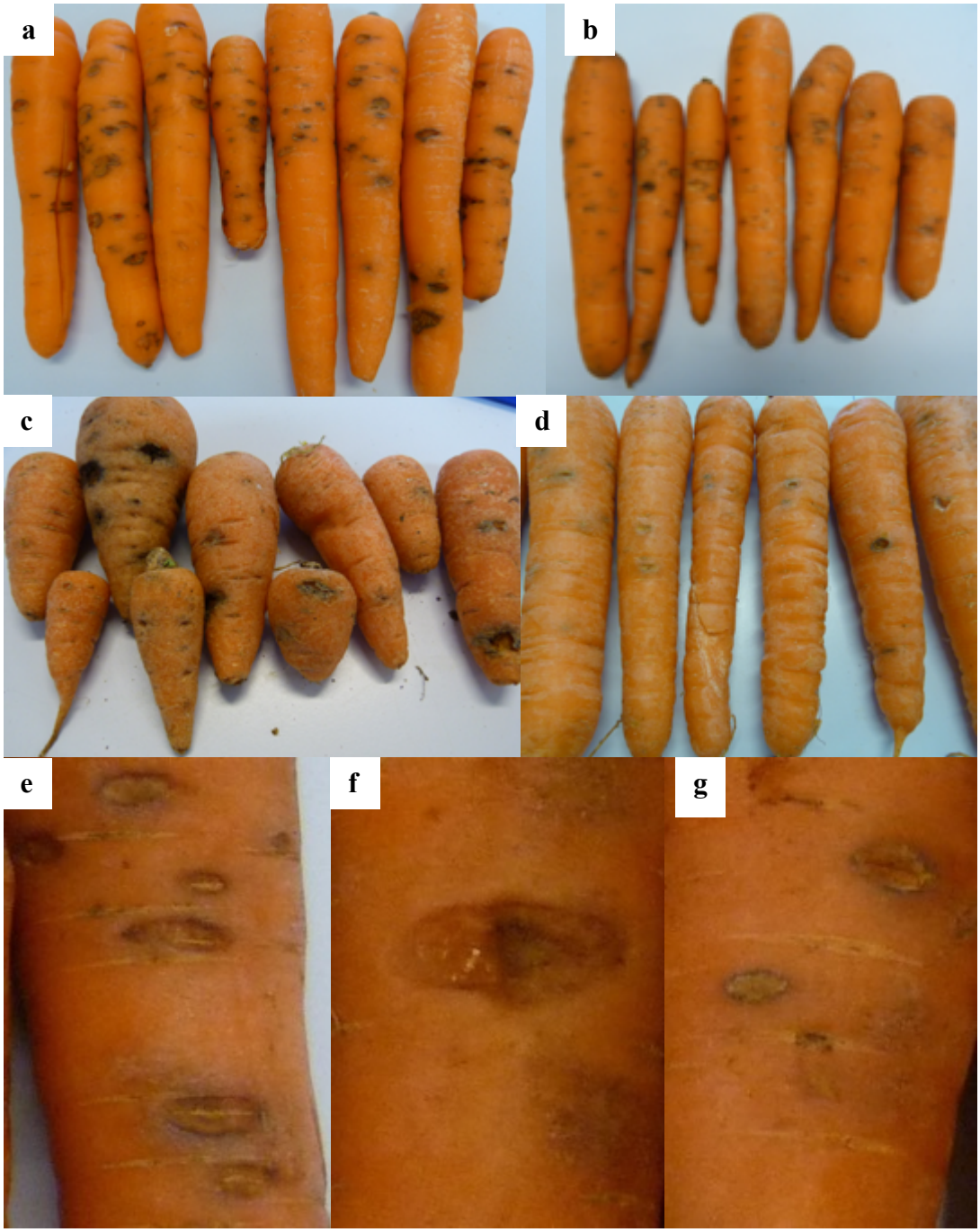


Figure 1.1 Symptoms of cavity spot. **a-d: Carrots from commercial carrot crops:** Roots from Yorkshire (Oct. 2014), lesions are dark, expanded and concentrated on the upper half of some roots (a); roots from Nottinghamshire (Oct. 2014), lesions are lighter in colour with some darkening/expansion (b); 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).

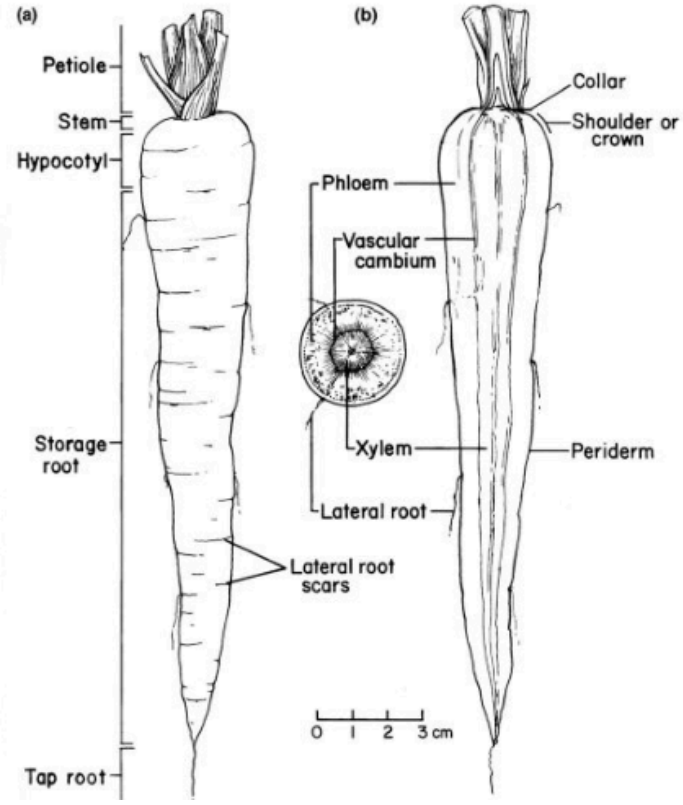


Figure 1.2 Carrot root anatomy exterior (a) and interior (b) as described by Kjellenberg (2007); reprinted from Rubatzsky *et al.* (1999).

1.2.3 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, and it was hypothesised that *Clostridium* bacteria may induce cavity spot symptoms in more anaerobic environments. Although the bacteria was not isolated from the majority of cavity spot lesions, poor soil aeration continued to be thought of as a possible influencing factor in cavity spot development (Goh & Ali, 1983) particularly in combination with environmental stress such as high temperatures (Soroker *et al.*, 1984). 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.

1.2.4 *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 1.1 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)

Suffert and Guibert (2007) studied the ecology of the *Pythium* community in cavity spot lesions and found slower growing species (*P. violae* and *P. sulcatum*) were often found individually in lesions, whereas faster growing species including *P. intermedium*, *P. sylvaticum* and *P. ultimum* were more often found as a complex. 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.

1.3 *Pythium*

1.3.1 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). There are a number of distinctions between the oomycota and the true fungi, including differences in cell wall composition, production of zoospores and flagella, and hyphal structure (Rossman & Palm, 2006). Oomycetes also produce oospores during sexual reproduction. These are thick-walled resting structures, formed by the fusion of an oogonium and an antheridium. True fungi do not produce oospores; sexual reproduction varies depending on the specific genus, however spores are often formed in large numbers inside complex structures (Fry & Grünwald, 2010).

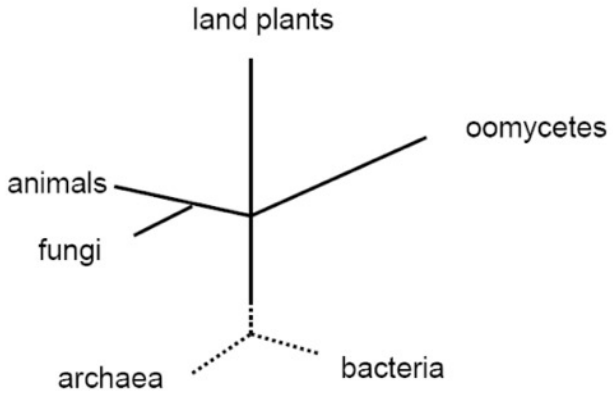


Figure 1.3 Schematic of the relationship between oomycetes, land plants, animals and fungi as described by Fry and Grünwald (2010); image adapted from http://home.planet.nl/~gkorthof/images/tree_of_life2.jpg.

1.3.2 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 1.2 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). The life cycle of *Pythium* has been described by Agrios (2005) (Figure 1.5). Asexual reproduction can occur via sporangia: these grow from the mycelia, and can germinate either via germ tubes or form vesicles which contain zoospores. A germ tube can penetrate the host tissue to initiate infection. Sexual reproduction occurs when the mycelium gives rise to (haploid) oogonia and antheridia, which connect with a fertilisation tube. The nucleus from the antheridium then moves to the nucleus in the oogonium where fusion occurs to form a diploid oospore. Oospores serve as long-term survival structures and usually require a resting period, after which they germinate, as described for sporangia.

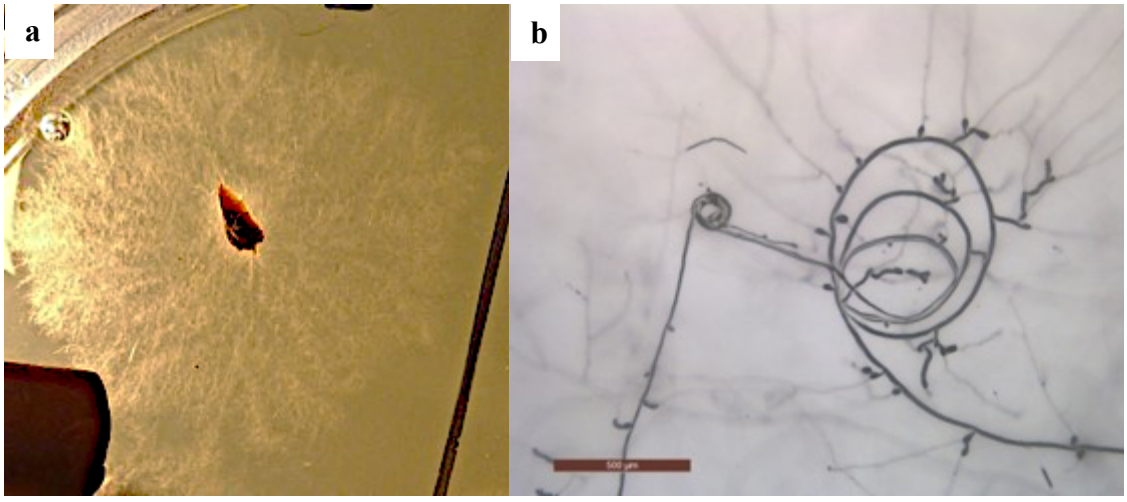


Figure 1.4 *Pythium violae* isolate P.10: mycelium growing on CMA + rifampicin (30mg L^{-1}) from a cavity spot lesion (a) and characteristic coil of mycelium growing on CMA (b).

1.3.3 *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).

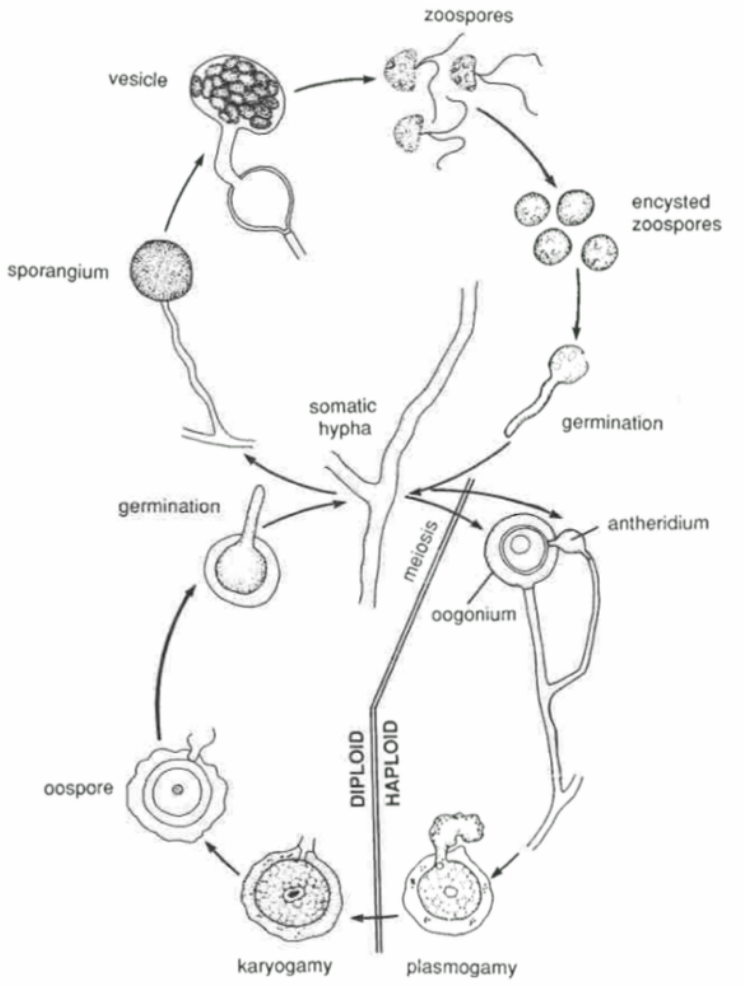


Figure 1.5 Life cycle of *Pythium* (Kendrick, 2000). Sexual reproduction occurs via fertilisation of the oogonium by the antheridium, and a diploid oospore is formed. Germination of the oospore produces mycelial growth, and oogonia can be formed from the hyphae. Asexual reproduction occurs via formation of sporangia from mycelial growth. *P. violae* has not been observed to produce zoospores.

1.3.4 *P. violae* host range and epidemiology

1.3.4.1 *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 (*Solanum nigrum*), prickly sow-thistle (*Sonchus asper*) and knot-grass (*Paspalum distichum*) (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).

1.3.4.2 *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. Suffert and Montfort (2007) comprehensively studied the development of cavity spot and the infection process, and concluded that that there are cycles of primary and secondary infection. Primary infection results from oospores, while under favourable environmental conditions, secondary infections occur through growth of mycelium along and between roots, contributing to further disease development (Phelps *et al.*, 1991; Suffert & Montfort, 2007).

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.

1.3.4.3 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.

1.3.4.4 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). For example, there has been conflicting evidence on the importance of soil nutrient levels in

relation to cavity spot disease development. When Guba *et al.* (1961) first described cavity spot, it was suggested there was a link between cavity spot and low nutrient levels in the soil. It has also been reported that low levels of calcium (caused by reduced uptake due to high potassium levels) caused cavity spot levels to increase (Hiltunen & White, 2002). However, a number of other studies have reported no association between cavity spot and any soil nutrient levels, including no relationship between cavity spot and calcium or potassium (Perry & Harrison, 1979; Soroker *et al.*, 1984; White, 1986; Vivoda *et al.*, 1991). Furthermore, it has been reported that there is no relationship between cavity spot incidence and a wide range of soil factors, including organic matter, soil electrical conductivity or moisture holding capacity (Hiltunen & White, 2002).

Similarly, there have been contradictory reports about the role of soil pH on cavity spot development. Perry and Harrison (1979) reported cavity spot incidence was at its lowest when soil pH was below 6.6. Since then, a number of studies have found cavity spot was reduced when the pH was above 7.0, and increased when below pH 6.5 or 5.5 (Scaife *et al.*, 1983; Perry & Groom, 1984). In addition, the influence of soil temperature on *P. violae* growth and cavity spot development has been debated. In the laboratory *P. violae* has been grown at "optimum" temperatures *in vitro* of both 17-20˚C (White, 1993; Schrandt *et al.*, 1994), and 25˚C (van der Plaats-Niterink, 1981). Similarly, although cavity spot is generally considered to develop at lower temperatures, it has been reported to be favoured at temperatures of 15˚C (Vivoda *et al.*, 1991), 20˚C (White *et al.*, 1993) and 28˚C (Hiltunen & White, 2002) in different countries. No consistent relationship has been found between temperature and *Pythium* spp. or isolate, and hence is thought to be less important than other environmental factors (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).

Growers regularly report that cavity spot is a disease that develops later in the season. Cavity spot is rarely seen before August, and the severity of cavity spot increases as plants mature (Montfort & Rouxel, 1988). In field experiments with different cultivars conducted by the National Institute for Agricultural Botany (NIAB), cultivars were often rated more susceptible when harvested later in the season (Hiltunen & White, 2002). It is unclear whether this increase in cavity spot disease severity is due to conditions favouring lesion development/expansion, or plants becoming more susceptible as they mature (Vivoda *et al.*, 1991). Furthermore, although very few studies have monitored disease development over time, White *et al.* (1995) found that carrots in some fields showed a gradual increase in cavity spot over the season (July-January) whilst in other fields incidence was less than 10% in July, but then did not increase beyond 14% incidence by the following February. It seems likely that some soils are more conducive to disease, and others act to suppress the disease depending on the community of microbes present in the soil, however the factors determining this have not been studied. 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.

1.4 Management and control of cavity spot

1.4.1 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.

1.4.2 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).

Many growers plant early crops in fields suspected to be conducive to cavity spot disease development to try to avoid disease by sowing and harvesting earlier in the season. In general, cavity spot develops from late August onwards and therefore crops that are sown in January or February, and harvested in July or August are much less likely to be affected than later sown crops (R. Hobson, Hobson Farming Ltd., UK, personal communication). Although the reasons for this effect are not clear, it is likely to be related to environmental conditions e.g. soil temperature. This strategy is more commonly used on land that either has been more recently cropped with carrots, or has the potential for poor soil drainage (R. Hobson, Hobson Farming Ltd., UK, personal communication). Many growers resort to regular sampling of carrot fields to determine disease levels, and harvest early to

minimise losses if cavity spot is detected (Pettitt & Gladders, 2003). This technique is also used to identify fields that can be strawed down for winter. However, this approach is not always reliable, as discussed above, fields with roots not showing any cavity spot disease pressure can develop significant cavity spot over the winter months. Furthermore, regular sampling is both time-consuming and has associated labour costs.

1.4.3 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.

1.4.4 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.

1.5 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.

1.6 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.

1.7 Structure of thesis

Chapter 2 – Identification, characterisation and pathogenicity of *Pythium* species causing cavity spot in the UK

This chapter describes the molecular identification and phylogenetic analysis of *Pythium* spp. collected from carrots with cavity spot from different locations in the UK and their pathogenicity on carrot roots in the laboratory.

Chapter 3 – Development of *P. violae* artificial inoculation systems for carrot

This chapter describes the development of an oospore inoculum production method for *P. violae* in both solid and liquid substrates, and the development of artificial inoculation systems for seedlings and mature plants in controlled environment, glasshouse and field.

Chapter 4 – Development of quantitative methods for detecting *P. violae*

This chapter describes the development of methods for oospore capture from soil, DNA extraction and qPCR for *P. violae* detection and quantification, and application using a field soil from a commercial carrot field.

Chapter 5 – General Discussion

This chapter concludes the thesis with a summary and discussion of all the results from the previous chapters, and outlines potential future work.

2 Identification, characterisation and pathogenicity of *Pythium* species causing cavity spot in the UK

2.1 Introduction

2.1.1 *Pythium* spp. causing cavity spot

Attempts to understand the cause of cavity spot had a significant breakthrough when Lyshol *et al.* (1984) found 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.

North America

McDonald (1994) found that *P. violae*, *P. ultimum* and *P. irregulare* could cause cavity spot in Ontario, Canada, whilst Benard and Punja (1995) identified eight *Pythium* spp. associated with the disease in British Columbia, Canada. Of these, *P. violae* and *P. sulcatum* were regarded as the most important, and *P. irregulare* and *P. ultimum* were thought of as less important, with *P. sylvaticum* being non-pathogenic. In Quebec, the majority of isolates identified from cavity spot infected roots were either *P. sulcatum* or *P. sylvaticum* (Allain-Bouléa *et al.*, 2004). *P. ultimum* and *P. violae* were reported to be the major species associated with cavity spot in California, USA (Vivoda *et al.*, 1991).

Australia

P. sulcatum was found to be the major cause of cavity spot in Australia (Davison & McKay, 1998), but *P. ultimum*, *P. coloratum* and *P. violae* have also been associated with the disease (El-Tarabily *et al.*, 1996; Davison & McKay, 1998).

Europe

Hermansen *et al.* (2007) reported that there were five *Pythium* spp. important in causing cavity spot in Norway. *P. intermedium* and *P. sulcatum* were predominant, whilst *P. violae*, *P. sylvaticum* and a potential new species *P. vipa* were isolated less often. In The Netherlands *P. sulcatum* was the main species associated with the disease (Wagenvoort *et al.*, 1989) while in France cavity spot was shown to be principally caused by *P. violae* and *P. sulcatum* (Montfort & Rouxel, 1988; Guerin *et al.*, 1994). *P. violae* was also shown to be associated with cavity spot in Israel (White *et al.*, 1993), alongside P. *irregulare* (Shelvin *et al.*, 1987).

UK

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.

2.1.2 Methods to isolate *Pythium* spp. from cavity spot lesions

The slow growth shown by *P. violae* and *P. sulcatum* has contributed to our limited understanding of them as pathogens. These *Pythium* spp. are easily out-competed in culture, making them very difficult to isolate from cavity spot lesions unless the lesions are young and newly formed (Hiltunen & White, 2002; Koike *et al.*, 2007; Kageyama, 2014). Antibiotics and anti-fungal compounds are routinely included to help allow slow-growing *Pythium* spp. to establish. A number of artificial media have been identified as more conducive to *Pythium* growth, including V8 vegetable juice agar (Guo & Ko, 1991)

and cornmeal agar containing anti-microbial compounds including pimaricin (100 mg^{-l}) and rifampicin (30 mg^{-l}) (White, 1988; Koike *et al.*, 2007).

2.1.3 Identification and characterisation of *Pythium* spp.

Traditionally, a range of morphological characteristics have been used for identification of *Pythium* spp. (Suffert & Guibert, 2007; Uzuhashi *et al.*, 2010; Kageyama, 2014), but the importance of different criterias have been debated. Kageyama (2014) reviewed the morphological and molecular taxonomy of the genus *Pythium*, highlighting that since the original taxonomies were proposed by Matthews (1931) and Sideris (1932), a number of more recent keys have been published, with each author emphasising differing reproductive/structural morphologies as the most important in separating species. The most commonly used of these is van der Plaats-Niterink (1981) 'Monograph of the genus *Pythium'* which considered that the characteristics of the oospore and oogonial wall, presence of reproductive structures, sporangial morphology and characteristics of the antheridium, were all central to classifying the members of this genus. However, it is commonly thought that the historical lack of consensus as well as the high variability in these morphological characteristics, including a high number of atypical isolates, variations in size and shape of structures, or no formation of structures on agar, which can occur within species, complicates accurate identification. These problems are particularly difficult for those with minimal experience with this genus (Levesque & de Cock, 2004; Suffert & Guibert, 2007; Paul *et al.*, 2008; Uzuhashi *et al.*, 2010; Kageyama, 2014).

More recently, molecular methods using DNA sequences and phylogenetic analyses have been developed to supplement morphological taxonomy and have allowed successful identification of isolates that were previously problematic to identify (Uzuhashi *et al.*, 2010; Kageyama, 2014). The ribosomal DNA (rDNA) region is routinely used for identification of fungi and oomycetes, primarily the Internal Transcribed Spacer (ITS) region (Levesque & de Cock, 2004; Uzuhashi *et al.*, 2010; Capote *et al.*, 2012). This region is amplified using a universal primer set developed by White *et al.* (1990) which amplifies this highly variable region throughout all fungi and oomycetes. Many phylogenetic analyses have been performed using the ITS region of *Pythium* which have revealed a much longer ITS region (750-1050bp) than the Eumycota, allowing greater sequence length for analysis. Levesque and de Cock (2004) conducted a comprehensive phylogeny at the genus level with 116 *Pythium* spp. using the ITS region of the rDNA

and concluded that this approach was robust, as nearly all *Pythium* spp. are easily resolved due to the increased length ITS 1 and 2 spacer regions, allowing identification of unknown isolates with greater accuracy.

The Cytochrome Oxidase Subunit II (CoxII) gene is another 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.

2.1.4 Pathogenicity experiments

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 posses 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.

2.1.5 Aims

The overall aim of the work in this chapter was to identify the current *Pythium* species associated with cavity spot in the UK, and build understanding of *Pythium* biology by investigating the phylogeny and virulence of a range of *Pythium* isolates. The objectives of the work described in this chapter were:

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

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

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

2.2 Materials and methods

2.2.1 Sampling of carrots and isolation of *Pythium* spp.

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 from third parties where the sampling location was unknown or confidential. The number of isolates collected from any location was based on the number of samples sent from that area. Most samples were obtained during the autumn and winter seasons, as this is when cavity spot was most prevalent. Location data and carrot cultivar were recorded for all isolates where possible.

Table 2.1 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

Pythium isolation and storage

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 2.2 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.

2.2.2 Molecular identification of *Pythium* isolates

To identify and characterise 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^{TM} (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^{TM}) 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.

To identify bacterial species, any typical bacterial growth was sub-cultured onto Lysogeny Broth (LB) media (ThermoFisher, UK) and incubated at 37˚C for two days. Once a pure culture was obtained, a single colony was isolated and spread onto LB media and incubated again at 37˚C for two days. DNA extraction was performed using DNeasy Blood & Tissue Kit (Qiagen, UK). Conventional PCR amplification (GeneAmp ® PCR system 9200) was performed using the primer pair fD1 (ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG) and rP1 (cccgggatccaagcttACGGTTACCTTGTTACGACTT) commonly used for bacterial identification (Weisburg *et al.*, 1991). Thermal cycling parameters consisted of 94˚C for 2 minutes; 35 cycles of 95˚C for 2 minutes, 42˚C for 30 seconds and 72˚C for 4 minutes; and 72˚C for 20 minutes. Each PCR reaction was carried out as described above and gel visualised under UV light to confirm successful amplification. PCR products were purified as above and 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 2.3 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

2.2.3 Molecular characterisation of *Pythium* isolates

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 2.4 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*

2.2.4 Carrot root inoculation with *Pythium* isolates

2.2.4.1 *Pythium* isolate pathogenicity experiments

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 2.1 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.

2.2.4.2 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 the DEFRA-funded Vegetable Genetics Resource Network project (VeGIN) based on differences in cavity spot incidence in a previous field experiment). 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 03/05/2017 (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 (22/05/2017) 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 in Section 2.2.4.1 (Figure 2.2).

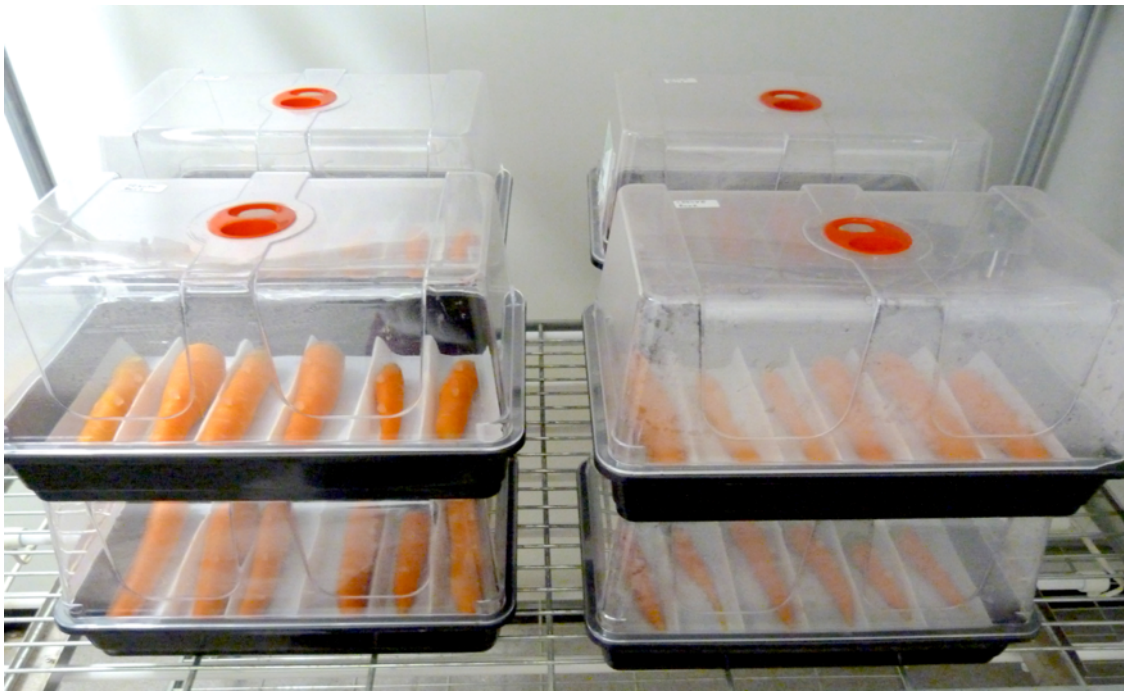


Figure 2.2 Carrot roots arranged on shelves in controlled environment room, inoculated with plugs of *P. violae* isolates P10.

2.3 Results

2.3.1 Molecular ID of *Pythium* isolates and distribution of species

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). A number of other fungal species were also consistently isolated alongside *Pythium* including *Rhizoctonia* spp., *Neonectria* spp., *Mucor* spp. and *Mycocentrospora acerina* (Figure 2.3 c/d). Bacterial species often associated with lesions included *Pseudomonas reinekei*, *Stentrophomonas maltophilia* and *Achromobacter xylosoxidans*).

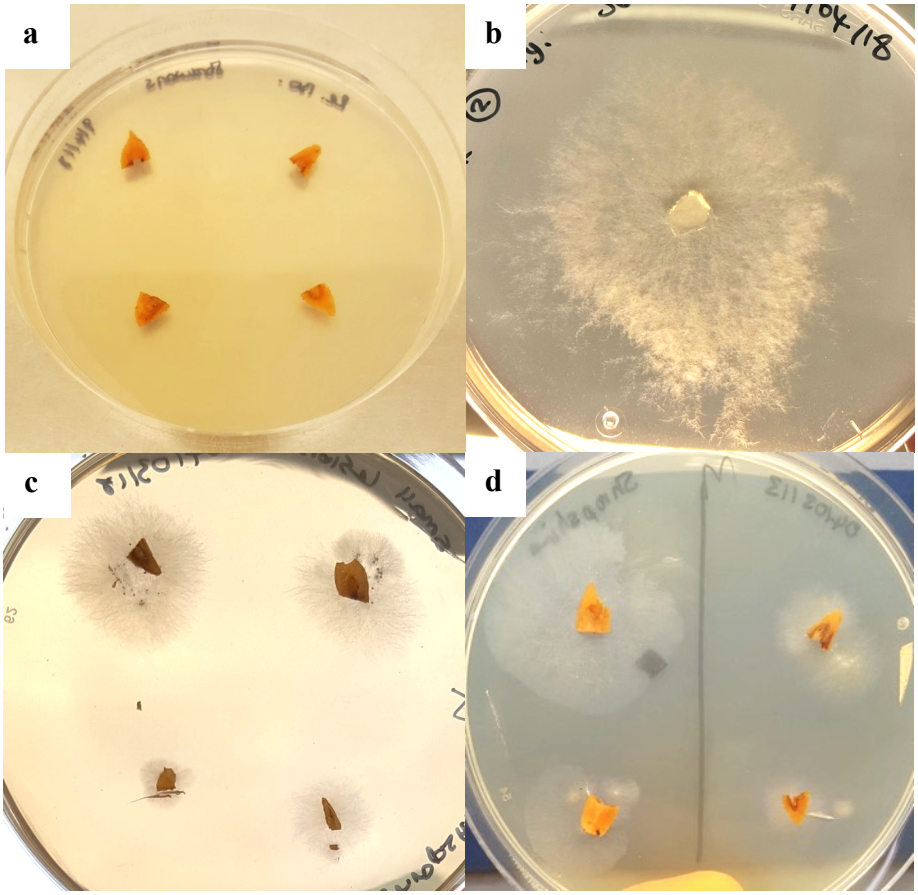


Figure 2.3 Cavity spot lesions placed on CMA plate for isolation of *Pythium* species (a); *P. violae* growing on CMA after isolation from a cavity spot lesion (b); a range of other fungal species growing out of cavity spot lesions on CMA plate (c)&(d).

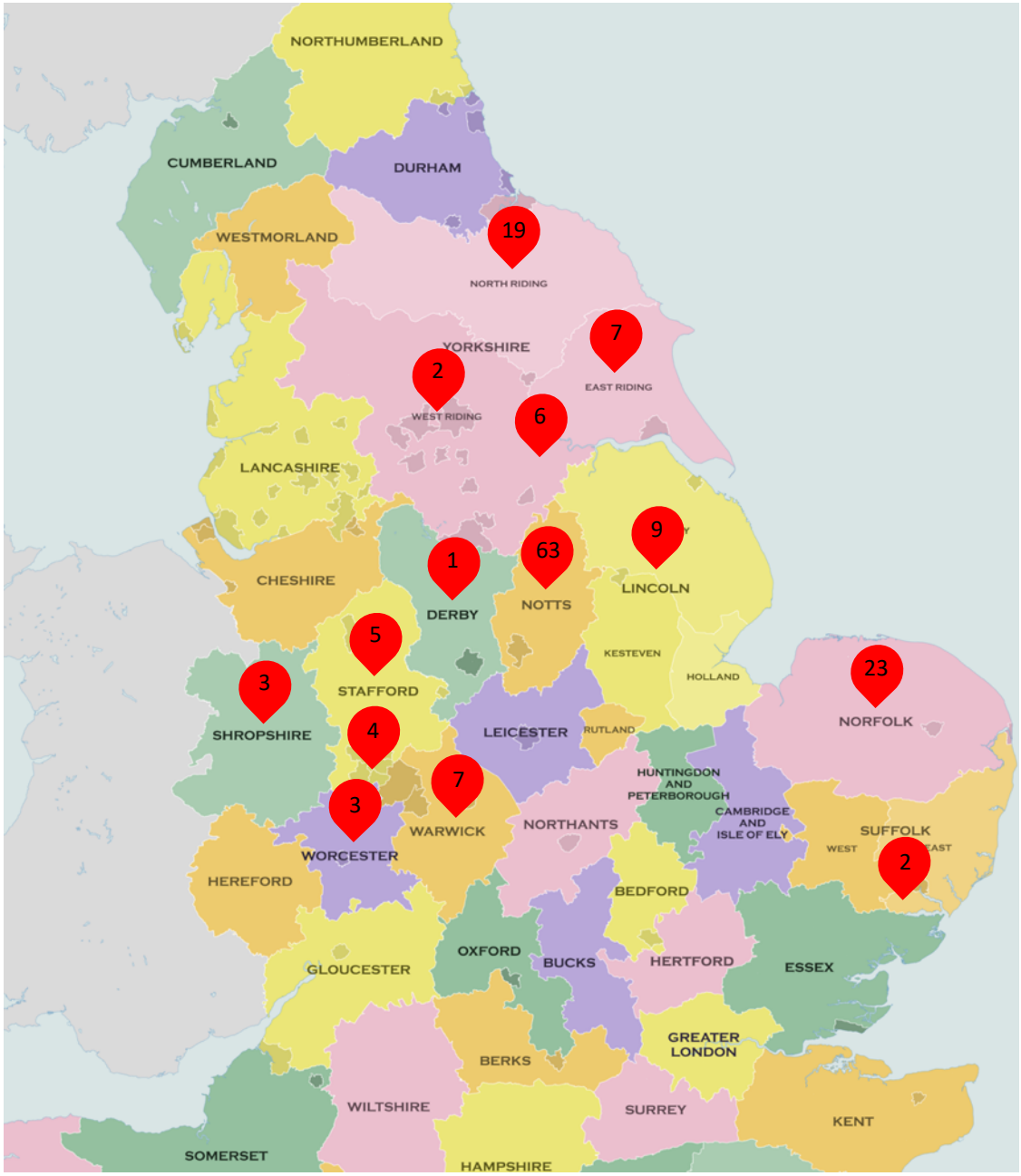


Figure 2.4 Map of *Pythium* isolates of known locations collected in UK by county (2013-2017; total 155). A total of 34 were collected from Yorkshire and the Humber, 74 from the East Midlands, 22 from the West Midlands, and 25 from the East of England. Nine UK collected isolates were of unknown or confidential locations.

Table 2.5 Carrot cultivar, location, collection date and identity of 178 *Pythium* isolates collected and examined in this study.

***Pythium* isolate**	**Cultivar**	**County**	**Region**	**Date**	**Identity**
P1	Vac 63	Norfolk	East of England	Oct.13	*P.violae*
P2	Elegance	Norfolk	East of England	Oct.13	*P.violae*
P4	Match	Norfolk	East of England	Oct.13	*P.violae*
P5	Stanford	Norfolk	East of England	Oct.13	*P.intermedium*
P7	Match	Norfolk	East of England	Oct.13	*P.violae*
P8	Bangor	North Yorkshire	Yorkshire and the Humber	Nov.13	*P.violae*
P10	Nairobi	Lincolnshire	East Midlands	Oct.13	*P.violae*
P11	Nairobi	Suffolk	East of England	Oct.13	*P.violae*
P12	Nairobi	East Yorkshire	Yorkshire and the Humber	Oct.13	*P.violae*
P14	Confidential	North Yorkshire	Yorkshire and the Humber	Oct.13	*P.intermedium*
P15	Nairobi	Norfolk	East of England	Nov.13	*P.violae*
P16	Nairobi	Suffolk	East of England	Nov.13	*P.violae*
P17	Nairobi	Norfolk	East of England	Dec.13	*P.violae*
P18	Unknown	South Yorkshire	Yorkshire and the Humber	Dec.13	*P.violae*
P19	Confidential	North Yorkshire	Yorkshire and the Humber	Jan.14	*P.violae*
P21	Nairobi	Warwickshire	West Midlands	Oct.07	*P.violae*
P23	Unknown	-	The Netherlands	Jan.14	*P.violae*
P27	Nairobi	North Yorkshire	Yorkshire and the Humber	Jan.14	*P.violae*
P29	Nairobi	Norfolk	East of England	July.14	*P.violae*
P30	Nairobi	East Yorkshire	Yorkshire and the Humber	Oct.14	*P.violae*
P31	Nairobi	East Yorkshire	Yorkshire and the Humber	Oct.14	*P.violae*
P32	Nairobi	South Yorkshire	Yorkshire and the Humber	Oct.14	*P.violae*
P33	Nairobi	South Yorkshire	Yorkshire and the Humber	Oct.14	*P.violae*
P34	Nairobi	South Yorkshire	Yorkshire and the Humber	Oct.14	*P.violae*
P36	Parsnip	Nottinghamshire	East Midlands	Oct.14	*P.violae*
P38	Nairobi	Lincolnshire	East Midlands	Oct.14	*P.violae*
P40	Nairobi	Nottinghamshire	East Midlands	Oct.14	*P.violae*
P41	Nairobi	Nottinghamshire	East Midlands	Oct.14	*P.violae*
P42	Nairobi	Nottinghamshire	East Midlands	Oct.14	*P.violae*
P43	Nairobi	Nottinghamshire	East Midlands	Oct.14	*P.sulcatum*
P44	Nairobi	Lincolnshire	East Midlands	Oct.14	*P.violae*
P45	Nairobi	Nottinghamshire	East Midlands	Oct.14	*P.violae*
P48	Nairobi	Nottinghamshire	East Midlands	Oct.14	*P.attrantheridium*
P50	Unknown	East Yorkshire	Yorkshire and the Humber	Oct.14	*P.violae*
P51	Unknown	Lincolnshire	East Midlands	Oct.14	*P.violae*
P52	Unknown	North Yorkshire	Yorkshire and the Humber	Oct.14	*P.violae*
P53	Unknown	North Yorkshire	Yorkshire and the Humber	Oct.14	*P.violae*
P54	Unknown	North Yorkshire	Yorkshire and the Humber	Oct.14	*P.violae*
P55	Norfolk	Norfolk	East of England	Oct.14	*P.violae*
P56	Newark	Norfolk	East of England	Oct.14	*P.violae*
P57	Nairobi	North Yorkshire	Yorkshire and the Humber	Nov.14	*P.violae*
P58	Norfolk	Norfolk	East of England	Nov.14	*P.violae*
P59	Nairobi	North Yorkshire	Yorkshire and the Humber	Oct.14	*P.violae*
P61	Chantenay	Nottinghamshire	East Midlands	Oct.14	*Pythium* spp.
P62	Chantenay	Staffordshire	West Midlands	Oct.14	*P.violae*
P63	Nairobi	Lincolnshire	East Midlands	Oct.14	*P.violae*
P64	Chantenay	Nottinghamshire	East Midlands	Oct.14	*P.violae*
P65	Chantenay	Staffordshire	West Midlands	Oct.14	*P.violae*
P67	Chantenay	Nottinghamshire	East Midlands	Oct.14	*P.sulcatum*
P70	Chantenay	Nottinghamshire	East Midlands	Oct.14	*P.violae*
P74	Chantenay	Nottinghamshire	East Midlands	Oct.14	*P.violae*
P78	Chantenay	Telford & Wrekin	West Midlands	Oct.14	*P.sulcatum*
P79	Chantenay	Nottinghamshire	East Midlands	Oct.14	*P.violae*
P80	Chantenay	Nottinghamshire	East Midlands	Oct.14	*P.intermedium*
P81	Unknown	Unknown	Unknown	Unknown	*P.violae*
P82	Unknown	Unknown	Unknown	Unknown	*P.violae*
P83	Unknown	Unknown	Unknown	Unknown	*Pythium* spp.
P86	Unknown	Unknown	Unknown	Unknown	*P.intermedium*
P87	Unknown	Unknown	Unknown	Unknown	*P.debaryanum*
P89	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.violae*
P90	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.violae*

P91	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.sulcatum*
P93	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.violae*
P95	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.intermedium*
P96	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.violae*
P97	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.violae*
P98	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.intermedium*
P100	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.sylvaticum*
P102	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.intermedium*
P103	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.violae*
P105	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.violae*
P106	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.violae*
P107	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.intermedium*
P108	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.sulcatum*
P109	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.sulcatum*
P110	Nairobi	Nottinghamshire	East Midlands	Nov.14	*P.sylvaticum*
P111	Chantenay	Nottinghamshire	East Midlands	Dec.14	*P.violae*
P112	Chantenay	Telford & Wrekin	West Midlands	Dec.14	*P.intermedium*
P119	Chantenay	Nottinghamshire	East Midlands	Dec.14	*P.violae*
P122	Nairobi	Lincolnshire	East Midlands	Dec.14	*P.intermedium*
P123	Chantenay	Nottinghamshire	East Midlands	Dec.14	*P.violae*
P124	Chantenay	Nottinghamshire	East Midlands	Dec.14	*P.violae*
P125	Chantenay	West Midlands	West Midlands	Dec.14	*P.violae*
P126	Chantenay	Nottinghamshire	East Midlands	Dec.14	*Pythium* spp.
P127	Chantenay	Kidderminster	Worcestershire	Dec.14	*P.sulcatum*
P128	Chantenay	Worcestershire	West Midlands	Dec.14	*P.intermedium*
P130	Chantenay	Nottinghamshire	East Midlands	Dec.14	*P.violae*
P131	Chantenay	Lincolnshire	East Midlands	Dec.14	*P.violae*
P132	Chantenay	Nottinghamshire	East Midlands	Dec.14	*P.sulcatum*
P134	Nairobi	Warwickshire	West Midlands	Dec.14	*P.violae*
P135	Nairobi	Warwickshire	West Midlands	Dec.14	*P.violae*
P136	Nairobi	Warwickshire	West Midlands	Dec.14	*P.sulcatum*
P137	Nairobi	East Yorkshire	Yorkshire and the Humber	Jan.15	*P.violae*
P138	Nairobi	North Yorkshire	Yorkshire and the Humber	Jan.15	*P.violae*
P139	Nairobi	North Yorkshire	Yorkshire and the Humber	Jan.15	*P.intermedium*
P140	Nairobi	East Yorkshire	Yorkshire and the Humber	Jan.15	*P.violae*
P142	Nairobi	North Yorkshire	Yorkshire and the Humber	Jan.15	*P.violae*
P143	Nairobi	East Yorkshire	Yorkshire and the Humber	Jan.15	*Pythium* spp.
P144	Nairobi	Lincolnshire	East Midlands	Jan.15	*P.violae*
P145	Nairobi	Nottinghamshire	East Midlands	Feb.15	*P.sylvaticum*
P146	Nairobi	Nottinghamshire	East Midlands	Feb.15	*P.sylvaticum*
P147	Nairobi	Nottinghamshire	East Midlands	Feb.15	*P.intermedium*
P148	Nairobi	Nottinghamshire	East Midlands	Feb.15	*P.intermedium*
P149	Nairobi	Derbyshire	East Midlands	Feb.15	*P.violae*
P150	Nairobi	Nottinghamshire	East Midlands	Feb.15	*P.violae*
P151	Nairobi	Nottinghamshire	East Midlands	Feb.15	*P.sulcatum*
P152	Nairobi	West Yorkshire	Yorkshire and the Humber	Feb.15	*P.violae*
P153	Nairobi	West Yorkshire	Yorkshire and the Humber	Feb.15	*P.sulcatum*
P154	Nairobi	Nottinghamshire	East Midlands	Feb.15	*P.sulcatum*
P155	Nairobi	Unknown	East Midlands	Feb.15	*P.sulcatum*
P156	Nairobi	Nottinghamshire	East Midlands	Feb.15	*P.sulcatum*
P158	Nairobi	Nottinghamshire	East Midlands	Feb.15	*P.sulcatum*
P159	Chantenay	Telford & Wrekin	West Midlands	Feb.15	*P.intermedium*
P160	Chantenay	Staffordshire	West Midlands	Feb.15	*P.intermedium*
P161	Chantenay	Worcestershire	West Midlands	Feb.15	*P.intermedium*
P162	Chantenay	Nottinghamshire	East Midlands	Feb.15	*P.irregulare*
P163	Chantenay	Nottinghamshire	East Midlands	Feb.15	*P.violae*
P164	Chantenay	Worcestershire	West Midlands	Feb.15	*P.violae*
P165	Chantenay	Nottinghamshire	East Midlands	Mar.15	*P.sulcatum*
P166	Chantenay	Nottinghamshire	East Midlands	Mar.15	*P.sulcatum*
P167	Chantenay	South Yorkshire	Yorkshire and the Humber	Mar.15	*P.intermedium*
P168	Chantenay	Nottinghamshire	East Midlands	Mar.15	*P.intermedium*
P169	Chantenay	Staffordshire	West Midlands	Mar.15	*P.intermedium*
P170	Unknown	Norfolk	East of England	Jan.14	*P.violae*
P173	Unknown	Unknown	Unknown	Unknown	Unknown
P174	Unknown	Unknown	Unknown	Unknown	*P.ultimum*
P175	Unknown	Unknown	Unknown	Unknown	*P.ultimum*

P180	Unknown	Unknown	The Netherlands	Apr.15	*P.sulcatum*
P181	Unknown	Unknown	The Netherlands	Apr.15	*Pythium* spp.
P182	Unknown	Unknown	The Netherlands	Apr.15	*Pythium* spp.
P183	Unknown	Unknown	The Netherlands	Apr.15	*Pythium* spp.
P184	Unknown	Unknown	The Netherlands	Apr.15	*Pythium* spp.
P185	Unknown	Unknown	The Netherlands	Apr.15	*P.sylvaticum*
P186	Unknown	Unknown	The Netherlands	Apr.15	*Pythium* spp.
P188	Confidential	Confidential	Confidential	Apr.15	*P.violae*
P191	Nairobi	Warwickshire	West Midlands	Apr.15	*P.sylvaticum*
PP192	Chantenay	Staffordshire	West Midlands	Apr.15	*P.intermedium*
P193	Nairobi	Warwickshire	West Midlands	Apr.15	*P.intermedium*
P195	Chantenay	Nottinghamshire	East Midlands	Apr.15	*P.intermedium*
P196	Unknown	Nottinghamshire	East Midlands	Apr.15	*P.sulcatum*
P197	Unknown	Unknown	The Netherlands	Apr.15	*P.irregulare*
P198	Unknown	Unknown	The Netherlands	Apr.15	*P.irregulare*
P199	Unknown	Unknown	The Netherlands	Apr.15	*Pythium* spp.
P201	Nairobi	Warwickshire	West Midlands	Apr.15	*P.sulcatum*
P203	Unknown	Unknown	The Netherlands	Apr.15	*P.sulcatum*
P204	Unknown	Unknown	The Netherlands	Apr.15	*P.sulcatum*
P205	Unknown	Unknown	The Netherlands	Apr.15	*P.sulcatum*
P207	Laguna	Nottinghamshire	East Midlands	Jul.15	*P.sylvaticum*
P208	Laguna	Nottinghamshire	East Midlands	Jul.15	*P.sylvaticum*
P209	Laguna	Nottinghamshire	East Midlands	Jul.15	*P.violae*
P210	Laguna	Nottinghamshire	East Midlands	Jul.15	*P.violae*
P211	Laguna	Nottinghamshire	East Midlands	Jul.15	*P.sylvaticum*
P212	Laguna	Nottinghamshire	East Midlands	Jul.15	*P.sylvaticum*
P213	Laguna	Nottinghamshire	East Midlands	Jul.15	*P.violae*
P214	Laguna	Nottinghamshire	East Midlands	Jul.15	*P.sylvaticum*
P216	Laguna	Nottinghamshire	East Midlands	Jul.15	*P.sylvaticum*
P217	Nairobi	North Yorkshire	Yorkshire and the Humber	Sep.15	*P.violae*
P218	Nairobi	Lincolnshire	East Midlands	Sep.15	*P.violae*
P219	Nairobi	Lincolnshire	East Midlands	Sep.15	*P.violae*
P220	Nairobi	North Yorkshire	Yorkshire and the Humber	Aug.15	*P.ultimum*
P221	Nairobi	North Yorkshire	Yorkshire and the Humber	Aug.15	*P.ultimum*
P222	Nairobi	North Yorkshire	Yorkshire and the Humber	Aug.15	*P.ultimum*
P223	Nairobi	North Yorkshire	Yorkshire and the Humber	Sep.15	*P.violae*
P224	Nairobi	North Yorkshire	Yorkshire and the Humber	Sep.15	*P.violae*
P225	Nairobi	North Yorkshire	Yorkshire and the Humber	Aug.15	*P.intermedium*
P227	Nairobi	Shropshire	West Midlands	May.16	*P.sylvaticum*
P228	Nairobi	Shropshire	West Midlands	May.16	*P.violae*
P231	Confidential	Norfolk	East of England	Feb.17	*P.intermedium*
P232	Nairobi	Norfolk	East of England	Feb.17	*P.violae*
P233	Nairobi	Norfolk	East of England	Feb.17	*P.violae*
P234	Confidential	Norfolk	East of England	Feb.17	*P.sylvaticum*
P235	Nairobi	Norfolk	East of England	Feb.17	*P.violae*
P236	Nairobi	Norfolk	East of England	Feb.17	*P.violae*
P237	Confidential	Norfolk	East of England	Feb.17	*P.violae*
P238	Confidential	Norfolk	East of England	Feb.17	*P.violae*
P239	Confidential	Norfolk	East of England	Feb.17	*P.sylvaticum*
P240	Confidential	Norfolk	East of England	Feb.17	*P.intermedium*
P241	Confidential	Norfolk	East of England	Feb.17	*P.violae*

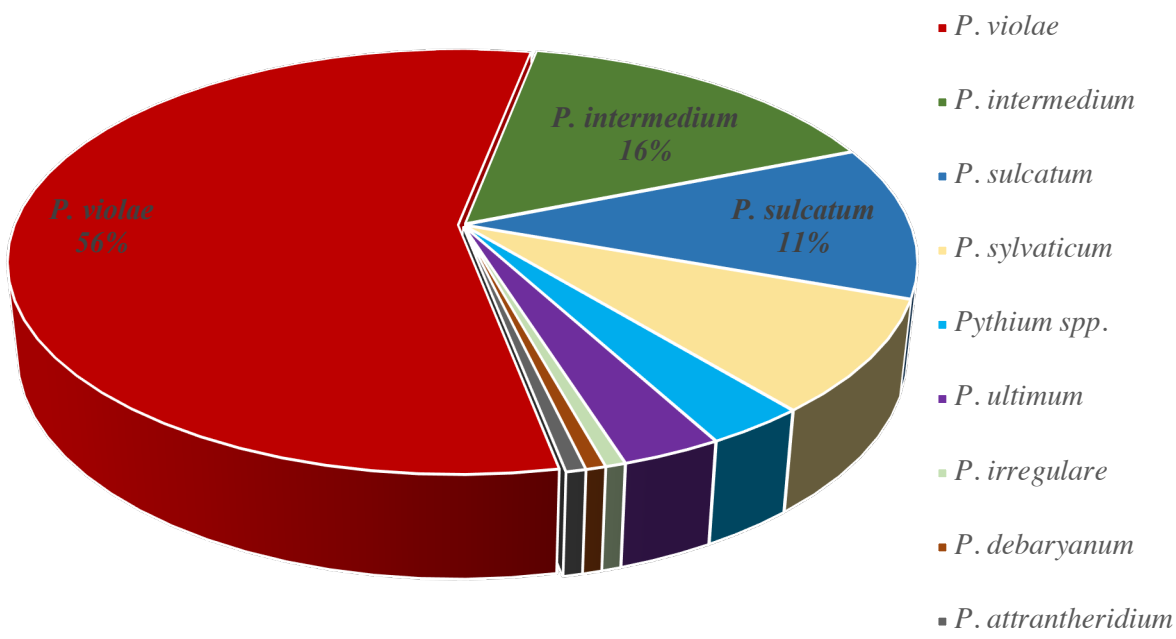


Figure 2.5 Relative proportions of different *Pythium* species identified from 164 UK isolates based on sequencing of the ITS regions of the rDNA.

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).

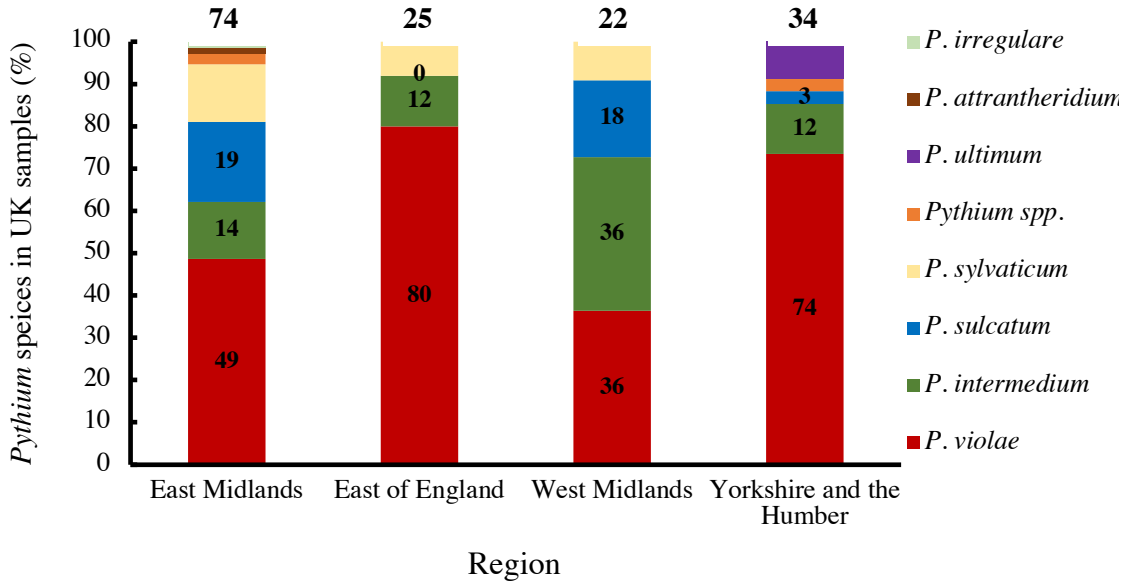


Figure 2.6 Percentage of different *Pythium* species isolated from samples of carrots with cavity spot lesions from commercial sites by region in England (total 155). The total number of samples collected for each region is shown above each percentage bar (bold).

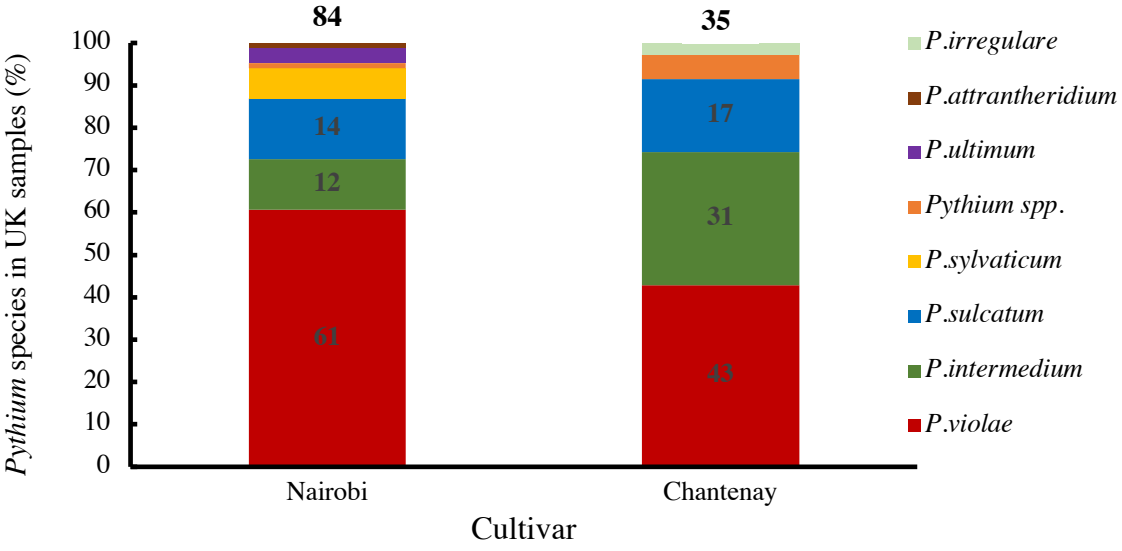


Figure 2.7 Percentage of different *Pythium* species isolated from two carrot varieties (Nairobi and Chantenay) from samples of carrots with cavity spot lesions from commercial sites in England (total 119). The total number of samples collected for each cultivar is shown above each percentage bar (bold).

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 2.6 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).

2.3.2 Molecular characterisation of *Pythium* species

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 2.7 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. All sequences associated with taxonomical reference isolates were obtained from Levesque and de Cock (2004).

Cytochrome Oxidase subunit II

Published sequences from Villa *et al.* (2006) and sequences published on NCBI from Lu *et al* (2012; paper unpublished) were used as taxonomical reference for the CoxII analysis. 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

No published sequences were available for *P. violae, P. intermedium, P. sulcatum* or *P. sylvaticum* for the NADH gene, but sequences from an unpublished study of cavity spot on carrot were available on NCBI (Lu et al; 2012) and added to the alignment (Figure 2.10). 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.

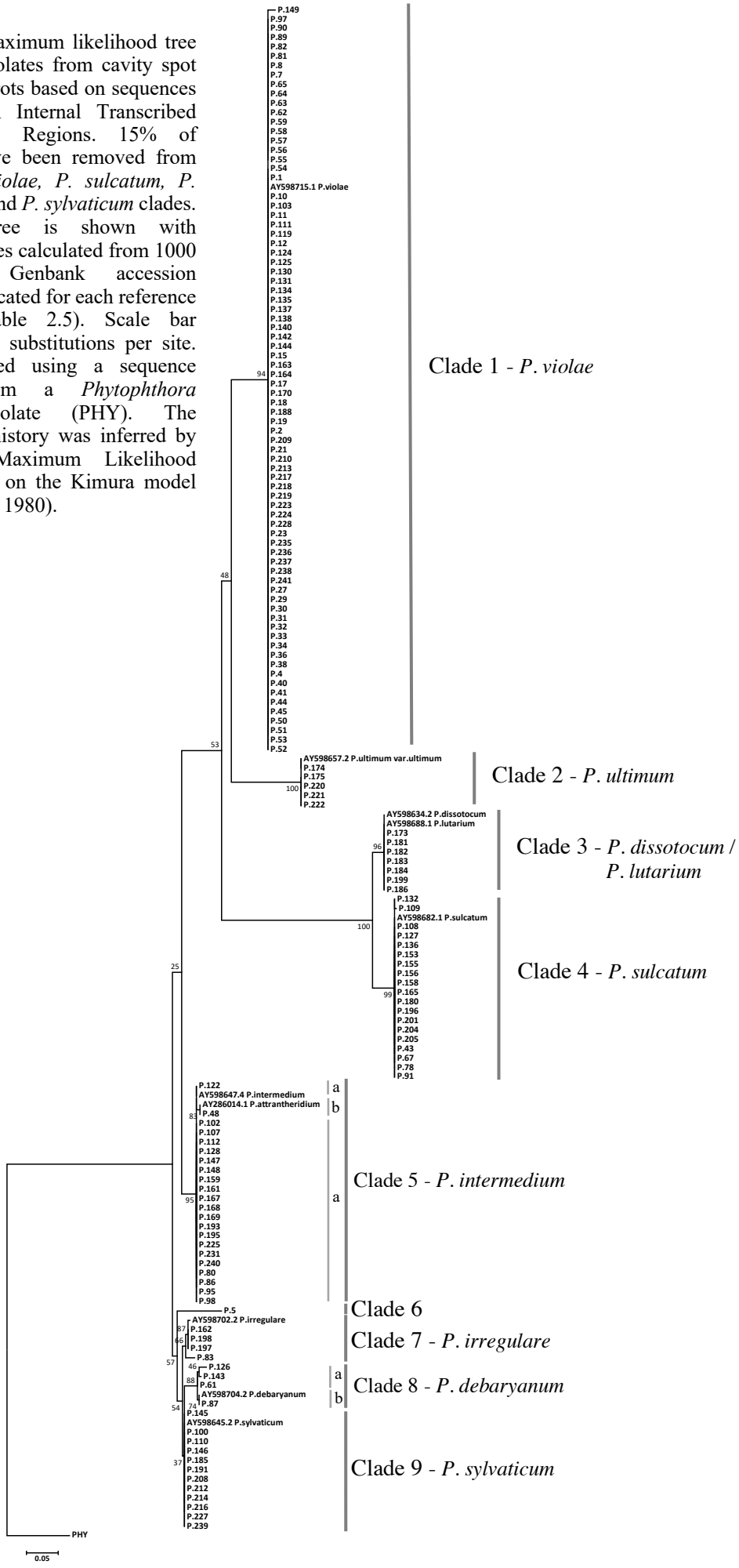
Concatenated alignment

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 variations, 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 isolates 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.

Additional trees for all individual gene alignments were constructed using Neighbour-Joining and Minimum Evolution methods, and similar topography was observed (data not shown).

Figure 2.8 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).



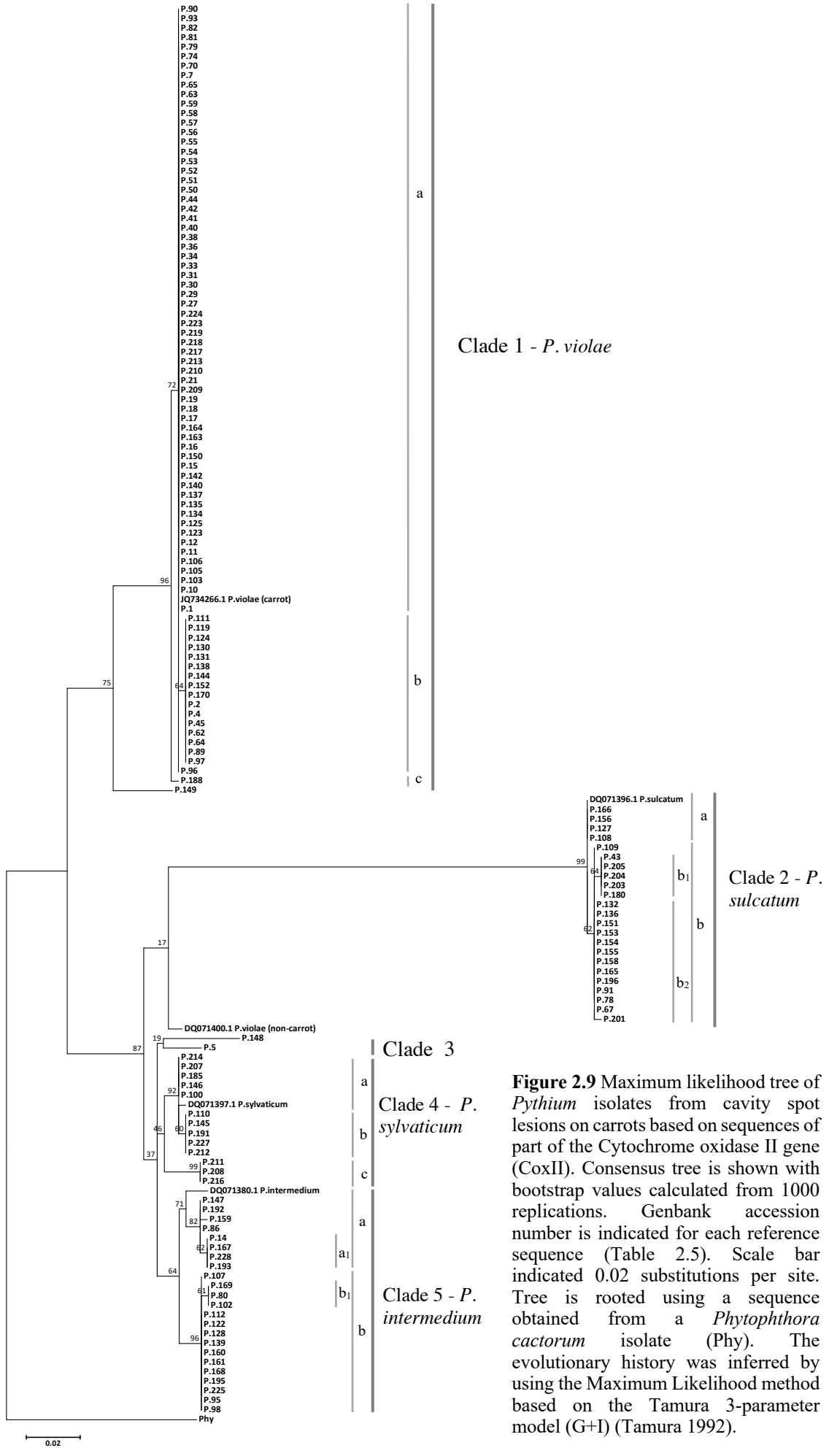


Figure 2.9 Maximum likelihood tree of *Pythium* isolates from cavity spot lesions on carrots based on sequences of part of the Cytochrome oxidase II gene (CoxII). Consensus tree is shown with bootstrap values calculated from 1000 replications. Genbank accession number is indicated for each reference 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).

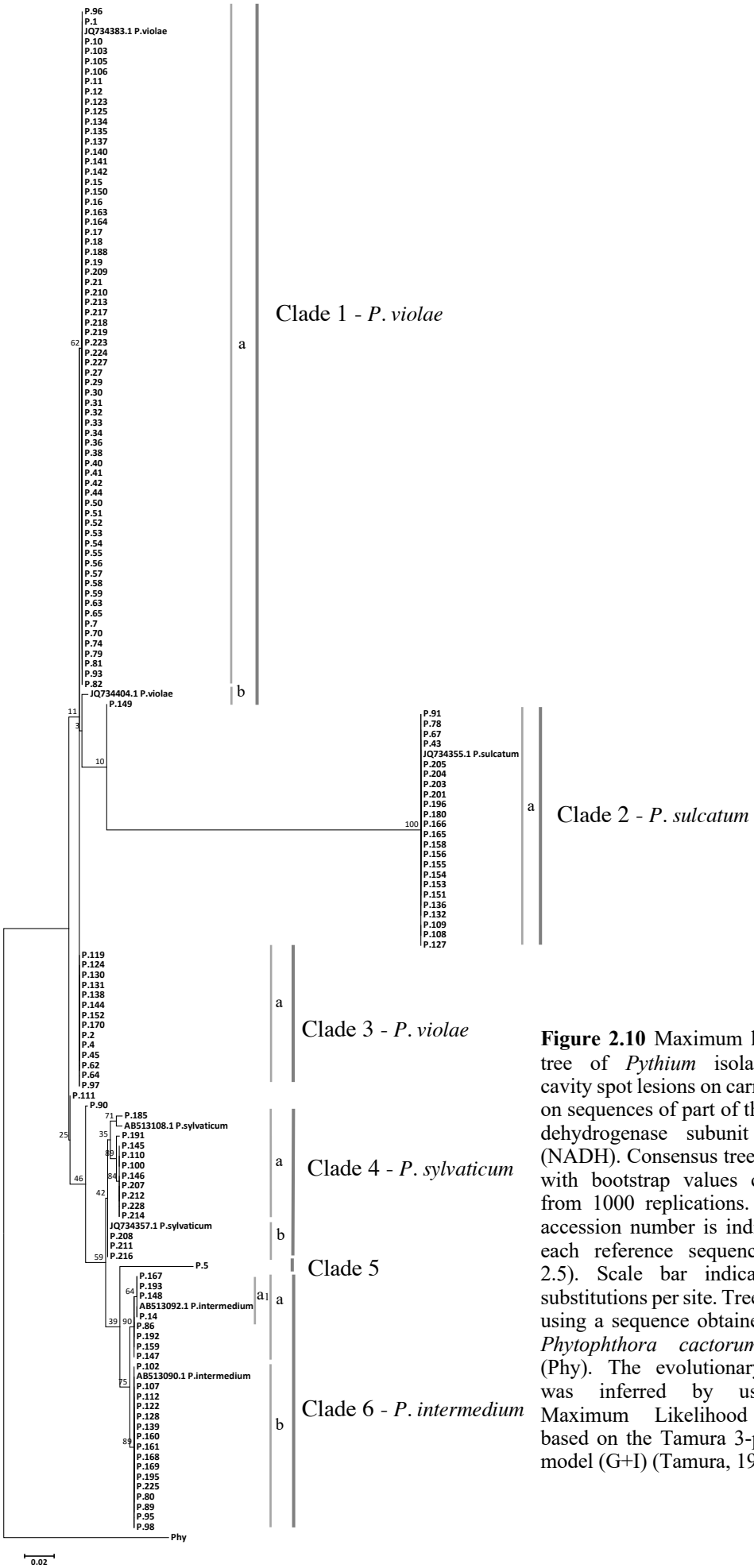


Figure 2.10 Maximum likelihood tree of *Pythium* isolates from cavity spot lesions on carrots based on sequences of part of the NADH dehydrogenase subunit 1 gene (NADH). Consensus tree is shown with bootstrap values calculated from 1000 replications. Genbank accession number is indicated for each reference 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).

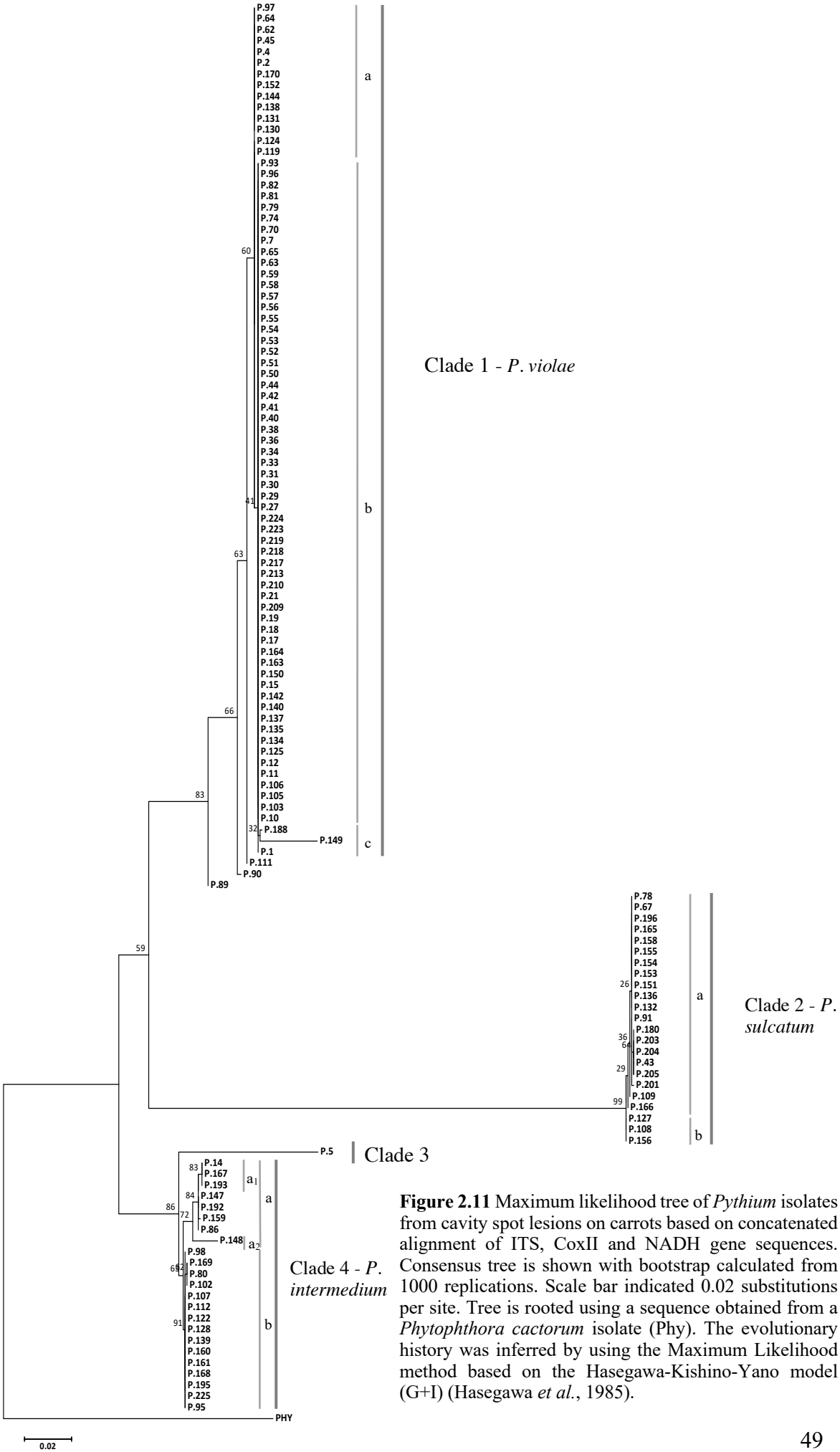


Figure 2.11 Maximum likelihood tree of *Pythium* isolates from cavity spot lesions on carrots based on concatenated alignment of ITS, CoxII and NADH gene sequences. Consensus tree is shown with bootstrap calculated from 1000 replications. Scale bar indicated 0.02 substitutions per site. Tree is rooted 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).

2.3.3 Carrot root inoculation

2.3.3.1 Pathogenicity of different *Pythium* isolates

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.

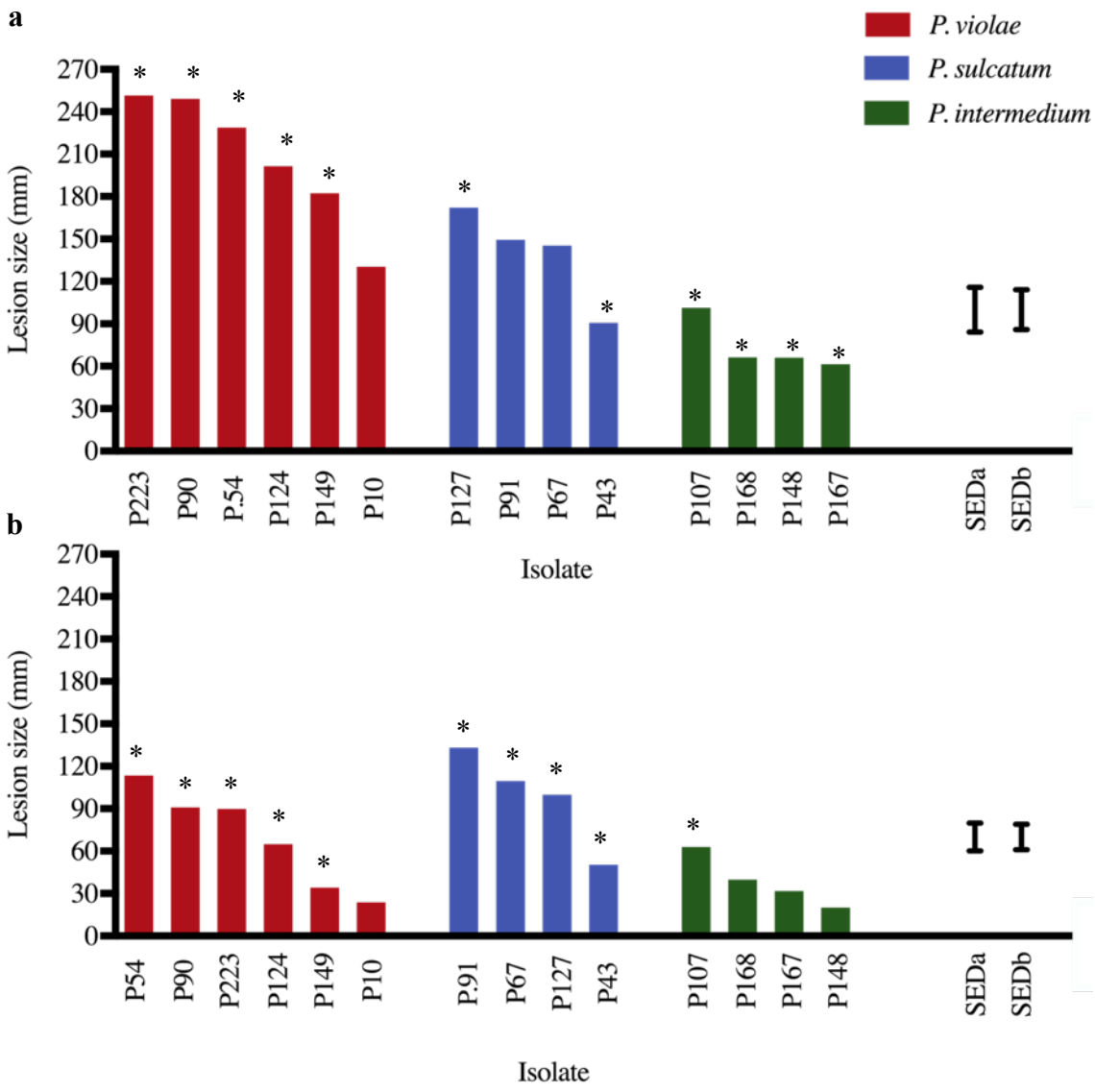


Figure 2.12 Pathogenicity of 14 *Pythium* isolates on carrot roots for Experiment 1 (a) and Experiment 2 (b); 6x *P. violae*, 4x *P. sulcatum*, 4x *P. intermedium*). Data are the mean lesion size produced on carrot roots nine days post inoculation at 15°C. Error bar represents the standard error of the 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 2.8 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 ($F_{1,9} = 4.32$, $p < 0.001$). 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 ($F_{3,80} = 17.01$, $p < 0.001$). 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.

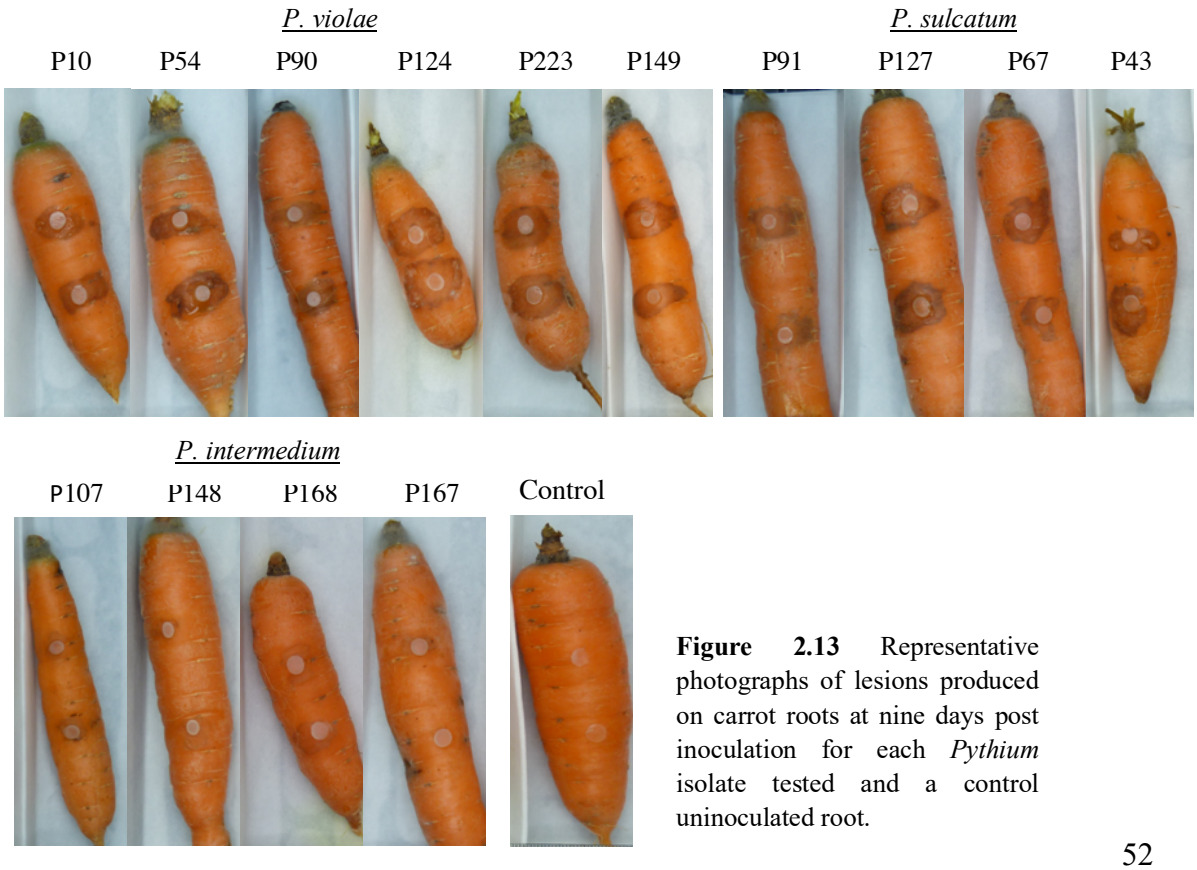


Figure 2.13 Representative photographs of lesions produced on carrot roots at nine days post inoculation for each *Pythium* isolate tested and a control uninoculated root.

2.3.3.2 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 2.9 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

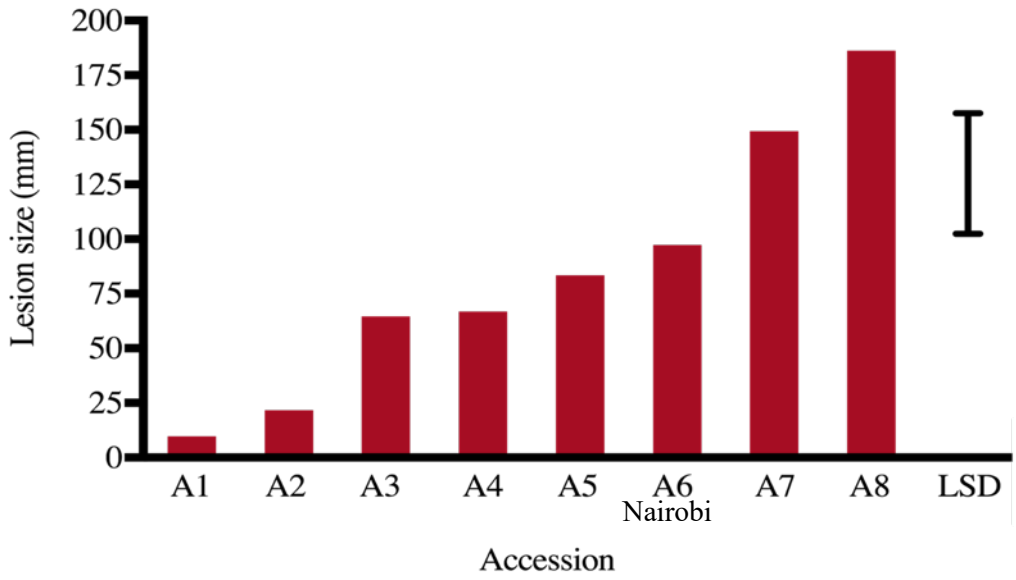


Figure 2.14 Lesion size produced by *Pythium violae* isolate P10 on eight carrot varieties. Data shown are the mean lesion size produced on whole carrot roots nine days post inoculation at 15˚C. Error bar represents the least significant difference (LSD) the 5% level.

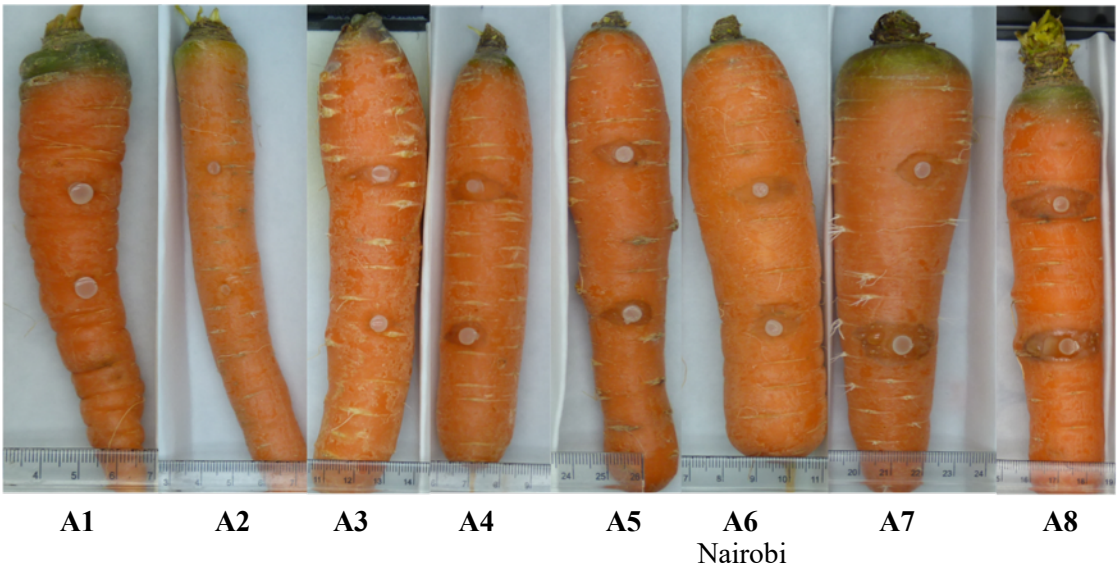


Figure 2.15 Representative photographs of lesions produced on carrot roots by *P. violae* P10 from different accessions at nine days post inoculation for each carrot accession tested.

2.4 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 (White *et al.*, 1993). For instance, in warmer climates, e.g. Australia or California, *P. sulcatum* and *P. ultimum* are commonly associated with cavity spot, although the disease is generally favoured by relatively low temperatures in the cooler winter months (Vivoda *et al.*, 1991; McDonald, 1994). 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.

All of the fungal species that were also occasionally isolated from cavity spot lesions alongside *Pythium* are ubiquitous soil dwelling species, with *Rhizoctonia* spp. and *Neonectria* spp. known to be plant pathogens, causing cankers and root rots (Mazzola, 1997; Chaverri *et al.*, 2011). *Mycocentrospora acerina* is known to cause infection in carrot, it is the causal agent of liquorice root rot disease, and triggers dark, water soaked cankers to form (Louarn *et al.*, 2012). These non-*Pythium* spp. were usually fast-growing and may have out-competed the growth of *Pythium* spp. in cultures. This is a common phenomenon and often cited as the main reason why *Pythium* spp. are difficult to isolate from lesions (Lyons & White, 1992; Hiltunen & White, 2002). 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. The pathogenicity tests conducted are necessary to show species isolated from cavity spot lesions have the potential to cause lesions and fulfil Koch's postulates. Previously *M. acerina* and more commonly *Cylindrocarpon destructans* have been isolated from 'atypical' lesions (DEFRA, 2009). These species sometimes occur alongside *P. violae*, and previously anaerobic bacteria have been implicated in cavity spot development (Perry & Harrison, 1979). It was postulated that some 'cavity spot' symptoms (particularly early cases) may be mistakenly attributed to *Pythium* spp. when ascomycete fungi may be involved. The bacterial species identified are all commonly found in wet environments

(Ca´mara *et al.*, 2007; Mahdi *et al.*, 2014; Wittmann *et al.*, 2014). Given the frequent association of these pathogens from the isolations in this study, this complex of species must be considered when reviewing the range of cavity spot symptoms and their potential cause.

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. Levesque and de Cock (2004) found there was no overall relationship between *Pythium* spp. and geographic location, however only two *P. violae* isolates from Australia and The Netherlands were included and the origin of isolates was not studied at field level. The two *P. violae* sequences included showed variation in ITS sequences, however there was question over the morphological identity of one of the isolates (Levesque & de Cock, 2004). 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. Furthermore, in fields where carrots were more intensively sampled, and in turn more lesions plated out, a wider range of *Pythium* species were identified in general, implying that a failure to obtain a *Pythium* culture from a lesion may be due to insufficient plating, rather than the *Pythium* spp. not being present. In addition, the lesions

excised from the carrot were not surface sterilised before plating onto agar. This decision was taken as previous work at Warwick Crop Centre had observed this technique allowed growth of more *Pythium* species than when lesions were sterilised. This effect may be due to the fragility of the *Pythium* mycelium (Hendrix & Campbell, 1973). However, lack of surface sterilisation may have contributed to the contamination issues, and also makes forming conclusions on the identity of the *Pythium* causing the lesion more difficult. The species that grew out from the lesion may have been secondary colonisers, but in future a range of sterilisation techniques could be examined to optimise *P. violae* isolation.

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. Guerin *et al.* (1998) identified that there may be cellular differences with respect to *Pythium* infection between susceptible and partially resistant carrots. In the susceptible cultivar Nanco, penetration of *P. violae* was intra-cellular with prominent damage, but with a partially resistant line, colonisation was limited to the pericycle. 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. in accordance with the taxonomic clades reported in Levesque and de Cock (2004). It was noted previously that there are a number of incorrect GenBank ITS *Pythium* species submitted, so using those submitted by Levesque and de Cock (2004) as reference sequences is considered reliable (McLeod *et al.*, 2009). 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. Similarly, the close relationship between *P. irregulare* and *P. cryptopiorregulare* has been documented by Matsumoto *et al.* (1999) although Garzon *et al.* (2007) concluded that they are separate species based on molecular data. This study corroborates these findings. For instance, *P. intermedium* isolates clustered with *P. attrantheridium* isolates, indicating a close relationship. Both these species have a 5 % divergence from one another for the ITS region (Levesque & de

Cock, 2004) and this study corroborates that based on ITS sequences alone, the distinction between these species is minimal.

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). Cox II has previously been shown to be suitable for exploring intraspecific relationships in *Pythium* by Martin (2000) who analysed the phylogenetic relationship of 24 species and found that they grouped into three major clades that reflected morphological characteristics. Similarly, Villa *et al.* (2006) completed an analysis of 39 *Pythium* spp. using ITS, Cox II and β-tubulin genes, and found the data added support to the growing body of literature emphasising the importance of sporangial morphology in separation within *Pythium* spp. Martin and Tooley (2003) completed a similar analysis of *Phytophthora* species, but found no consistent relationship between clades identified through phylogenetic analysis and morphological characteristics or pathogenicity.

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. Unlike the Cox II analysis, the *P. sulcatum* isolates formed no sub-clades with the NADH sequence analysis. However, the *P. violae* isolates were more genetically distinct, with a number isolates forming a separate clade, and a number of individual isolates showing greater variation from the main *P. violae* clade. The NADH gene clearly identifies greater intra-specific variation within the *P. violae* isolates than the Cox II gene alignment, 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. Although previous studies have evaluated the virulence of multiple *P. violae* isolates on carrot (Guerin *et al.*, 1994; Zamski & Petretz, 1995; El-Tarabily *et al.*, 1996; El-Tarabily *et al.*, 2004; Hermansen *et al.*, 2007; Suffert & Guibert, 2007), the selection of those isolates has not previously been based on genetic evaluation, therefore 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. Guerin *et al.* (1994) found similar results, with growth rate on agar being negatively correlated with aggressiveness on carrot in tests using a range of *Pythium* spp. including *P. violae, P. sulcatum, P. sylvaticum, P. irregulare, P. ultimum*, and *P. intermedium. P. violae* and *P. sulcatum* were identified as slow-growing and produced a larger average lesion size than *P. intermedium* isolates, which were classified as fast-growing. This is comparable with the results from this PhD study, where consistently *P. intermedium* produced smaller lesions than *P. violae* and *P. sulcatum*.

There have been conflicting results in studies that have investigated the relative virulence of *P. violae*, *P. sulcatum* and *P. intermedium*. El-Tarabily *et al.* (2004) studied the causes of cavity spot in Egypt and tested 223 *Pythium* isolates for pathogenicity. Isolates were classified as 'slow-growing' (*P. sulcatum*) or 'fast-growing' (*P. ultimum*). *P. sulcatum* isolates (165) were more aggressive, and produced large lesions (> 30 mm diameter), but only 44 of the 58 *P. ultimum* isolates produced lesions, which were all small (< 15 mm). Suffert and Guibert (2007) used wounded roots to test the ability 33 isolates of *Pythium* spp. (*P. violae, P. sulcatum, P. intermedium, P. sylvaticum, P. irregulare, P. coloratum,* and *P. ultimum*) to cause lesions. They classified the species as 'major' pathogenic

species (*P. violae* and *P. sulcatum*), and 'minor' pathogenic species (*P. intermedium, P. sylvaticum*, *P. irregulare, P. ultimum* and *P. coloratum*). The results from the current pathogenicity experiments correlate well with these findings, where *P. sulcatum* and *P. violae* were more pathogenic across the two experiments than *P. intermedium*.

In contrast to the studies above, 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. Similarly, Hermansen *et al.* (2007) conducted comparable experiments on carrot testing a total of 62 isolates over six experiments. *P. vipa* and *P. sylvaticum* resulted in the most severe lesions, whilst *P. violae* and *P. sulcatum* produced the smallest lesions and *P. intermedium* produced an intermediate size lesion. 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. Furthermore, *P. intermedium* isolates P107 and P167 produced the largest lesion sizes of the *P. intermedium* isolates tested across both experiments, both isolates grouped together within the same clade. The other two *P. intermedium* isolates tested grouped together in a separate clade, and both produced smaller lesions.

P. violae P10 was the standard isolate that was used in all experiments throughout this PhD project, 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 project, it is possible that the loss of virulence is associated with repeated use (Smith *et al.*, 2008; Songe *et al.*, 2014). The variation in the particular environmental conditions, or other unidentified factors in different experiments, may have also contributed to the change in virulence.

Previous work has examined the relationship between enzymes that degrade cell wall polysaccharides, and development or progression of cavity spot disease symptoms. El-Tarabily *et al.* (2004) reported that the greater pathogenicity of *P. sulcatum*, as compared to *P. ultimum* (two causal agents of cavity spot in Egypt), could be associated with its ability to produce a greater array, and higher activity level, of cell-wall-degrading enzymes. A wide array of cell-wall-degrading enzymes was also associated with *P. violae* isolates ability to penetrate and degrade carrot cell walls in a study from Israel (Zamski

& Petretz, 1995). However, the production of enzymes that degrade cell-wall polysaccharides does not always correlate with the virulence of *Pythium* spp.. Campion *et al.* (1997) noted that isolates which were highly pathogenic on carrot did not produce high levels of cell wall degrading enzymes, and therefore additional studies of how enzyme production may relate to pathogenicity, need to be conducted before further conclusions can be drawn.

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.

3 Development of *P. violae* artificial inoculation systems for carrot

3.1 Introduction

3.1.1 Requirement for a *P. violae* artificial inoculation system

Management of cavity spot involves a variety of techniques including rotation, early harvesting and fungicides but none of these are effective alone (Chapter 1, Section 1.4). Growers rely heavily on the fungicide metalaxyl, but its efficacy is variable. It has been hypothesised that this is due to enhanced microbial degradation in some fields (Davison & McKay, 1999; Kenny *et al.*, 2001). Gladders (2014), as part of AHDB project FV491, tested soil for enhanced degradation of metalaxyl in 32 fields in 2011, and found that in 15 samples, the half-life was less than 10 days. This is a breakdown rate previously associated with failure to control cavity spot (Davison & McKay, 1999). There are major concerns for the industry about the future control of cavity spot because of the reliance on this single fungicide, the reduced efficacy in disease control, and the potential withdrawal of approval in the near future. Consequently, 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. Recently, AHDBFV491 (Gladders, 2014) tested a range of potential new fungicides, biological control treatments, and pre-planting calcium applications using susceptible carrot varieties. This field-based project relied on naturally-occurring high disease levels to allow treatment efficacy to be evaluated effectively and with statistical significance. However over two years of testing, no significant results could be identified due to low levels of natural disease. Similarly, AHDB project FV405 (Clarkson, 2014) investigated the potential of biofumigation in cavity spot suppression. Despite best efforts to identify high risk carrot growing sites for these experiments and encouraging disease by supplementing irrigation, no, or low levels of cavity spot developed in many experimental fields, consequently hindering the ability to obtain significant results. These problems have resulted in a failure so far to reliably identify any new active ingredients or approaches to control cavity spot.

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.

3.1.2 Production of *P. violae* inoculum

Pythium infection can occur via germination of long-lasting oospores (Hendrix & Campbell, 1973; Mitchell, 1978) and mycelial growth (Suffert & Montfort, 2007). There is currently no optimised protocol for production of *P. violae* inoculum, but a variety of standard methods exist for production of inoculum for many other oomycete plant pathogens. Previous research on *Phytophthora* species found that oospores are typically formed in the dark, and at a temperature lower than the optimum temperature for mycelial growth (typically 20˚C or lower, rather than 22-25˚C for optimal mycelial growth; (Jeffers, 2006). In addition, the growing medium used (Vegetable Broth Agar; VBA) was amended with sterols to increase oospore production (Jeffers, 2006). Although many media, including corn meal agar (CMA; as used in this study for culturing *P. violae*), contain a certain amount of sterols, VBA has been the most widely used in *Pythium* research and is considered a good medium for oospore production (Guo & Ko, 1993). When oospores are produced on agar it is difficult to separate oospores from mycelium and agar due to submerged mycelial growth (van der Plaats-Niterink, 1981; O'Sullivan & Kavanagh, 1992; Botha & Coetzer, 1996).

Cultures of many oomycetes grow readily in V8 juice broth (V8B) and this liquid medium has been widely used for oospore production for many *Pythium* and *Phytophthora* species (Ayers & Lumsden, 1975; Sneh *et al.*, 1977; Pettitt *et al.*, 2002; Raftoyannis & Dick, 2002). Cultures are generally grown for 4-7 weeks, then air-dried, macerated and filtered to remove mycelial fragments in order to leave free oospores to apply as inoculum. Mitchell (1975) produced *Pythium* oospores by a similar procedure in a synthetic liquid medium, and mycelial mats were homogenized, then sonicated to free oospores and eliminate viable mycelial fragments.

Production of *Pythium* oospores in solid media is less well established. Paulitz and Baker (1987) grew *P. nunn* for one week in potato-dextrose broth (PDB), and added this to

autoclaved soil with rolled oats, followed by incubation for a further three weeks at 26˚C. They produced inoculum at a concentration of 300 cfu g^{-1}. Using this method, a mixture of sporangia and oospores were produced, with only very small amounts of visible hyphae. In this instance, *P. nunn* was being tested as a biological control for the suppression of *P. ultimum*, and they were successful in reducing disease pressure and supressing damping-off in cucumber plants. A number of researchers have used vermiculite amended with V8B inoculated with agar plugs, and incubated for 1-3 weeks to produce *Pythium* oospores (*P. acanthicum, P. afterile, P. coloratum, P. irregulare, P. ultimum*; Hawthorne, 1988; *P. violae, P. ultimum*; Vivoda *et al.*, 1991; *P. aphanidermatum*; Wheeler *et al.*, 2017). Higginbotham *et al.* (2004) studied the virulence of nine different *Pythium* spp. isolated from wheat and produced inoculum in 1 L glass jars containing soil amended with 1% (w/w) ground rolled oats, inoculated with ten PDA culture disks of *Pythium* and incubated at 22˚C for three weeks, followed by 8-12 weeks at 4˚C. Most of these methods have proved successful, although quantifying the concentration of inoculum has sometimes proved difficult.

3.1.3 Methods for artificial inoculation of carrot with *P. violae*

Significantly, no standard inoculation method has been adopted for *P. violae*. Several researchers have attempted inoculation with a range of techniques, with variable results. One technique that has been routinely used for studying the pathogenicity of *Pythium* spp. associated with cavity spot involves inoculating freshly harvested carrot roots with agar plugs of mycelium (Guerin *et al.*, 1994; Zamski & Petretz, 1995; El-Tarabily *et al.*, 1996; El-Tarabily *et al.*, 2004; Hermansen *et al.*, 2007; Suffert & Guibert, 2007). Although this approach may give an indication of the pathogenicity of different isolates, it does not always result in typical cavity spot lesions (Vivoda *et al.*, 1991), nor is it appropriate or practical for testing control treatments such as fungicides or biological control agents. A more realistic approach is to artificially inoculate a growing medium with *P. violae* to try and induce disease in carrot seedlings or mature plants. Pettitt *et al.* (2002) used two different techniques to assess the effect of inoculum on carrot seedlings. First, oospores were produced in V8B at 15˚C for eight weeks, and the mycelial mat washed, dried, macerated and filtered to remove mycelial fragments. A 1 ml aliquot of the remaining oospore suspension was added to modular cells each containing two carrot seeds. Secondly, three agar plugs of a *P. violae* culture were added to a solid substrate mixture of sand, oats and SDW, and incubated at 20˚C for two weeks. The inoculum was mixed with sterile sand (1:5) and used to fill the modular cells. The first liquid oospore inoculum

resulted in slightly reduced carrot seedling emergence as well as *P. violae* colonisation of roots (Pettitt *et al*., 2002). In contrast, there was no emergence of any seedlings from the solid inoculum mixture, possibly because the inoculum rate was too high (Pettitt *et al*., 2002). However, in both instances, the inoculum concentration was not quantified, and therefore the concentrations required to achieve different levels of disease in the seedlings were not defined.

Another approach is to use soil from commercial fields naturally infested with *P. violae*. This approach for inducing cavity spot was developed by White (1986) and tested by Pettitt *et al.* (2002) in an attempt to induce cavity spot lesions in pot-grown carrots. Whilst this approach was an improvement over relying solely on naturally infected fields, which often yield no disease, this technique, in the absence of quantifying inoculum potential, yielded highly variable and unpredictable results. An alternative method is to use an organic substrate such as barley grains inoculated with *P. violae* for field inoculation (Suffert & Guibert, 2007). There has been some success with this method, however there were also some problems surrounding the viability of the inoculum, and it was established that the inoculum potential declined over time in parallel pot tests. Furthermore, the amount of *P. violae* inoculum was difficult to standardise as the amount of pathogen growth on the barley grains varied. Research by Kretzschmar (2010) also used a variation of the method above, where wheat grains inoculated with *P. violae* agar plugs were used in pot experiments. However, results were disappointing, with the incidence and severity of cavity spot lesions only slightly higher in inoculated pots compared to uninoculated controls. Finally, El-Tarabily *et al.* (2004) used a similar method but employed millet seed as the organic substrate. This resulted in cavity spot lesions in pot-grown carrots for *P. sulcatum* isolates, but was ineffective for *P. ultimum* isolates. The differences in inoculum type (mycelium vs. oospore) may partly account for the different infectivity of different *Pythium* spp. in different experiments.

3.1.4 Quantification of *P. violae* inoculum

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). Attempts to quantify *P. violae* inoculum from soil via isolation on soil dilution plates has been challenging as *P. violae* is commonly outcompeted by faster-growing fungal or oomycete species (Phelps *et al.*, 1991; Pettitt *et al.*, 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. Mitchell (1978) identified that oospores were an important form of inoculum for several *Pythium* spp. on different hosts in field soil. Oospores of *P. aphanidermatum*, *P. myriotylum* and *P. polymatsum* were produced by incubating agar plugs of the isolates in liquid culture in the dark at 25˚C for three weeks. Mycelial mats were rinsed, homogenised and sonicated to leave free oospores as the only viable propagules. Oospores were mixed in sand and sown with a range of species. It was found that seedling percentage infection increased as inoculum concentration increased, and that between 15 and 43 oospores g^{-1} of soil resulted in 50% infection of the range of seedling species by the *Pythium* spp. tested. However, the number of *P. violae* oospores required to initiate infection and cause economically damaging levels of cavity spot on carrots is unknown and a relationship between oospore number and disease severity/incidence has yet to be defined.

In this PhD project, 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. Preliminary experiments prior to and at the beginning of this PhD project with *P. violae* found that producing inoculum in a solid substrate could produce a greater number of oospores than in liquid culture, and made mass production less time-consuming and labour intensive. Visual examination of the inoculum also revealed there was considerable reduction in mycelial growth in comparison to oospore production in liquid culture. This allowed for easier and more accurate quantification of oospores and gives more confidence that disease is being initiated by germination of oospores rather than by mycelial fragments. Although *Pythium* oospores can be produced in large quantities, the quality of those oospores (i.e. their ability to germinate and/or dormancy requirements) is unknown.

The inability to consistently produce and quantify inoculum, as well as the wide variation in infection rates, means there is 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.

3.1.5 Aims

The overall aim of this project was to develop a reliable method for producing *P. violae* inoculum, and determine the potential of this inoculum to cause cavity spot disease in carrot seedlings and mature plants in glasshouse and field experiments. In addition, the effect of different oospore concentrations on subsequent disease was explored. The specific objectives were to:

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

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

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

4. Test the efficacy of different concentrations of *P. violae* inoculum on cavity spot symptom development in field grown carrots.

3.2 Materials and Methods

3.2.1 *P. violae* isolate selection and oospore production

A solid medium comprised of horticultural sand (J. Arthur Bowers, UK) and an oat-based substrate was selected for oospore production. The solid substrate was prepared in 250 ml (125 g sand, 1.25 g milled oats), 500 ml (250 g sand, 2.5 g milled oats) or 1 L (500 g sand, 5 g milled oats) conical flasks and SDW added to achieve a final moisture content of approximately 13-15% w/w. Flasks were autoclaved twice for 15 minutes at 121˚C with an interval of 24 hours between each cycle. Flasks were inoculated with 5 (250 ml flasks), 10 (500 ml flasks) or 15 (1 L flasks) agar plugs from the edge of actively growing cultures of two *P. violae* isolates (P4 and P10; grown on CMA) and incubated at 14-17˚C in the dark for 8-20 weeks (Figure 3.1).

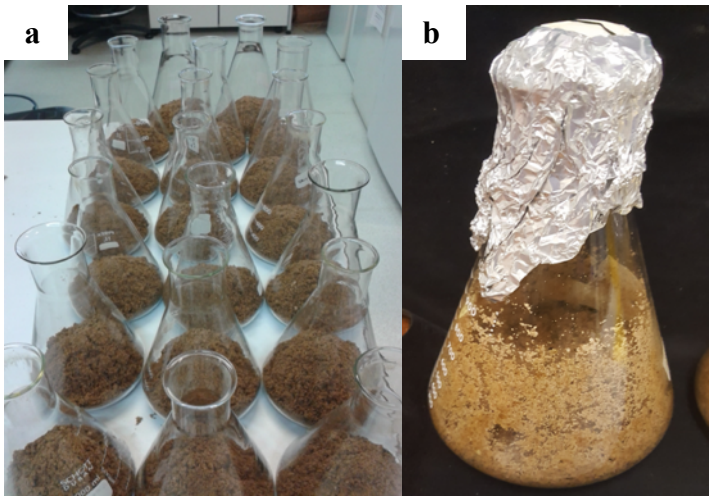


Figure 3.1 Flasks of sand/oat inoculum being prepared (a) and flask of *P. violae* solid inoculum (b).

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 they consistently produced high (P10) or low (P4) numbers of oospores on agar and caused higher (P10) or lower (P4) levels of damping-off in carrot seedlings. In addition, both isolates caused cavity spot lesions on carrot roots when inoculated with agar plugs of mycelium. The identity of isolates P4 and P10 was previously confirmed as *P. violae* by sequencing of the ITS regions of the rDNA gene (Chapter 2, Section 2.2.2).

3.2.2 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 above (Section 3.2.1). 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.

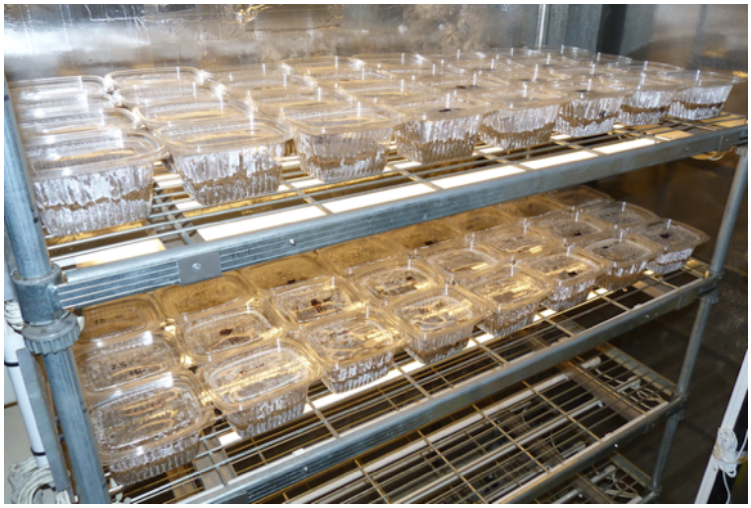


Figure 3.2 Seedling experiment boxes in randomised design on shelving in controlled environment.

Table 3.1 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)	Temperature (°C)	Isolate	Replicate boxes / concentration	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

3.2.3 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 x 10^5 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 3.2 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

3.2.4 Mature carrot plant experiments: solid *P. violae* inoculum

Between April 2016 and June 2017, three glasshouse experiments were conducted to develop a glasshouse-based artificial inoculation system to induce cavity spot symptoms in pot-grown carrots. These experiments were carried out jointly with Nicole Pereira and were part of AHDB projects FV391a and FV391b (Clarkson *et al.*, 2016, 2018). Experiments 1 and 2 tested six different concentrations of the solid *P. violae* inoculum (0-75 oospores g^{-1}), while Experiment 3 investigated the effect of four oospore concentrations (0-100 oospores g^{-1}) and also tested the effect of metalaxyl-treated seed.

3.2.5 Inoculation with *P. violae*, experimental set-up and maintenance

Mature Plant Experiments: 1 and 2

Experiments were conducted with a standard growing medium: a 50:50 (v/v) mix of sieved compost (John Innes No. 3, Erin, UK) and horticultural grade sharp sand (Westland, UK). This mixture was selected as this substrate is free of large particulates and free-draining, but still retains adequate water for carrot growth, whilst the high compost content is nutrient rich to support long-term plant growth. Solid substrate

inoculum of *P. violae* isolate P10 (4-8 months old) was prepared as outlined above (Section 3.2.1) in 1 L flasks. The contents of four flasks were decanted into a large sterile bag to give approximately 2 kg of starting material and thoroughly mixed by hand for 10 minutes to ensure the mix was homogenous. Oospore concentration was initially estimated by vortexing 1 g of this inoculum in 10 ml SDW for 1 minute and oospores counted in a 1 ml counting chamber (Sedgewick-Rafter). Two replicate counts for each of four 1 g samples were recorded. This starting inoculum was diluted appropriately in horticultural sand and mixed in a cement mixer to obtain a bulk batch of diluted inoculum at a concentration of approximately 5000 oospores g^{-1}. This was checked by a further oospore count, after which a final dilution was made to obtain another bulk batch of inoculum with a concentration of approximately 1000 oospores g^{-1}. Appropriate amounts of the 1000 and 5000 oospore g^{-1} inoculum were then mixed with the compost/sand growing medium in a cement mixer to obtain final *P. violae* oospore concentrations of 5, 10, 25, 50 and 75 oospores g^{-1} (5.4, 10.8, 27.0, 54 & 81.0 oospores/cm^3). All growing medium/inoculum mixtures were prepared such that the compost/sand ratio was always 50:50 (v/v). Plastic pots (5.5 L capacity, 20 cm diameter, 27.5 cm high) were filled with the inoculated growing medium (5.65 kg/pot) at each of the five oospore concentrations. Control pots containing a mixture of 50:50 (v/v) compost/sand only were also included. In total over 600 litres of mixture was prepared for each experiment.

Pots were placed in deep saucers within a glasshouse compartment (max. 18˚C, min. 16˚C; supplementary lighting from 05:00-20:00 h when day length was reduced) and watered to a high moisture content before sowing with untreated carrot seed (10 seeds/pot, cv. Nairobi, Elsoms Seeds Ltd, UK). Initially, the growing medium was kept damp on the surface by gentle overhead watering while carrot seedlings emerged, with additional weekly watering in the saucers. After 6-7 weeks, where there were more than five seedlings remaining, seedlings were thinned to five per pot. From 7-8 weeks onwards, watering was increased from both above and below via the saucers to keep the growing medium damp. Carrot plants also received watering with a nutrient solution via the saucers from nine weeks post sowing (w.p.s), alternating weekly with 2N:1P:4K (Vitax Vitafeed) and 0N:1P:3K (Solufeed) feeds. Aphiline, Amblyline, Encarline, Exhibitline & Hypoline biocontrol agents (Bioline AgroSciences, UK) were routinely used for controlling aphids, thrips, whitefly and sciarid flies. In total, 16 replicate pots were prepared for each oospore concentration in a randomised block design consisting of four blocks each containing four replicate pots of each treatment. Two experiments were set

up (Figure 3.3): Experiment 1 on 28/04/2016 (21 weeks duration) and Experiment 2 on 16/06/2016 (23 weeks duration).

Figure 3.3 Pot-grown carrots inoculated with *P. violae* in Experiment 1 (right) and 2 (left) at 13 and 6 w.p.s respectively; July 2016.

Mature Plant Experiment: 3

This experiment was designed to test the effect of both *P. violae* oospore concentration and a metalaxyl seed treatment on cavity spot lesion development. Experimental set up was as described above for Experiments 1 and 2, but with oospore concentrations of 1, 10, 50 and 100 oospores g^{-1} and using both untreated and metalaxyl-treated carrot seed (cv. Nairobi, Elsoms Seeds Ltd, UK). Eight replicate pots for each *P. violae* oospore concentration for each seed type were included, arranged in 12 blocks over two benches in a randomised block design (Figure 3.4).

Figure 3.4 Pot-grown carrots inoculated with *P. violae* in Experiment 3 (February 2017) at eight w.p.s.

3.2.6 Assessment of carrot plant growth, cavity spot symptoms and infection by *P. violae*

Mature Plant Experiments: 1, 2 and 3

In each experiment, carrot seedling emergence (germination) and seedling mortality (damping-off), were recorded weekly for the first 6-7 weeks. The fresh and dry weight of the seedlings that were removed as part of thinning were recorded to assess the effect of *P. violae* on seedling growth at an early stage. Both mean shoot fresh and dry weight per plant, and total shoot fresh and dry weight per pot, were recorded. Carrot plants were harvested at 21, 23 and 26 w.p.s for Experiments 1, 2 and 3 respectively. The top-growth (foliage) from the five carrots in each pot was removed and the total fresh and dry weight per pot, as well as the mean fresh and dry weight per plant, was recorded. Carrots were gently loosened and removed from the growing medium to reduce damage to the long tap roots, washed, and both mean weight per root and total root weight per pot were recorded. Each carrot was assessed and scored for cavity spot incidence (presence of one or more cavity spot lesions on each carrot) and cavity spot severity (the number of lesions per carrot). Severity was measured across all roots (including those unaffected) as well as across infected roots only.

To confirm infection by *P. violae*, one carrot was selected from two of the four pots from each oospore concentration treatment in each block (total eight roots per treatment), the long thin tap root excised and stored at 5˚C in SDW until used. Roots were surface sterilised in 70% ethanol for 1 minute and washed twice in SDW after which they were cut into three sections of roughly equal length (top: nearest the base of the carrot, middle and bottom; total 24 root pieces per treatment). A 1-2 cm length of each section was plated onto CMA amended with rifampicin (30 mg L^{-1}; pH 6), incubated in the dark at 17˚C and examined for *P. violae* growth after 5-10 days. Furthermore, small pieces of carrot tissue excised from selected cavity spot lesions in each treatment were plated directly onto CMA amended with rifampicin and also monitored for *P. violae* growth.

Statistical analysis was carried out with the support of Andrew Mead (Rothamsted Research) in Genstat® (18.1 edition, VSN International Ltd). All data recorded were subjected to statistical analysis using ANOVA with angular transformation of percentage germination, percentage seedling mortality (damping-off, calculated as a percentage of those which germinated) and cavity spot incidence. A $\log_{10}$ transformation of cavity spot

severity was performed for severity across all roots. No statistical analysis was possible for severity across infected roots only due to a large number of missing values (i.e. unaffected roots). In each of Experiment 1 and 2, one pot was removed for mean shoot weight per seedling and total shoot weight per pot at seedling stage due to very poor germination affecting the growth of the remaining seedling(s). Further outliers and anomalies were removed from the analyses as necessary. Linear and quadratic polynomial contrasts were included within the oospore concentration effect to assess for smooth trends in the response across concentration (i.e. regression lines were fit to assess if there was a dose-response relationship). Assessment of *P. violae* infection from the subset of root pieces excised from the carrot tap roots was carried out using a Chi-squared (χ^2) analysis. Where applicable, all data presented in figures are the back-transformed values, and data presented in tables are the transformed values used for statistical analysis.

3.2.7 Field Experiments

Two field experiments were carried out from May 2016-February 2017, and May 2017-March 2018 in 24 'macrocosm' plots located in the Wellesbourne quarantine field (Figure 3.5, Appendix B). The macrocosms comprised sunken concrete tubes 100 cm in diameter and 60 cm deep. Each macrocosm was initially filled with a 10 cm gravel layer, followed by a bottom 20 cm layer of sandy silt loam ('Wick' series, Wellesbourne, UK; 225 kg, Appendix B) mixed with 40 kg horticultural sand (Westland, UK). A 30 cm top layer consisting of 328 kg sieved soil (Wick Series, Wellesbourne) and 60 kg horticultural sand was then added to provide a suitable sandy loam substrate for carrot growth. *P. violae* isolate P10 was used to produce solid inoculum in 1 L flasks (as described in Section 3.2.1) and decanted into a large sterile bag to give approximately 4 kg of starting material. After determining the oospore concentration, dilutions were made with sand using a cement mixer to obtain bulk batches of inoculum at concentrations of approximately 5000 and 1000 oospores g^{-1}. The inoculum was further diluted in sand to provide 5 kg batches of inoculum, which were raked into the top 10 cm of the macrocosms on 26th May 2016 to give final oospore concentrations of 5, 10, 20, 30 and 50 oospores g^{-1} in the top 10 cm of the macrocosm. Uninoculated control macrocosms were also set up and received 5 kg sand only.

Each treatment was replicated across four macrocosms in a randomised block design and each sown with approximately 280 carrot seeds (cv. Nairobi, Elsoms Seeds Ltd, UK) which were then covered with a 1-2 cm layer of the sieved sandy loam soil.

Approximately two weeks after sowing in the May 2016 season, thunderstorms produced large volumes of rain that temporarily flooded the macrocosms. When germination was visually assessed four weeks after sowing, some plots showed significantly reduced germination, likely due to this flooding, and so were re-sown with approximately 100 carrot seed. The experiment was repeated with plots re-inoculated on 24th May 2017 with inoculum of the same oospore concentrations and each macrocosm again sown with approximately 280 carrot seeds (cv. Nairobi).

In both 2016 and 2017 macrocosms were irrigated regularly throughout the growing season and treated with Hallmark (lambda-cyhalothrin) to reduce damage from carrot root fly. Macrocosms and surrounding area were also treated with Glyphosate pre-sowing and after strawing each year to remove weeds, and hand-weeded regularly throughout the growing season. Carrots sown in May 2016 and 2017 were covered with fleece/straw in November 2016 and early December 2017 respectively to prevent frost damage over the winter period (Figure 3.6).

Carrots were harvested on 23rd February 2017 and 12th March 2018 (eight months after inoculation) and carrot yield was recorded (mean root weight per carrot, total root weight per macrocosm and total number of roots per macrocosm). Cavity spot incidence and severity (as defined in Section 3.2.6) were recorded. All data were subjected to statistical analysis using ANOVA with angular transformation of cavity spot incidence and a $\log_{10}$ transformation of the number of lesions per root (cavity spot severity).

Figure 3.5 Macrocosm plots used for *P. violae* inoculation of carrots, 10 w.p.s (July 2016).

Figure 3.6 Macrocosm plots used for *P. violae* inoculation of carrots insulated with fleece and straw for the winter (November 2016).

3.3 Results

3.3.1 *P. violae* oospore production in solid and liquid media

P. violae isolates P4 and P10 grew well in the sand/oat solid substrate and in V8 liquid media (P10 only) with large numbers of oospores produced (Figure 3.7a). Different types of oospores were observed which were classified as 'immature' or 'mature'. Immature oospores had no double wall and a granular/grainy appearance (Figure 3.7b). These were generally slightly smaller in size than mature oospores, with a diameter of less than 25 µm. Mature oospores all had a distinct double wall with space between the oospore wall and the oogonium (aplerotic), as well as a darker internal body, but varied in size: 'small mature' oospores (from approx. 20 µm diameter) were observed as well as 'large mature' oospores (30-40 µm diameter; Figure 3.7c/d). It was assumed that only mature *P. violae* oospores would have the ability to germinate and cause infection and therefore data is presented for mature (small and large) oospores only. In V8B liquid culture, *P. violae* isolate P10 oospore production ranged from 7,498-11,747 oospores ml^{-1}. In the sand/oat solid substrate, the number of oospores ranged between 10,240-47,639 oospores g^{-1}. Over the initial four weeks of storage, fine *P. violae* mycelium was observed growing on the side of the flasks, but was more difficult to observe thereafter. Visual examination of the inoculum under the microscope revealed very few mycelial fragments.

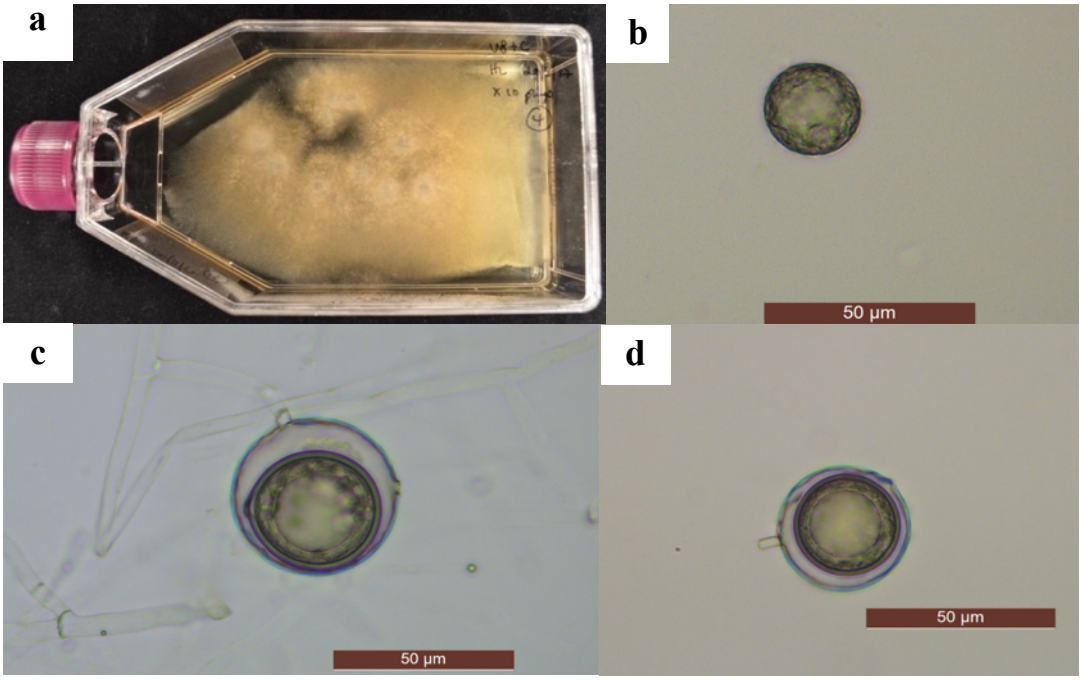


Figure 3.7 *P. violae* mycelial mat grown in V8B+C for five weeks (a); immature *P. violae* oospore (b); mature *P. violae* oospores with an aplerotic double wall (c+d).

3.3.2 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).

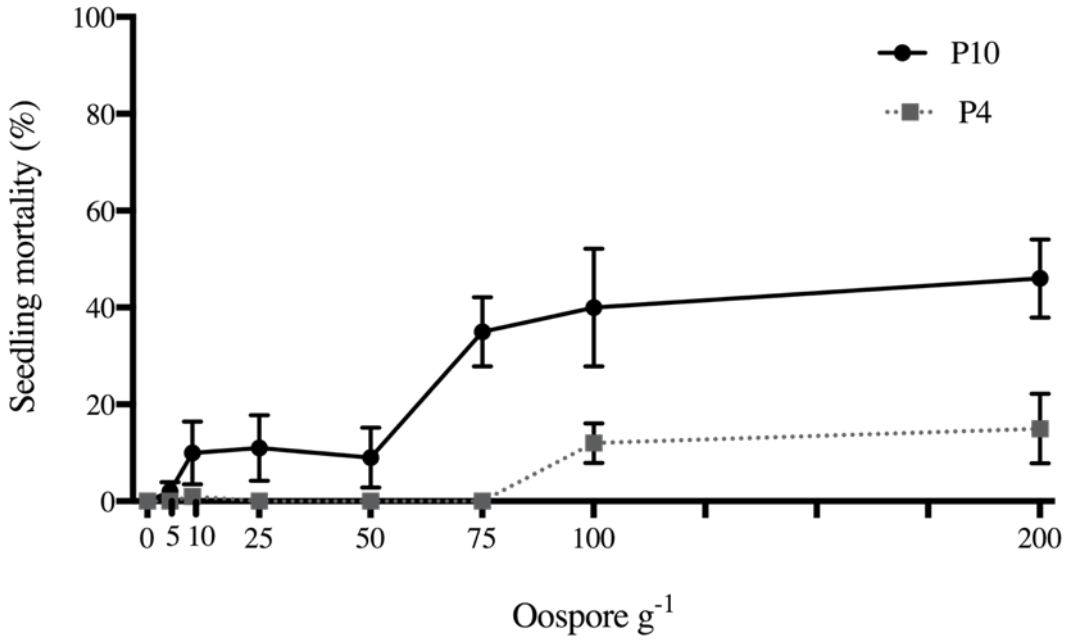


Figure 3.8 Seedling Experiment 3: effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on carrot seedling mortality at 10 w.p.s for isolates P10 (black solid line) and P4 (grey dashed line).

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).

3.3.3 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.

3.3.4 Mature carrot plant experiments: solid *P. violae* inoculum

3.3.4.1 Experiments 1 and 2

Effect of *P. violae* inoculation on seed germination, seedling survival and carrot growth

In Experiment 1, pots inoculated with *P. violae* P10 oospores produced in the solid medium resulted in carrot seedling germination between 86-92% and was similar to the 90% germination recorded for the uninoculated control treatment. In Experiment 2, germination was reduced to 75-80% for the *P. violae* treatments compared to 84% in the control (Figure 3.9). However, these differences were not significant (Expt. 1: $F_{5,87} = 0.46$, $p = 0.807$; Expt. 2: $F_{5,87} = 0.57$, $p = 0.872$; Table 3.3).

Some post-emergence damping-off of carrot seedlings was observed in both experiments with a maximum of 3% and 2% in Experiments 1 and 2 respectively (Figure 3.10, Table 4.3). In Experiment 1, there was weak evidence of differences between treatments ($F_{5,87} = 2.18$, $p = 0.064$) with significant evidence of a linear dose-response relationship ($F_{1,87} = 9.15$, $p = 0.003$); i.e. the percentage of seedlings surviving decreased with increasing *P. violae* oospore concentration. Significant differences in damping-off were found between both the 50 and 75 oospore g^{-1} *P. violae* concentrations and the uninoculated control (Figure 3.10). In contrast, there was no evidence of differences in carrot seedling survival between the different oospore concentrations and the uninoculated control for Experiment 2 ($F_{5,87} = 1.43$, $p = 0.223$; Table 3.3).

In both experiments, there was evidence that total shoot dry weight per pot differed significantly between the treatments for the carrot seedlings that were removed as they were thinned out at six and seven w.p.s respectively (Expt. 1: $F_{5,82} = 10.52$, $p < 0.001$; Expt. 2: $F_{5,72} = 2.35$, $p = 0.049$; Table 3.3). The 25, 50 and 75 oospore g^{-1} *P. violae* concentrations in Experiment 1 resulted in significantly reduced total seedling weight compared with the uninoculated control, with significant evidence for a linear dose-response relationship ($F_{1,82} = 46.95$, $p < 0.001$; Figure 3.11a; Table 3.3). However, in Experiment 2, the 5 and 10 oospore g^{-1} concentrations were the only *P. violae* oospore concentrations that resulted in significantly reduced seedling weight compared to the uninoculated control (Figure 3.11a; Table 3.3).

Mean shoot dry weight for the carrot seedlings thinned also differed significantly between the treatments for Experiment 1 ($F_{5,81} = 11.2$, $p < 0.001$); however there was no significant difference in Experiment 2 ($F_{5,71} = 1.84$, $p = 0.116$; Table 3.3). As with the total shoot dry weight, in Experiment 1, the mean shoot dry weight for *P. violae* oospore concentrations of 25, 50 and 75 oospores g^{-1} were significantly reduced compared to the uninoculated control, with significant evidence for a linear dose-response relationship ($F_{1,81} = 48.18$, $p < 0.001$; Figure 3.11b; Table 3.3). This was clearly visually noticeable at 6-9 w.p.s, with a marked reduction in carrot foliage for the *P. violae* inoculated treatments compared to the uninoculated control. The amount of foliage increased with decreasing oospore concentration (Figure 3.12). However, this effect was not evident in Experiment 2 and for both experiments, any differences in top-growth had evened-out by harvest, where there were no significant differences in carrot foliage wet or dry weight across any of the treatments (Table 3.3).

At harvest the most noticeable effect of the different *P. violae* treatments on carrot growth and development was on root shape and size. All *P. violae* treatments resulted in carrot roots which were distinctly stunted. Roots were small, stubby and characterised by a long, brown tap root with increased lateral root formation, many of which were collapsed (Figure 3.13). *P. violae* treatments therefore had a significant effect on total carrot weight per pot across the different oospore concentrations in both experiments (Expt. 1: $F_{5,85} = 11.34$, $p < 0.001$; Expt. 2: $F_{5,86} = 8.14$, $p < 0.001$; Figure 3.14a, Table 3.3). In both experiments, all inoculated treatments had significantly reduced total root weight per pot compared to the uninoculated control. Mean weight per carrot in the *P. violae* treatments was also significantly different across all oospore concentrations in both experiments (Expt. 1: $F_{5,85} = 11.55$, $p < 0.001$, Expt. 2: $F_{5,86} = 6.25$, $p < 0.001$; Figure 3.14b) and

significantly reduced compared to the uninoculated control. Individual carrot weight ranged between 41-45 g and 34-42 g for Experiments 1 and 2 respectively for inoculated treatments compared to a mean of 61 g and 48 g in the corresponding untreated controls (Figure 3.14b; Table 3.3).

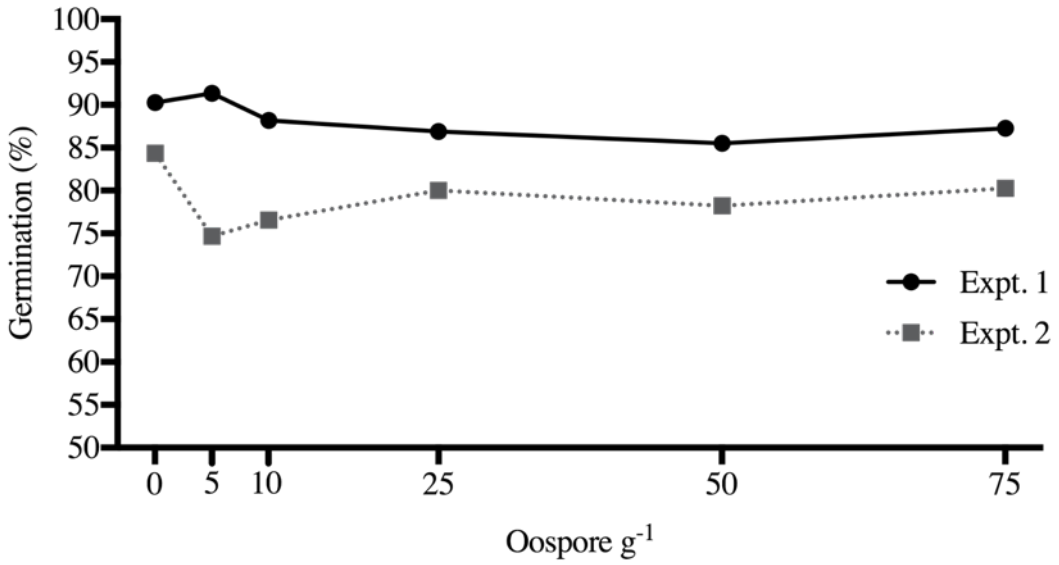


Figure 3.9 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on mean percentage carrot seed germination in Experiment 1 (black line with circles) and Experiment 2 (grey dashed line with squares) at six and seven w.p.s respectively.

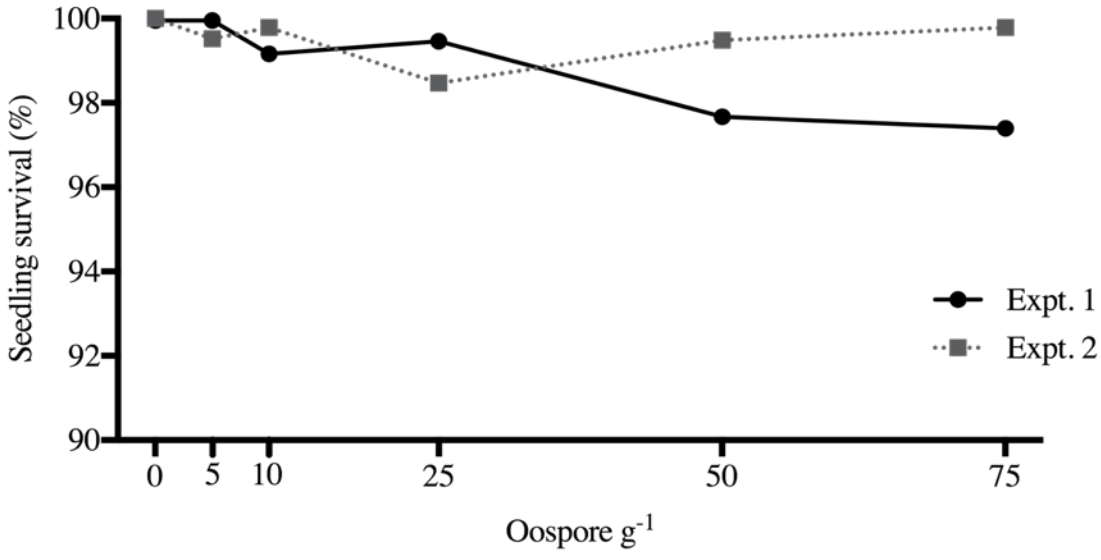


Figure 3.10 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on mean percentage seedling survival in Experiment 1 (black line with circles) and Experiment 2 (grey dashed line with squares) at six and seven w.p.s respectively.

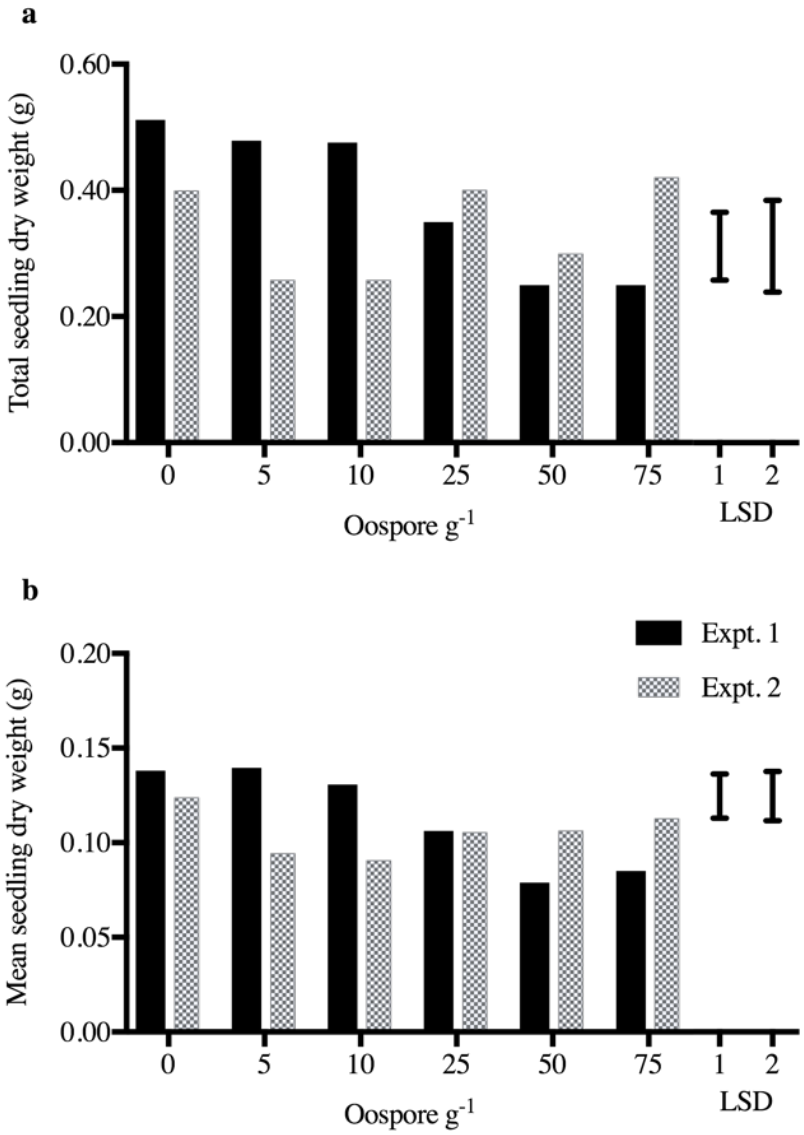


Figure 3.11 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on total seedling dry weight (a) and mean seedling dry weight (b) in Experiment 1 (black solid bars) and Experiment 2 (grey hashed bars) at six and seven w.p.s respectively. Error bars represent the least significant difference between treatments (LSD) at the 5% level. LSD 1 is for Expt.1, LSD 2 for Expt 2.

Figure 3.12 Photograph of the effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on growth of carrot foliage six w.p.s in Experiment. 1. From left to right: uninoculated control, 5, 10, 25, 50 and 75 oospores g^{-1}

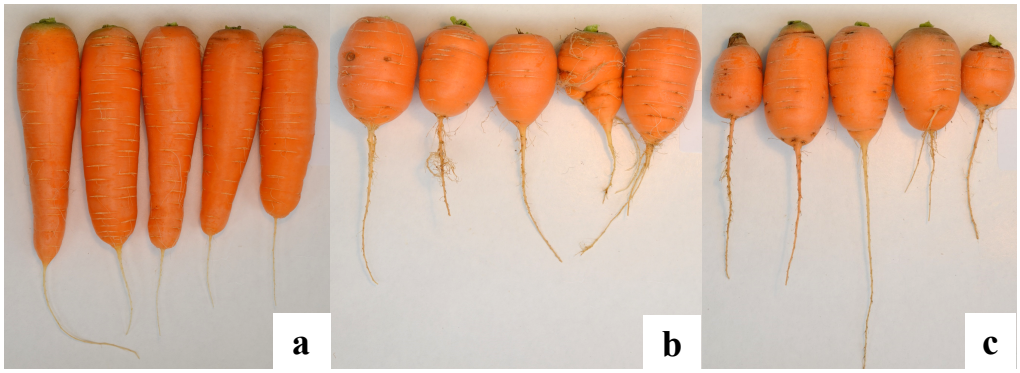


Figure 3.13 Representative photographs of carrot roots demonstrating the effect of *P. violae* inoculation on root growth in Experiment 1 for control (a), 25 oospores g^{-1} (b), and 50 oospores g^{-1} (c). Carrots grown in medium inoculated with *P. violae* (b+c) has stunted growth and larger tap roots compared to control (a).

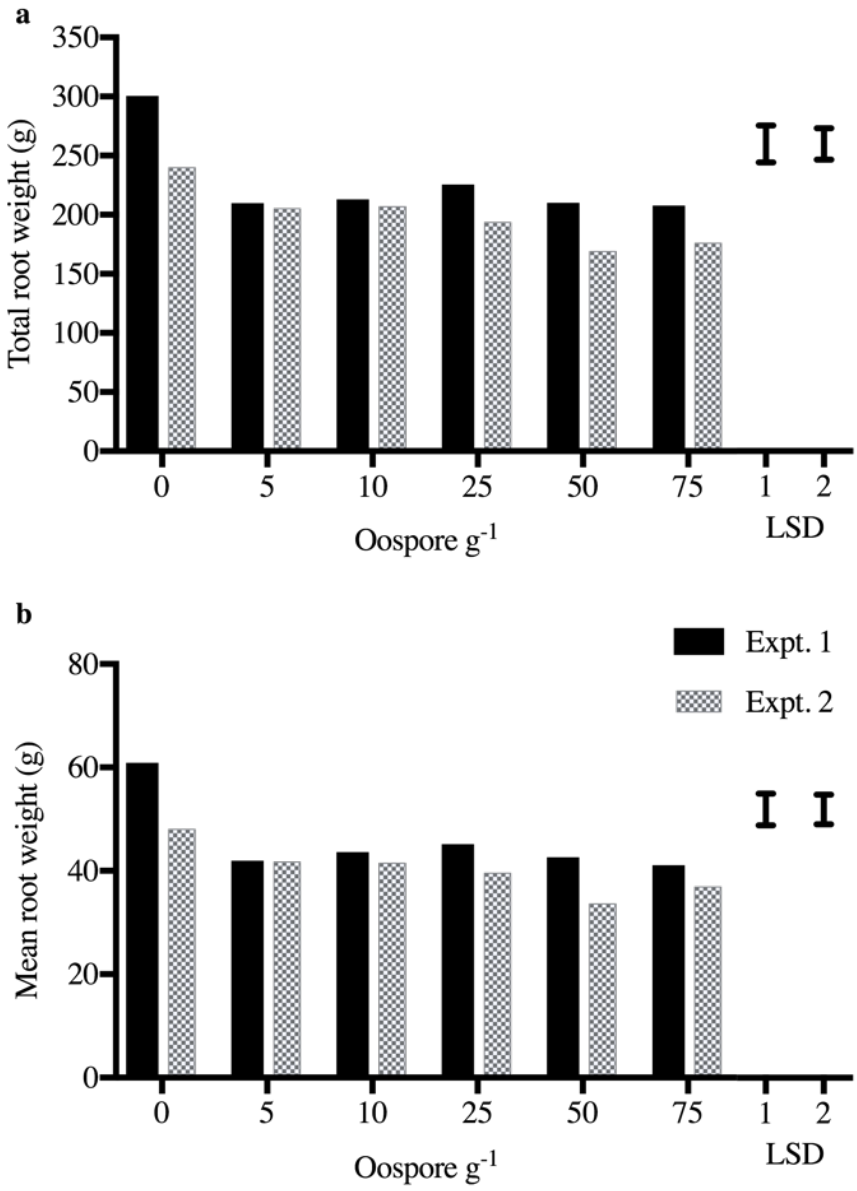


Figure 3.14 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on carrot root weight: mean root weight per carrot (a) and total root weight per pot (b) for Experiment 1 (black solid bars) and Experiment 2 (grey hashed bars) at six and seven w.p.s respectively. Error bars represent the LSD at the 5% level. LSD 1 is for Expt.1, LSD 2 for Experiment 2.

Effect of *P. violae* inoculation on cavity spot incidence and severity

Characteristic cavity spot symptoms were observed on carrot roots for all *P. violae* treatments in Experiments 1 and 2 (Figure 3.15), and incidence varied between 10-20% and 5-13% respectively (Figure 3.16, Table 3.3). No cavity spot symptoms were observed in uninoculated control treatments. The effect of *P. violae* inoculation on cavity spot incidence across all treatments was significant for Experiment 1 ($F_{5,87} = 6.42, p < 0.001$), and although non-significant, was approaching the 5% level of significance for Experiment 2 ($F_{5,87} = 2.19$, $p = 0.063$). In Experiment 1, all inoculated treatments were significantly different to the uninoculated control whilst only concentrations of 10 and 75 oospores g^{-1} were statistically different from the uninoculated control in Experiment 2.

The severity of cavity spot was calculated across all carrot roots (including those unaffected) and also only for those with symptoms. Severity across all roots was low in both experiments and ranged from 0-0.32 lesions per carrot in Experiment 1 and 0-0.09 lesions per carrot in Experiment 2 (Figure 3.17; Table 3.3). Nonetheless the effect of *P. violae* inoculation on the number of cavity spot lesions across all concentrations was significant for Experiment 1 ($F_{5,85} = 5.31$, $p < 0.001$), though non-significant for Experiment 2 ($F_{5,87} = 1.99$, $p = 0.088$). When only carrots with cavity spot symptoms were considered, the number of lesions per carrot in inoculated treatments ranged between 1.84-2.35 in Experiment 1 and 1.11-1.80 in Experiment 2 (Figure 4.17, Table 4.3; statistical analysis not possible due to large number of missing values i.e. unaffected carrots). The maximum number of cavity spot lesions ranged from 4-6 and 2-5 in Experiments 1 and 2 respectively (Table 3.3).

Figure 3.15 Representative photographs of cavity spot lesions on carrot roots inoculated with *P. violae* solid oat/sand substrate in Experiment 1.

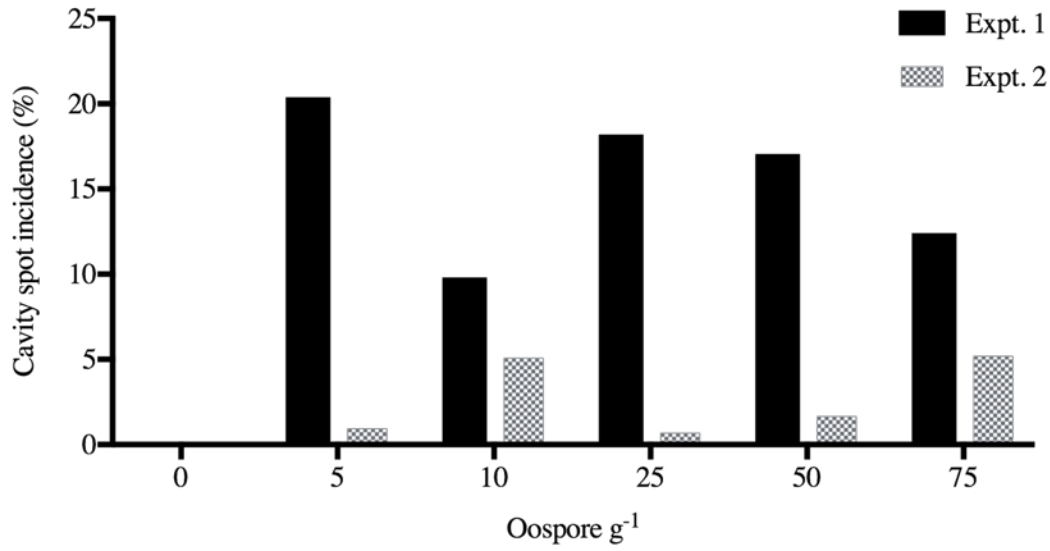


Figure 3.16 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on cavity spot incidence for Experiment 1 (black solid bars) and Experiment 2 (grey hashed bars) at harvest.

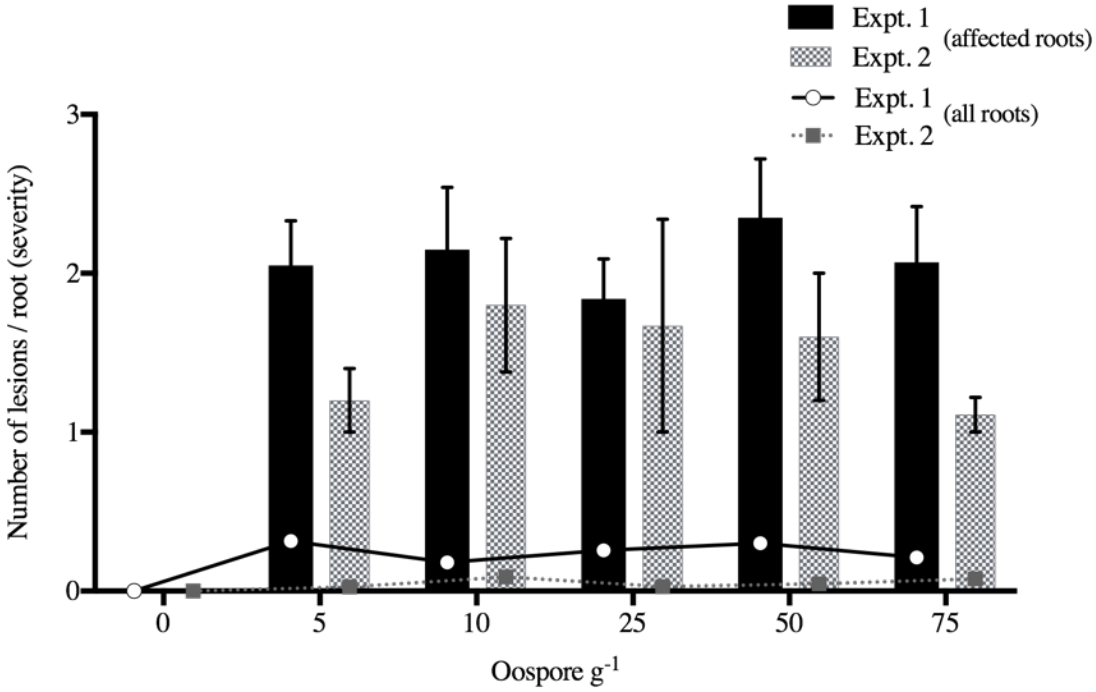


Figure 3.17 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on the number of lesions per root (cavity spot severity) calculated across all roots (Experiment 1: black line with circles and Experiment 2: grey dashed line with squares) and across affected roots only (Experiment 1: black solid bars and Experiment 2: grey hashed bars. Error bars represent the standard error of the mean (SEM).

Isolation of *P. violae* from roots and cavity spot lesions

Carrot tap root pieces from *P. violae* treatments plated on to CMA yielded *P. violae* colonies (Figure 3.18) demonstrating that they had been infected by the pathogen. For Experiment 1, the χ^2 analysis revealed a significant difference in the proportion of infected root pieces between the uninoculated controls and *P. violae* inoculated treatments (χ^2 (5, n = 24) = 19.87, p = 0.001) with the highest proportion for the 5 oospores g^{-1} concentration (14 of 24, 58%). There was no clear pattern of infection across the different oospore concentrations, although all had a higher proportion of roots infected than the uninoculated control (no infection). For Experiment 2, the χ^2 test was not significant (χ^2 (5, n = 24) = 7.43, p = 0.220), although inspection of the data showed an increased proportion of roots infected with *P. violae* for the five different oospore concentrations (maximum 6 out of 24 infected pieces) compared with the uninoculated control (no infection).

There was a high percentage of carrots with deformed roots in Experiment 1 (Appendix C).

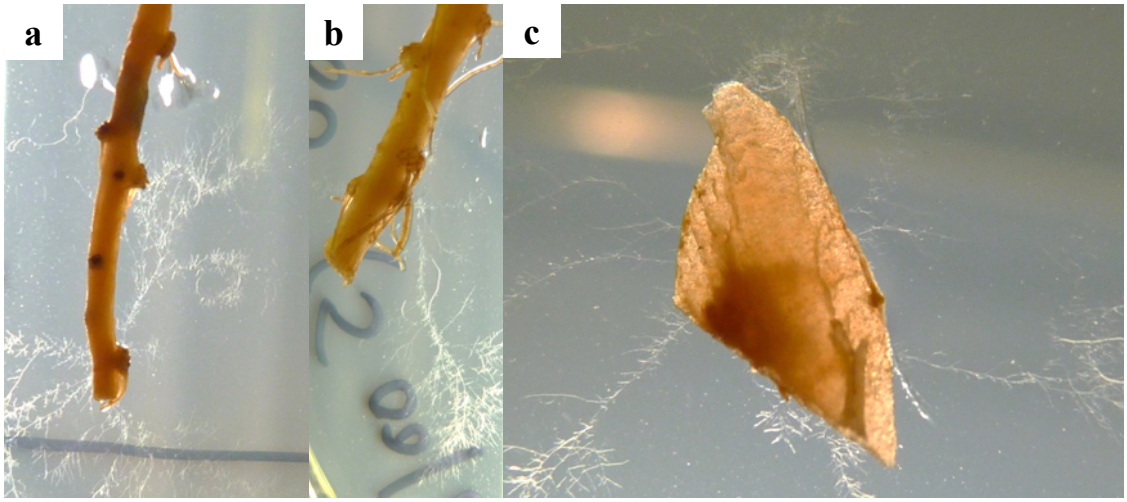


Figure 3.18 Representative examples of *P. violae* growing out from carrot tap root pieces (a, b) and cavity spot lesion (c) on CMA.

Table 3.3 Summary of results and statistical analyses for parameters measured for carrot growth and cavity spot development in Experiments 1 and 2 examining the effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on pot-grown carrots.

Variable measured	Experiment 1								Experiment 2							
	Concentration (oospores g^{-1})						LSD (5%)	*p* value	Concentration (oospores g^{-1})						LSD (5%)	*p* value
	0	5	10	25	50	75			0	5	10	25	50	75		
Seed germination (%)	71.8	72.9	69.9	68.8	67.6	69.1	8.2	NS	66.7	59.8	61.1	63.4	62.2	63.6	8.9	NS
Seedling survival (%)	1.2	1.2	5.3	4.2	8.8	9.2	6.7	0.064	0	4.0	2.7	7.1	4.1	2.7	5.5	0.223
Seedling total dry weight (g)	0.51	0.48	0.48	0.35	0.25	0.25	0.10	<0.001	0.40	0.26	0.26	0.40	0.30	0.42	0.14	0.049
Seeding mean dry weight (g)	0.14	0.14	0.13	0.11	0.08	0.09	0.02	<0.001	0.12	0.09	0.09	0.11	0.11	0.11	0.03	0.116
Harvest total dry weight (g)	10.82	11.29	10.98	10.42	11.25	11.40	1.147	0.545	10.02	9.57	9.35	8.74	8.92	9.17	0.938	0.099
Harvest mean dry weight (g)	2.17	2.26	2.25	2.08	2.28	2.26	0.225	0.490	2.00	1.94	1.88	1.79	1.78	1.92	0.202	0.207
Carrot total root weight (g)	300.7	209.9	213.2	225.7	210.2	207.7	30.1	<0.001	240.3	205.4	207.1	193.8	169.4	176.3	25.3	<0.001
Carrot mean root weight (g)	60.9	42.0	43.6	45.2	42.6	41.1	5.9	<0.001	48.1	41.7	41.6	39.6	33.7	36.9	5.5	<0.001
Cavity spot incidence (%)	0.00	26.8	18.3	25.3	24.4	20.6	11.0	<0.001	0.00	5.6	13.1	4.8	7.4	13.2	9.7	0.063
Cavity spot severity (all roots)	-0.99*	-0.38	-0.55	-0.45	-0.40	-0.51	0.28	<0.001	-1.0	-0.90	-0.72	-0.89	-0.83	-0.75	0.20	0.088
Cavity spot severity (infected roots)	0.00	2.05	2.15	1.84	2.35	2.07	N/A	N/A	0.00	1.20	1.80	1.67	1.60	1.11	N/A	N/A
Deformed roots (%)	3.8	21.2	14.6	10.8	6.6	17.9	11.55	0.029	0.00	1.88	0.00	0.00	0.00	0.00	2.152	0.423

Angular transformed means are shown for all percentage data (germination, seedling survival, cavity spot incidence and deformed roots). Log_{10} transformed means are shown for cavity spot severity (this resulted in values of less than 1).

*The value for the 0 oospores g^{-1} for the number of lesions per root (cavity spot severity) across all roots in Experiment 1 was adjusted for a missing value (a pot where all roots had rotted), and therefore after the log_{10} transformation was applied, a value less than 0 was obtained (a value of -1 would have indicated no lesions were observed). Therefore, once data was back-transformed, a value of greater than 0 was obtained, however this is represented as 0 in the figure (Figure 3.17) because all observed values were zero.

3.3.4.2 Experiment 3

Effect of *P. violae* inoculation and metalaxyl seed treatment on seed germination, seedling survival and carrot growth

Across all the analyses performed, there was no effect of seed treatment (metalaxyl-treated/untreated seed) on any of the carrot growth or cavity spot disease variables measured.

Germination for metalaxyl-treated carrot seed ranged from 77-94% in the *P. violae* inoculated treatments which was greater than the mean germination of 74% in uninoculated control pots (Figure 3.19, Table 3.4). In contrast, germination for untreated carrot seed ranged from 73-91% in *P. violae* inoculated treatments, which was less than the germination in uninoculated control pots with a mean of 93% (Figure 4.19). This resulted in a significant interaction between *P. violae* oospore concentration and seed treatment ($F_{4,99} = 3.60$, $p = 0.009$), with the effect of seed treatment being inconsistent between concentrations (Figure 3.19, Table 3.4). For the control, germination was significantly lower for the metalaxyl-treated seed compared with the untreated seed while conversely, the germination in the 1 oospore g^{-1} treatment was significantly greater in the metalaxyl-treated seed than in the untreated seed. For the untreated seed, the greatest differences in germination were observed between the lower *P. violae* oospore concentrations and the uninoculated controls (concentrations of 1 and 10 oospore g^{-1} for untreated seed were the only treatments significantly different from the uninoculated control, Table 3.4). Across all oospore concentrations, there was a significant and contrasting effect on germination between the metalaxyl-treated and untreated seed for the uninoculated control and 1 oospore g^{-1} treatments (Figure 3.19).

Very little post-emergence damping-off occurred in this experiment with less than 1% of carrot seedlings affected across both seed treatments. There was no evidence for variation in damping-off between any of the treatments (Figure 3.20, Table 3.4).

In contrast to Experiments 1 and 2, there was no effect across all treatments on seedling total dry weight per pot for those that were thinned out at seven w.p.s ($F_{4,90} = 1.68$, $p = 0.160$; Figure 3.21a, Table 3.4). However, there was weak evidence of an interaction between oospore concentration and seed treatment ($F_{4,90} = 2.32$, $p = 0.063$). This interaction was mainly driven by the uninoculated control pots: in the control pots, the metalaxyl seed treatment resulted in significantly reduced total seedling dry weight

compared to the untreated seed (Figure 3.21a). For the inoculated *P. violae* treatments, there were no significant differences in seedling weight between the metalaxyl-treated and untreated seed (Figure 3.21a). Furthermore, within the metalaxyl-treated seed, all *P. violae* inoculated treatments resulted in an increase in total seedling dry weight compared to the uninoculated control, but was only significant for the 1 and 50 oospores g^{-1} concentrations. The mean seedling dry weight revealed no interaction between oospore concentration and seed treatment ($F_{4,89} = 1.56$, $p = 0.193$; Figure 3.21b), but there was weak evidence of differences across all *P. violae* inoculated treatments and the uninoculated control ($F_{4,89} = 2.28$, $p = 0.067$; Table 3.4). Only the 100 oospore g^{-1} concentration showed a significant difference to the uninoculated control, but this was a reduction in seedling weight.

At harvest, there was a main effect of treatment on both total carrot foliage dry weight per pot and mean foliage dry weight per carrot (total: $F_{4,99} = 4.91$, $p = 0.001$; mean: $F_{4,99} = 4.74$, $p = 0.002$; Table 3.4). For both variables, the dry weight at harvest was significantly reduced for all *P. violae* oospore concentrations compared to the uninoculated controls (data not shown). There was no evidence of an oospore concentration by seed treatment interaction (Table 3.4).

Although the effect of *P. violae* treatments on carrot root shape and size was less pronounced than in Experiments 1 and 2, many of the oospore concentration treatments resulted in carrot roots which were stunted and smaller than roots from the uninoculated controls. Most roots were also still characterised by a long, brown tap root with increased lateral root formation (Figure 3.22, 3.23). There was, therefore, a significant main effect of *P. violae* treatment on for total root weight per pot ($F_{4,99} = 2.97$, $p = 0.023$, Table 3.4), but there was no significant interaction with seed treatment ($F_{4, 99} = 0.39$, $p = 0.814$). *P. violae* oospore concentrations of 1 and 100 oospores g^{-1} resulted in the smallest root weights compared to the uninoculated control treatment (Figure 3.24a). A similar pattern was observed for mean carrot root weight, with a significant main effect of *P. violae* treatment ($F_{4,99} = 3.32$, $p = 0.013$, Table 3.4) but no evidence of a significant interaction with seed treatment ($F_{4,99} = 0.55$, $p = 0.697$). Again, *P. violae* oospore concentrations of 1 and 100 oospores g^{-1} resulted in the smallest mean root weight compared to the uninoculated control treatments (Figure 3.24b).

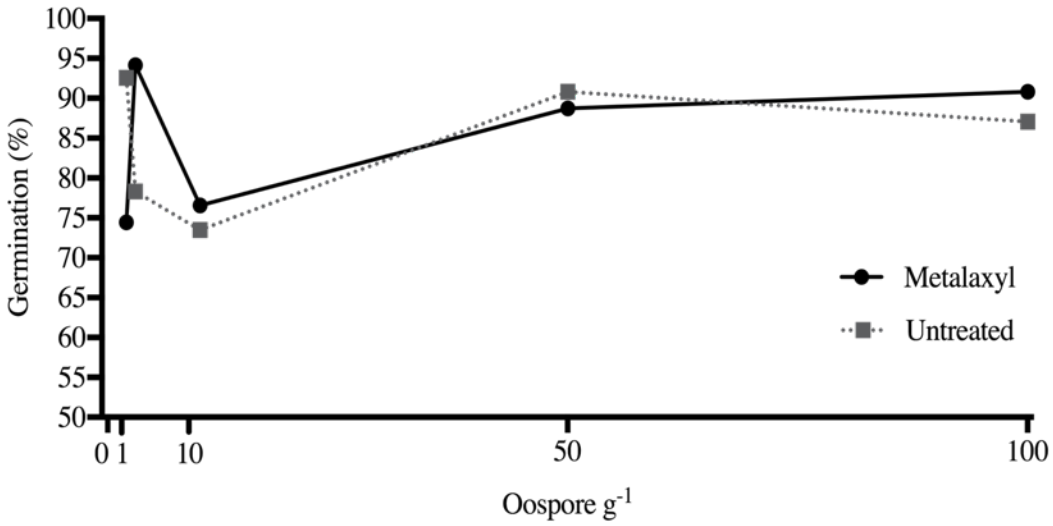


Figure 3.19 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on mean percentage carrot seed germination in metalaxyl-treated (black line with circles) and untreated seed (grey dashed line with squares) at seven w.p.s.

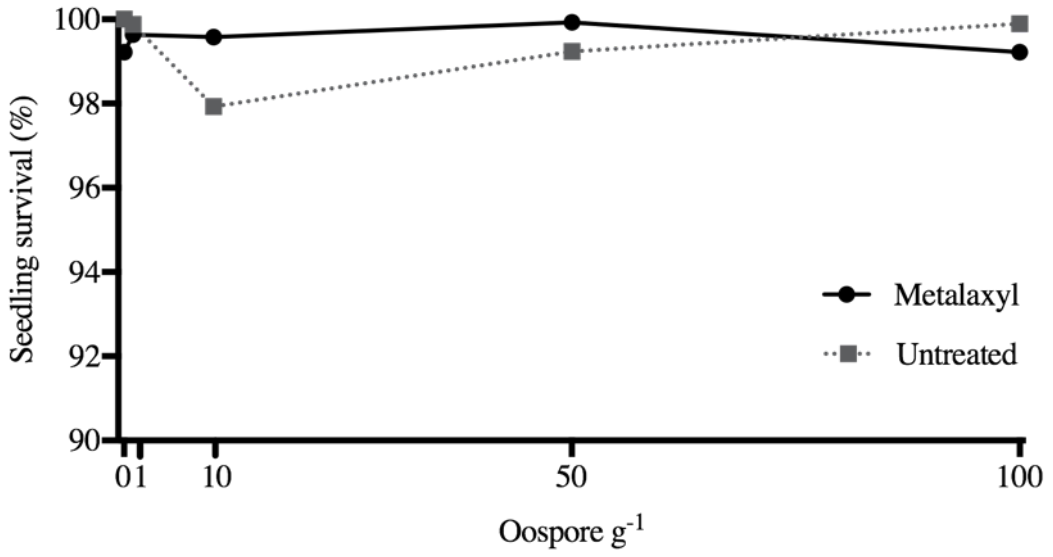


Figure 3.20 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on mean percentage carrot seedling survival for metalaxyl-treated (black line with circles) and untreated seed (grey dashed line with squares) at seven w.p.s.

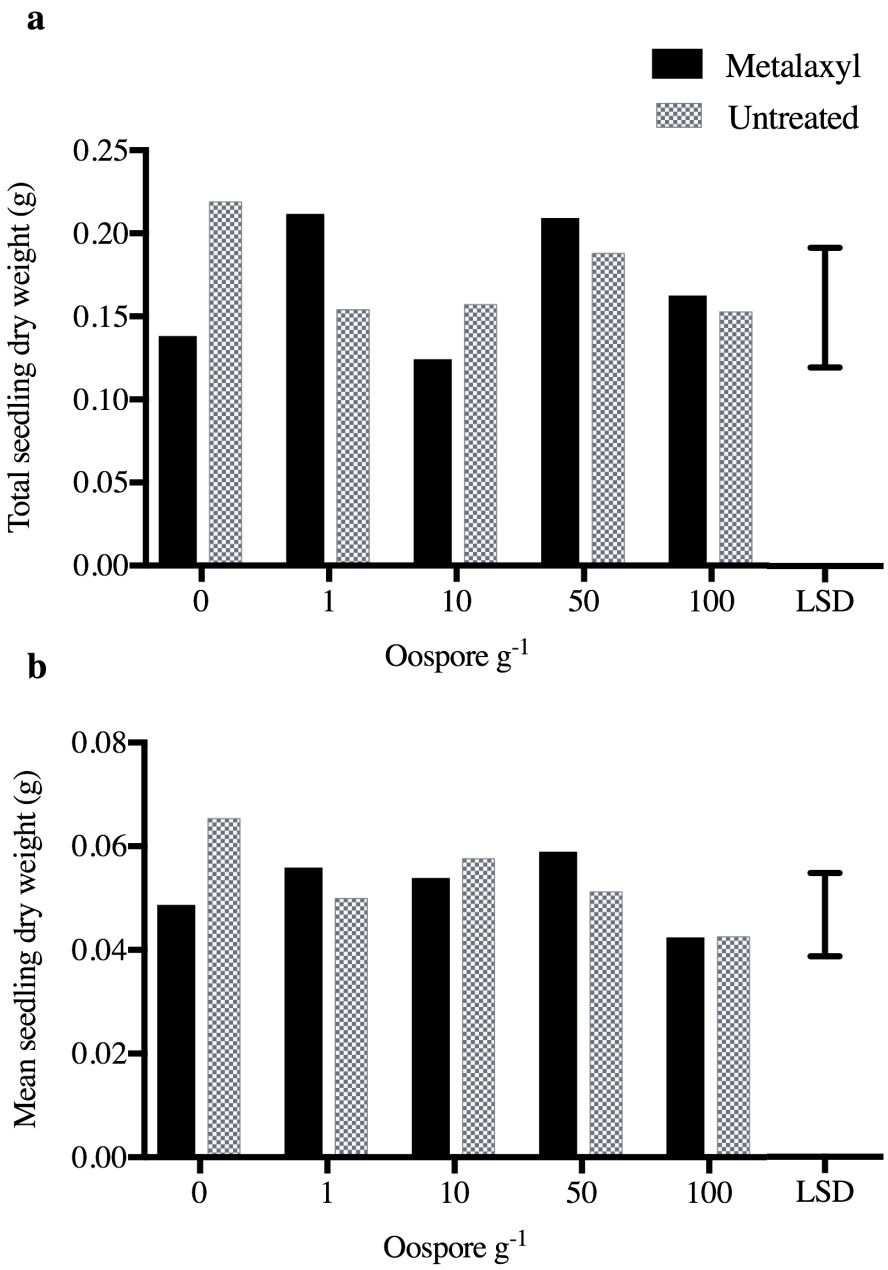


Figure 3.21 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on total carrot seedling dry weight per pot (a) and mean carrot seedling dry weight (b) in metalaxyl-treated (black solid bars) and untreated seed (grey hashed bars) at seven w.p.s. Error bars represent the LSD at the 5% level.

Figure 3.22 Representative photographs demonstrating the effect of *P. violae* on carrot root growth from untreated seed in uninoculated control (a), 10 (b) and 100 (c) oospore g^{-1} treatments

Figure 3.23 Representative photographs showing the effect of *P. violae* on carrot tap roots for metalaxyl-treated seed.

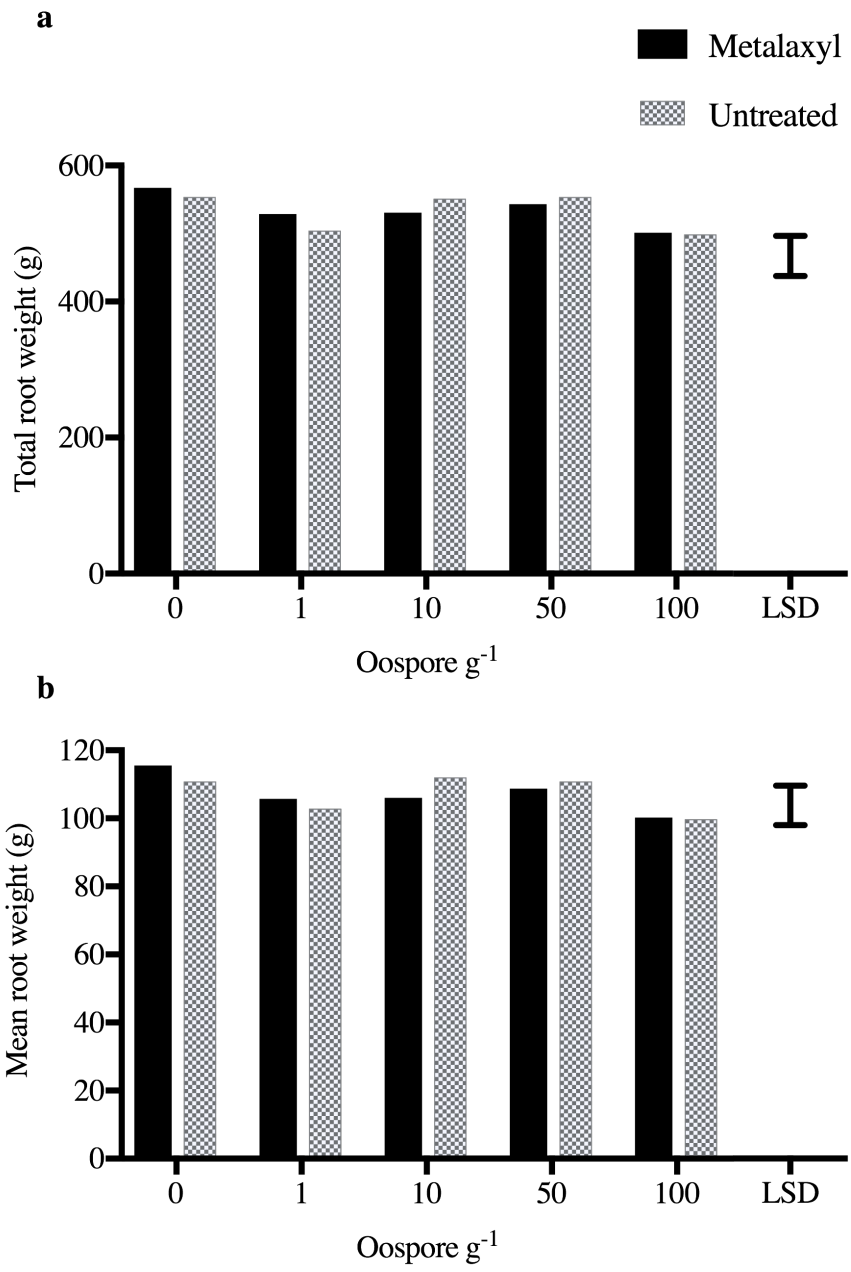


Figure 3.24 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on mean total root weight per pot (a) and mean carrot root weight (b) for metalaxyl-treated (black solid bars) and untreated seed (grey hashed bars) at seven w.p.s. Error bars represent the LSD at the 5% level.

Effect of *P. violae* inoculation on cavity spot incidence and severity

Cavity spot symptoms were observed in all *P. violae* treatments (Figure 3.25), with incidence varying between 3% and 23% across different oospore concentrations (Figure 3.26). However, a very small amount of cavity spot was also observed in uninoculated control pots for the metalaxyl-treated seed (3%). Overall, there was a main effect of *P. violae* treatment on cavity spot incidence ($F_{4,99} = 4.61$, $p = 0.002$; Table 3.4) but no significant interaction with seed treatment ($F_{4,99} = 1.69$, $p = 0.158$). As with root weight, the significant differences in cavity spot incidence between the uninoculated control and *P. violae* inoculated treatments were observed for oospore concentrations of 1 and 100 oospores g^{-1} but not for the intermediate oospore concentrations (10 and 50 oospores g^{-1}; Figure 3.26, Table 3.4).

The severity of cavity spot was calculated across all carrot roots (including those unaffected) and also only for those with symptoms. The number of cavity spot lesions across all roots was low and ranged from 0.06-0.2 lesions per carrot in metalaxyl-treated seed and 0-0.4 lesions per carrot in untreated seed. (Figure 3.27; Table 3.4). Nonetheless there was a significant interaction between *P. violae* oospore concentration and seed treatment ($F_{4,99} = 2.80$, $p = 0.030$), and the effect of concentration was not consistent across the seed treatments (Figure 3.27). Within the metalaxyl-treated seed, there were no differences in the number of cavity spot lesions between the different oospore concentrations, but as observed with both carrot root weight and cavity spot incidence, within the untreated seed, only concentrations of 1 and 100 oospore g^{-1} resulted in significantly greater numbers of lesions than the uninoculated control (Figure 3.27). There was also a significant main effect of *P. violae* treatment on cavity spot severity across all roots ($F_{4,99} = 4.73$, $p = 0.002$; Table 3.4). A similar pattern was observed here, where a significantly greater number of lesions was observed for the 1 and 100 oospore g^{-1} concentrations compared with the uninoculated control, but not for the intermediate oospore concentrations (10 and 50 oospores g^{-1}; Figure 3.27). This shows that when looking at cavity spot severity across different oospore concentrations, the increased severity in the 1 and 100 oospore g^{-1} treatments was sufficient to result in significant variation even when data from both seed treatment conditions was combined.

When only infected carrots were considered, the mean number of cavity spot lesions per carrot ranged between 1.3-2.5 lesions per root for metalaxyl-treated seed and 0-2.3 lesions per root for untreated seed (Figure 3.27, Table 3.4; statistical analysis not possible due to

large number of missing values i.e. unaffected carrots). Visual interpretation of the data revealed no consistent pattern of treatments on severity, i.e. if roots were infected, the number of lesions on each root was comparable across all *P. violae* oospore concentrations (Figure 3.27). The number of cavity spot lesions on a root ranged from 0-6 and 0-7 in metalaxyl-treated seed and untreated seed respectively.

There was evidence of a high percentage of carrots with deformed or forked roots, which increased as oospore concentration increased (Appendix C).

Figure 3.25 Representative photos of cavity spot lesions on carrot roots from *P. violae* inoculated treatments in Experiment 3.

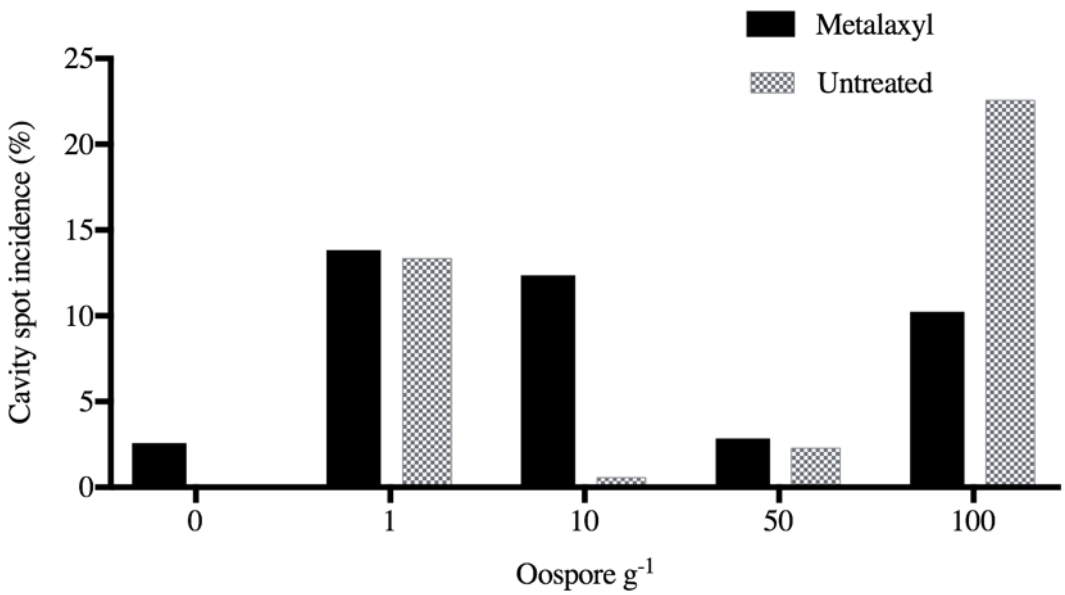


Figure 3.26 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on cavity spot incidence (proportion of roots affected) for metalaxyl-treated (black solid bars) and untreated seed (grey hashed bars) at harvest.

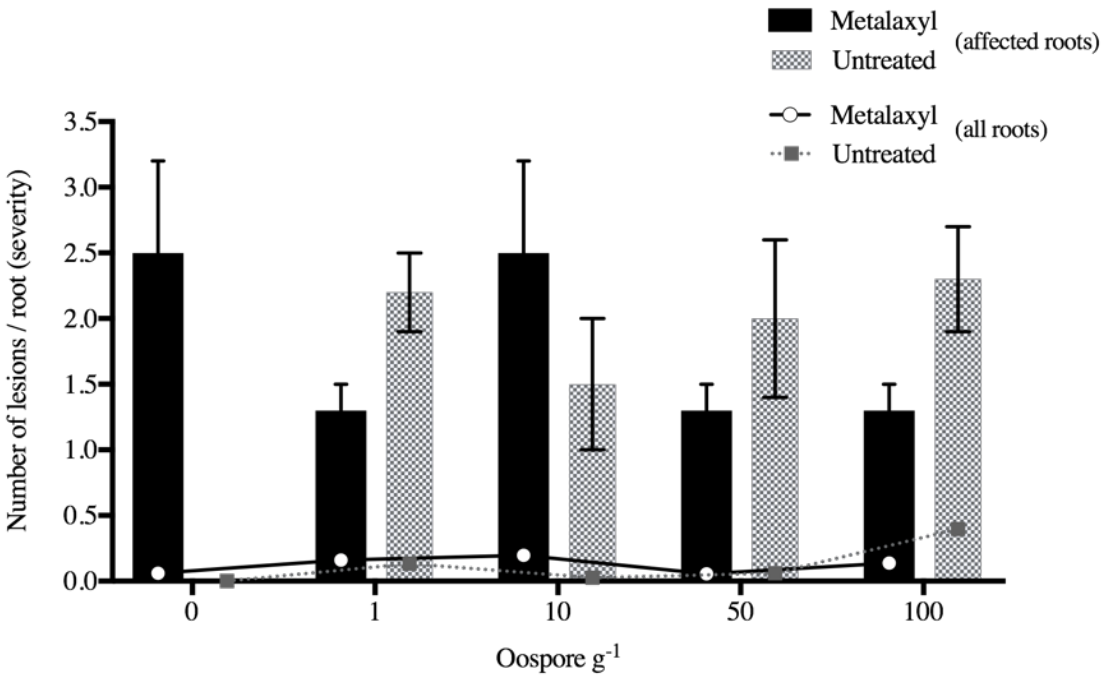


Figure 3.27 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on the number of lesions per root (cavity spot severity) calculated across all roots (metalaxyl-treated seed: black line with circles and untreated seed: grey dashed line with squares) and across infected roots only (metalaxyl-treated seed: black solid bars and untreated seed: grey hashed bars) at harvest. Error bars represent the SEM.

Isolation of *P. violae* from roots

Carrot tap root pieces from *P. violae* treatments plated on to CMA yielded *P. violae* colonies demonstrating that they had been infected by the pathogen. The χ^2 analysis revealed a significant difference in the proportion of infected roots between the uninoculated control and *P. violae* inoculated treatments across all root pieces tested (χ^2 (n = 9) = 68.968, $p < 0.001$). A similar pattern to root weight, incidence and severity was observed in that concentrations of 1 and 100 oospores g^{-1} resulted in the greatest proportion of infected root pieces with a mean of 14 out of 24 for both treatments, whilst the intermediate rates of 10 and 50 oospores g^{-1} resulted in lower levels of infection (a mean of 2 out of 24, and 7 out of 24 respectively). All *P. violae* inoculated treatments were different from the uninoculated control (0 out of 24 pieces infected for untreated seed, 1 out of 24 root pieces infected for metalaxyl-treated seed) but no clear differences were observed between carrot root pieces derived from metalaxyl-treated and untreated seed.

Table 3.4 Summary of results and statistical analyses for parameters measured for carrot growth and cavity spot development in Experiment 3 examining the effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium, and the effect of a seed treatment, on pot-grown carrots.

Variable measured	Concentration (oospores g^{-1})											
	***P. violae* oospore concentration**										**LSD (5%)**	***p* value**
Overall *P. violae* oospore conc.	**0**	**1**	**10**	**50**	**100**	**0**	**1**	**10**	**50**	**100**		
Germination (%) (conc)	66.9	69.1	60.0	71.4	7.57						7.57	0.027
Seedling survival (%) (conc)	2.54	2.73	6.00	3.27	4.62						4.62	0.586
Seedling total dry wt. (g) (conc)	0.179	0.183	0.141	0.199	0.049						0.049	0.160
Seeding mean dry wt. (g) (conc)	0.057	0.053	0.056	0.055	0.011						0.011	0.193
Harvest mean shoot dry wt. (g) (conc)	3.367	3.063	3.026	3.201	0.174						0.174	0.002
Harvest mean dry wt. (g) (conc)	16.69	15.16	14.99	16.01	0.869						0.869	0.001
Carrot total root wt. (g) (conc)	560.6	516.5	540.9	548.6	40.22						40.22	0.023
Carrot mean root wt. (g) (conc)	113.1	104.3	109.1	109.7	7.93						7.93	0.013
Cavity spot incidence (%)	4.62	21.64	12.51	9.23	10.57						10.57	0.002
Cavity spot severity (conc)	-0.897	-0.610	-0.711	-0.802	0.217						0.217	0.002
Deformed roots (%) (conc)	0.00	1.107	0.00	9.962	5.592						5.592	<0.001
Forked roots (%) (conc)	1.107	2.214	5.483	11.07	6.28						6.28	0.007
Combined oospore conc+seed treatment	**Metalaxyl**					**Untreated seed**						
Germination (%) (conc.seed)	59.6	76.0	61.0	70.4	72.6	92.6	78.3	73.5	90.8	87.1	10.7	0.009
Seedling survivial (%) (conc.seed)	5.07	3.45	3.70	1.54	5.07	0	2.01	8.28	5.01	1.85	6.54	0.173
Seedling total dry wt. (g) (conc.seed)	0.138	0.212	0.124	0.209	0.163	0.219	0.154	0.157	0.188	0.153	0.069	0.063
Seeding mean dry wt. (g) (conc.seed)	0.049	0.056	0.054	0.059	0.042	0.065	0.050	0.058	0.051	0.043	0.015	0.067
Harvest mean shoot dry wt. (g) (conc.seed)	3.399	2.951	2.918	3.180	3.201	3.335	3.175	3.135	3.222	3.039	0.247	0.120
Harvest total dry wt. (g) (conc.seed)	16.71	14.75	14.59	15.90	16.00	16.67	15.57	15.39	16.11	15.20	1.229	0.325
Carrot total root wt. (g) (conc.seed)	567.2	528.7	530.7	543.4	501.0	553.9	504.3	551.2	553.8	498.7	56.88	0.814
Carrot mean root wt. (g) (conc.seed)	115.5	105.7	106.1	108.7	100.2	110.8	102.8	112.1	110.8	99.7	11.22	0.697
Cavity spot incidence (%) (conc.seed)	9.23	21.84	20.59	9.71	18.66	0.00	21.44	4.43	8.75	28.38	14.94	0.158
Cavity spot severity (all roots) (conc.seed)	-0.793	-0.586	-0.526	-0.810	-0.623	-1.00	-0.633	-0.895	-0.794	-0.398	0.307	0.030
Deformed roots (%) (conc.seed)	0.00	0.00	0.00	8.855	14.235	0.00	2.214	0.00	11.069	19.72	7.908	0.865
Forked roots (%) (conc.seed)	0.00	4.428	8.752	13.283	7.697	2.214	0.00	2.214	8.855	11.069	8.88	0.419

Angular transformed means are shown for all percentage data (germination, seedling survival, cavity spot incidence, deformed and forked roots). Log_{10} transformed means are shown for cavity spot severity (this resulted in values of less than 1). [1] Top half of table represents data from analysis averaged across the seed treatment factors (conc); bottom half of table represents data from analysis with combined oospore concentration and seed treatments factors (conc.seed).

3.3.5 Field Experiments

The field macrocosms inoculated with solid inoculum of *P. violae* at different oospore concentrations in May 2016 and 2017 yielded a large number of mature carrot roots. All inoculated treatments resulted in characteristic cavity spot lesions forming on roots as would be observed in a commercial crop (Figures 3.28, 3.29). Observations during both growing seasons revealed no apparent effect of *P. violae* inoculation on seed germination, seedling survival, or top-growth (foliage) across any of the macrocosm plots.

All *P. violae* inoculated treatments resulted in characteristic cavity spot lesions (Figure 3.28, 3.29) with incidence ranging between 24-39% in 2017 and 30-36% in 2018 (Figure 3.31). Statistical analysis revealed a significant difference in cavity spot incidence across all treatments in both years (2017: $F_{5,15} = 26.65, p < 0.001$; 2018: $F_{5,15} = 32.26, p < 0.001$; Figure 3.31, Table 3.5). In 2017, all *P. violae* treatments resulted in significantly greater cavity spot incidence than the uninoculated control, with strong evidence of a linear dose response (increased oospore concentration resulting in increased cavity spot incidence; $F_{5,15} = 68.98, p < 0.001$; Table 3.5). Similarly, in 2018, all roots showed significantly greater cavity spot incidence than the uninoculated control, and there was still strong evidence of a linear dose response. However, only a small amount of the variation observed across treatments was explained by this ($F_{5,15} = 40.60, p < 0.001$, Table 3.5).

Despite a high incidence of cavity spot, disease severity measured across all roots was low, with 0.46-0.72 lesions per root in *P. violae* inoculated plots in 2017 and 0.69-0.86 lesions per root in 2018 (Figure 3.32, Table 3.5). However, there was a main effect of oospore concentration on cavity spot severity in both years (2017: $F_{5,15} = 42.96, p < 0.001$; 2018: $F_{5,15} = 16.00, p < 0.001$) and all *P. violae* inoculated plots resulted in significantly greater cavity spot severity than the uninoculated controls (Table 3.5). When severity was calculated across cavity spot affected roots only, the number of lesions per root in *P. violae* inoculated plots ranged between 1.9-2.4 in 2017, and 2.2-2.6 in 2018 (Figure 3.32). In 2017, there were significant differences in severity across infected roots only between different oospore concentrations ($F_{5,15} = 4.26, p = 0.008$; Table 3.5), and all *P. violae* inoculated treatments showed significantly greater cavity spot severity compared to the uninoculated control. However, in 2018 cavity spot severity across affected roots did not vary significantly between treatments ($F_{5,15} = 2.19, p = 0.109$). This reflects the greater cavity spot severity in the uninoculated control plots compared to 2017 (Figure 3.32).

Figure 3.28 Representative photographs of cavity spot symptoms on carrot roots harvested from field macrocosm plots in 2017 in uninoculated control (a), 20 oospores g^{-1} (b), 30 oospores g^{-1} (c) and 50 oospores g^{-1} (d).

Figure 3.29 Representative photographs of cavity spot symptoms on carrot roots harvested from field macrocosm plots in 2018 in uninoculated control (a), 5 oospores g^{-1} (b), 30 oospores g^{-1} (c) and 50 oospores g^{-1} (d).

At harvest in both 2017 and 2018, there were no significant differences in mean total carrot root weight, root weight per carrot, or total number of roots per macrocosm across any of the treatments (Table 3.5). When comparing across the two years, total carrot root fresh weight yielded per macrocosm was similar, however there was a difference in how this total weight was obtained. In 2017, mean root weight per carrot was greater (62.0-84.6 g) than in 2018 (30.5-39.8 g; Table 3.5). In contrast, the total number of roots was greater in 2018 than 2017, with 103-151 roots per plot in 2017, and 197-244 roots per plot in 2018 (Table 3.5; Appendix D).

At the first harvest in 2017, a few carrots exhibited cavity spot-type lesions in the uninoculated control treatments (Figure 3.30). In the following harvest in 2018, the levels of cavity spot in the control plots had increased (Figure 3.30). In 2018, although there was an increase in 'typical' cavity spot, many of the lesions were atypical: they had a darker and less sunken appearance than a 'typical' cavity spot lesion (Figure 3.29). A small number of atypical lesions were also observed in the *P. violae* inoculated treatments, however across all treatments, any lesions observed as atypical were not recorded as cavity spot. In 2018, samples of a range of cavity spot lesions from control plots were plated out on CMA to attempt to differentiate between typical and atypical cavity spot lesions. DNA was extracted from pure cultures and isolates were identified through sequencing of the ITS region as in Chapter 2, Section 2.2.3. Of the six cultures obtained from atypical lesions, four were identified as an *Ilyonectria* spp., one was identified as *P. sulcatum*, and another as a zygomycete, most likely a *Mortierella* spp. Of the four cultures obtained from 'typical lesions', sequencing identified them as *P. intermedium, P. violae, P. sulcatum* and an *Ilyonectria* spp.

Figure 3.30 Representative photographs of 'atypical' cavity spot lesions observed on carrot roots from uninoculated control treatments from field macrocosms in 2018.

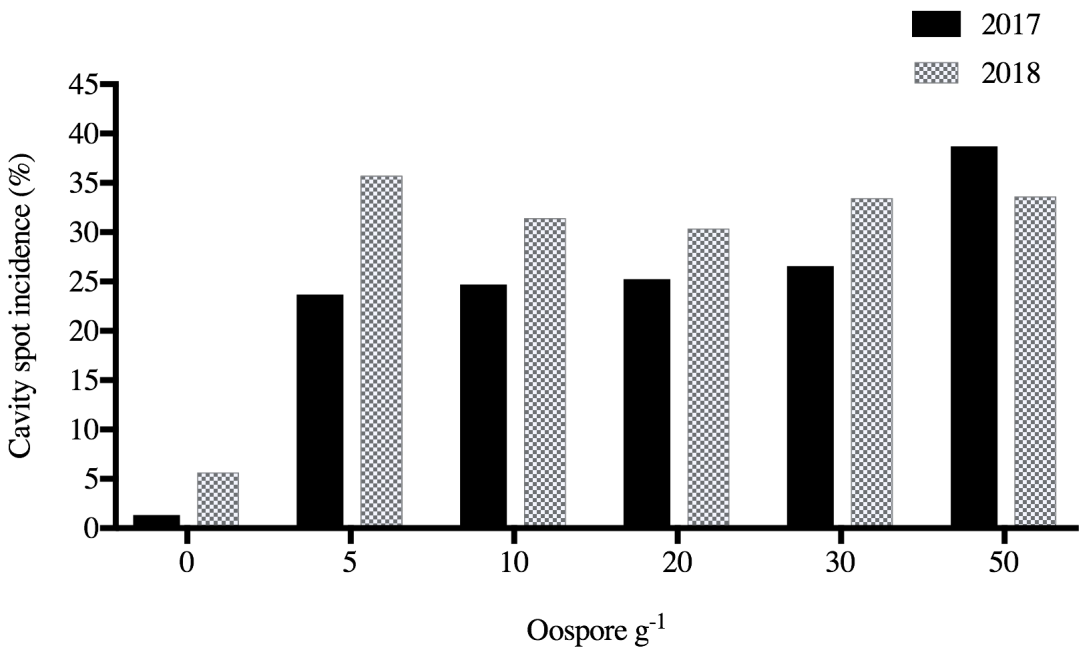


Figure 3.31 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on cavity spot incidence for 2017 (black solid bars) and 2018 (grey hashed bars) in field macrocosms at harvest.

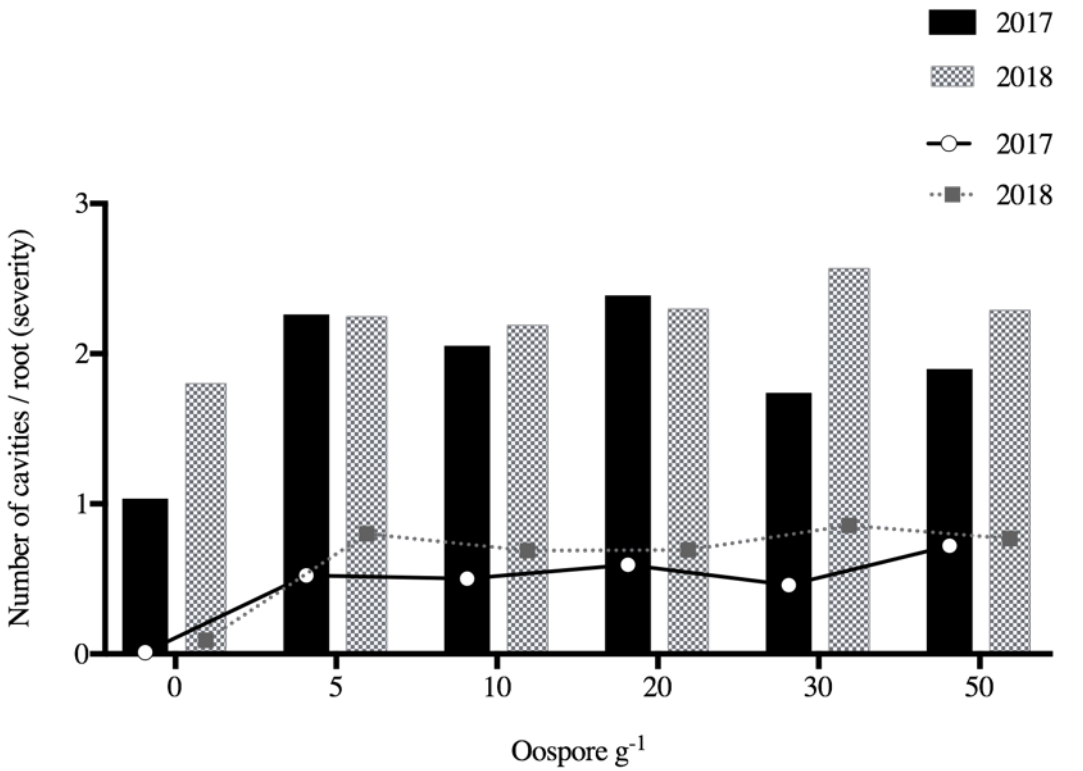


Figure 3.32 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on number of lesions per root (cavity spot severity) calculated across all roots (2017: black line with circles and 2018: grey dashed line with squares) and across cavity spot affected roots only (2017: black solid bars and 2018: grey hashed bars) in field macrocosms at harvest.

Table 3.5 Summary of results and statistical analyses for parameters measured for carrot growth and cavity spot development examining the effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on pot-grown carrots and in field-grown 'macrocosm' carrots in 2017 and 2018.

Variable measured	2017								2018							
	Concentration (oospores g^{-1})						LSD (5%)	*p* value	Concentration (oospores g^{-1})						LSD (5%)	*p* value
	0	5	10	25	50	75			0	5	10	25	50	75		
Total carrot root weight per macrocosm (g)	8.17	8.50	8.20	8.80	7.93	7.84	1.457	0.735	8.28	7.27	8.15	8.17	7.31	7.45	1.342	0.381
Mean root weight per carrot (g)	65.3	62.0	81.6	84.6	70.3	80.8	40.28	0.770	36.6	37.8	39.8	38.2	38.5	30.5	8.72	0.316
Total number of roots per macrocosm	147.8	150.5	112.2	116.5	117.5	102.8	64.99	0.542	226.2	198.5	204.2	216.0	197.0	243.5	43.78	0.230
Cavity spot incidence (%)	6.67	29.12	29.81	30.17	31.03	38.47	6.421	<0.001	13.74	36.71	34.10	33.44	35.33	35.44	4.646	<0.001
Cavity spot severity (all roots)	-1.91	-0.28	-0.30	-0.23	-0.34	-0.14	0.312	<0.001	-1.04	-0.09	-0.16	-0.16	-0.07	-0.11	0.284	<0.001
Cavity spot severity (infected roots)	0.015	0.354	0.313	0.378	0.240	0.278	0.182	0.008	0.256	0.352	0.341	0.362	0.410	0.360	0.102	0.109

Angular transformed means are shown for percentage data: cavity spot incidence. Log_{10} transformed means are shown for cavity spot severity (this resulted in values of less than 1).

3.4 Discussion

This is the first study to report the successful artificial inoculation of carrots with a *P. violae* solid substrate sand/oat inoculum in both glasshouse and field environments. Following successful large-scale production of inoculum, inoculation in field macrocosms resulted in up to 40% of carrot roots being affected by cavity spot lesions that were typical of those found in commercial production. In addition to lesions also being formed in pot-based glasshouse experiments, this is the first report of solid substrate *P. violae* oospore inoculum causing significant changes in carrot root development through severe stunting, reduced carrot root weight and formation of dark tap roots with necrosis.

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}. Quantification of solid *P. violae* inoculum has previously been determined by weight (El-Tarabily *et al.*, 2004; Suffert & Guibert, 2007), which can be difficult to standardise across inoculum batches. Variation in growth rate and ability to colonise the growing substrate between *P. violae* isolates can also lead to considerable variation in active/viable inoculum, which again is difficult to quantify. In this study, there was some variability between different batches of inoculum despite being produced under standard conditions, with inoculum density ranging from 10,000-40,000 oospores g^{-1} for *P. violae* isolate P10. Furthermore, variation between isolates, incubation temperature, and source of the growing substrate (sand) also affected oospore production (data not shown). However, all batches were quantified and adjusted to be comparable across experiments, and very little mycelial growth was observed under light microscopy during quantification.

P. violae inoculum was also successfully produced in V8 liquid medium and this medium supported the production of large numbers of oospores. Estimations of oospore numbers in a liquid culture mycelial mat has previously proved difficult, as oospores are strongly attached to mycelium and standard agitation does not liberate enough free oospores for infection. However, this work demonstrated that maceration, sonication and subsequent filtration resulted in *P. violae* oospore numbers of up to 11,747 spores ml^{-1} in liquid V8B.

By comparison, Ayers and Lumsden (1975) achieved oospore numbers of over 300,000 oospores ml^{-1} in liquid V8B for *P. aphanidermatum*, *P. ultimum* and *P. myriotylum*. The lower number of oospores produced in this PhD study may be attributed to innate differences between individual *Pythium* species, which are known to vary significantly in their ability to produce oospores on different media (van der Plaats-Niterink, 1981). In the same study by Ayers and Lumsden (1975), *P. aphanidermatum* produced 1,146,000 oospores ml^{-1}, almost four times that of *P. ultimum* and *P. myriotylum* (Ayers & Lumsden, 1975). In other experiments during this PhD project, as part of AHDB project FV391a, it was found that *P. sulcatum* produced significantly greater numbers of oospores in V8 liquid culture than *P. violae*.

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. In addition to *P. ultimum*, several other *Pythium* spp. have previously been shown to induce damping-off in carrot. Howard *et al.* (1978) reported damping-off and root die back in carrot seedlings following artificial inoculation with 11 different *Pythium* spp. (but not *P. violae*) including *P. sulcatum* and *P. irregulare* (both associated with cavity spot in the UK) using a corn meal/sand inoculum and a concentration of 1000 propagules g^{-1}. Hawthorne (1988) inoculated soil with *P. ultimum*, *P. irregulare* and *P. acanthicum* grown on 30 g vermiculite amended with 350 ml potato-carrot extract and reported that alfalfa seedling germination was reduced by approx. 30% compared to uninoculated controls. Wheeler *et al.* (2017) grew *P. aphanidermatum* in V8 amended vermiculite and noted this inoculum caused extensive mortality in petunia plants in under 24 hours, whereas half the volume

of inoculum caused good disease pressure without widespread mortality, however the inoculum was not quantified. These experiments all used inoculum of a much younger age than used for *P. violae* in this study (10-64 weeks) and therefore infection may have been initiated from mycelium rather than oospores.

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. 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 the mature plant experiments where carrots were grown in pots in the glasshouse, the stunted carrot roots and abnormal tap roots were confirmed to be due to *P. violae* as the pathogen was isolated from a large proportion of the roots. Although *P. violae* has not generally been associated with stunted carrot roots in the field, a number of researchers have reported this symptom during artificial inoculation. In addition, cavity spot incidence in the three glasshouse experiments in this PhD study, where 20, 13 and 23% of roots were affected in Experiments 1, 2 and 3 respectively, is comparable to previous studies. White (1986) sowed carrot seedlings into naturally *P. violae* infested field soil in pots in the glasshouse, and applied a range of treatments to attempt to control cavity spot. It was found that for all sowings where a soil drench containing metalaxyl was applied (which eliminated cavity spot lesions), numerous healthy white feeder roots were observed and these carrots were the largest compared to less effective treatments and an uninfected control. In treatments where disease was present, roots showed necrosis of feeder roots as well as a decreased root weight, alongside incidence of cavity spot lesions of up to 27% (White, 1986). In the experiment by White (1986), 36% of roots resulted in *P. violae* colonies following isolations from lesions on infected roots. Comparatively, in this PhD project, root infection was variable, but resulted in 58, 25 and 58% *P. violae* colonies isolated from roots in Experiments 1, 2 and 3 respectively.

There have been a number of other successful artificial inoculation attempts before, however these experiments have been with mycelium inoculation rather than oospore based inoculum. Some other inoculation methods have resulted in very high levels of cavity spot incidence. Sowing seeds and transplanting mature roots into a growing medium amended with vermiculite soaked in V8 liquid cultures of *P. violae* and *P. ultimum* at 500 cfu g^{-1} caused roots to develop typical cavity spot lesions (Vivoda *et al.*, 1991). Similarly, artificial inoculation using millet seed colonised with different *Pythium* spp. (but not *P. violae*) caused multiple lesions to develop on carrot roots (El-Tarabily *et al.*, 1996; El-Tarabily *et al.*, 2004). Although both of these methods were successful in causing cavity spot symptoms, they both use mycelium inoculum rather than oospores, the more natural and quantifiable approach used in this PhD study.

Suffert and Montfort (2007) attempted artificial inoculation using three methods including: (i) mixing *P. violae* mycelium from an agar culture into soil; (ii) cutting out

cavity spot lesions and mixing with soil, and (iii) using infected carrot roots as an inoculum source for transplanted clean roots. Cavity spot incidence was high for all methods ((i) and (iii), >80%; method (ii), <50%) (Suffert & Montfort, 2007). This incidence is greater than seen in any of the experiments conducted in this PhD project. No dose-response effect was observed for methods (i) or (iii), matching the lack of dose-response seen in this PhD project in the pot experiments. There was some indication of a dose-response (increasing incidence with increasing inoculum weight) using method (ii), but the results were highly variable. Method (iii) also resulted in carrot root weight decreasing significantly with increasing amount of *P. violae* inoculum (Suffert & Montfort, 2007), similar to the data in this PhD project. As discussed with the methods above, it is likely that some infection resulted from mycelium growth, rather than oospore germination.

In the third mature plant experiment with the solid *P. violae* inoculum, a metalaxyl seed treatment was also included with the aim of preventing early infection and damping-off of carrot seedlings and potentially allowing roots to develop normally without showing stunted growth. Contrary to expectation, there was no significant overall effect of the metalaxyl seed treatment across all variables measured. This surprising result should be further examined. Few previous studies have evaluated metalaxyl for *P. violae* control using artificial inoculation. White (1986) examined the effect of different treatments on cavity spot incidence for carrot roots grown both in naturally-infested and non-infested field soils. In the naturally-infested soil, treatments included untreated seed, metalaxyl-treated seed, as well as a soil sterilisation treatment with methyl bromide. Soil sterilisation resulted in the most significant reduction in cavity spot incidence but the metalaxyl seed treatment also showed significantly reduced incidence compared to the untreated seed controls. The isolate used in this study was not tested *in vitro* for metalaxyl sensitivity, and therefore it is possible that this isolate was unaffected by the metalaxyl seed treatment. Investigating this possibility for a wider range of isolates could potentially provide growers with a more detailed understanding of how metalaxyl sensitivity has altered for *P. violae* isolates across UK fields since the treatment was first introduced.
Metalaxyl seed treatment resulted in increased seed germination in *P. violae* inoculated treatments, but resulted in a reduction in germination in the absence of the pathogen (in the uninoculated control). This difference was also evident for seedling dry weight, indicating that the metalaxyl seed treatment may only be beneficial in the presence of *P. violae*. Root weight, cavity spot incidence/severity, and root-plating all followed a similar pattern in this experiment: the intermediate oospore concentrations (10 and 50 oospores

g^{-1}) resulted in less infection and bigger roots than the lowest and highest oospore concentrations (1 and 100 oospores g^{-1}).

The lack of a dose response effect across most of the variables in all three glasshouse experiments is difficult to explain. In our experiments, inoculum was prepared and aged to encourage oospore production and proactively reduce mycelial growth. This means the viability of any remaining mycelial fragments is likely to have been low. However, a small number of mycelium fragments were visible during inoculum quantification. If different amounts of surviving mycelium ended up in different pots, and these were viable, it may be some of the infection seen was due to mycelium fragment growth rather than germinating oospores. Furthermore, this may explain the general lack of a dose-response effect in the glasshouse experiments compared to the field macrocosm experiments. It is generally thought that mycelium of *Pythium* spp. is unable to compete in a microbiologically diverse environment, as it lyses easily and is out-competed by other species (Hendrix & Campbell, 1973). Holmes *et al.* (1998) found that the ability of the biological control agent *P. oligandrum* to control damping-off in sugar beet by *P. ultimum* varied depending the ability of *P. oligandrum* to colonise and become established in the compost growing medium. In these experiments mycelial preparations, in their actively growing state, were best for control. This work was conducted in sterile soil, and so mycelium growth (and therefore infection) may only be relevant in less microbiologically diverse settings where there is less competition with other species e.g. in compost / sand. In the field macrocosm experiments, the microbiological diversity of the field soil may have out-competed *Pythium* mycelium, meaning infection was driven mostly by oospore germination, thus showing the expected dose-response relationship. Another possible contributing factor to the lack of dose-response relates to oospore germination. Given that the conditions required for *P. violae* oospore germination have not yet been established, it is possible that the number of oospores that germinated was inconsistent across treatments, meaning the amount of *P. violae* infection was not necessarily linked to the number of oospores applied.

This is also the first report of consistent successful artificial inoculation with *P. violae* in field macrocosm plots. A high incidence of cavity spot (up to 40%) was observed over two years. In contrast to the glasshouse experiments, there was no obvious effect on seed germination or carrot foliage, although these variables were not specifically measured. Carrot seedling germination and damping-off is not usually a problem in commercial fields (I. Holmes, Strawsons Ltd, UK, personal communication), therefore a high

percentage emergence does not necessarily mean *Pythium* infection hasn't occurred, just that it is not affecting the plant at that time (Higginbotham *et al.*, 2004). The high incidence of cavity spot was evident across the different oospore concentrations, with clear differences between the inoculated plots and the uninoculated control. In both years there was significant evidence of a dose-response effect, although in 2018 the mean percentage of carrots with cavity spot was more comparable across the different oospore concentrations than in 2017. This may be because infection from the previous year provided additional inoculum. *P. violae* oospores are known to form and survive in the soil (Mitchell, 1978) and the number produced and surviving from 2017 may not have been related to the initial inoculum concentration applied, hence reducing any dose-response effect of the second inoculation for cavity spot symptoms observed in 2018. Over the two years, cavity spot incidence was similar, but severity increased in 2018. Again, this result may be due to additive effects of infection from the previous year and production of further inoculum, or may be related to different environmental conditions between the two years.

Cavity spot lesions were also observed in the uninoculated control macrocosm plots in 2018 although the majority of these were 'atypical' compared to those observed in the inoculated plots. From the variety of fungal species identified from sequencing DNA extracted from the lesions, the most common were *Ilyonectria* spp.. These are known root pathogens that cause root rots in a number of crops such as grapevine, ginseng and parsnip (Chaverri *et al.*, 2011). The zygomyecte possibly identified as a *Moriterella* spp. is common in most soil environments, and is often found as a saprophyte (Yadav *et al.*, 2015), therefore is likely to have been living off the decaying lesions. The only *Pythium* spp. identified from these atypical lesions in control plots was *P. sulcatum*, which presumably was a natural component of the field soil used in the macrocosms when they were established. *P. intermedium*, *P. sulcatum*, *P. violae* and *Ilyoncectria* were identified following isolation from the four 'typical' cavity spot lesions on control roots. These results highlight the fact that different fungi can be associated with cavity spot type lesions and that many of these may already be present in soil.

Cavity spot lesions observed on carrot roots grown in the *P. violae* inoculated macrocosms were generally larger and more typical of those observed in commercial production compared to the pot experiments. Although it is difficult to elucidate the specific factors involved, contributing factors may include: a greater variation of, or lower, soil temperatures; the roots being grown for longer; or more variable soil moisture

(Vivoda *et al.*, 1991). In addition, the presence of a different range of microorganisms in the field soil may have contributed to the expansion of lesions (Howard *et al.*, 2017).

Overall, the high variation in variables measured throughout the seedling, pot and field experiments clearly indicates that one or more additional factors are required for reproducible cavity spot infection. These may relate to inoculum production/viability, or sub-optimal environmental conditions (Ayers & Lumsden, 1975; Hiltunen & White, 2002) and could include:

- *P. violae* was not applied at a high enough concentration
- *P. violae* oospores were not viable
- *P. violae* oospores were dormant/did not germinate due to insufficient development or lack of correct environmental conditions
- Variation in *P. violae* isolates (i.e. some isolates less virulent or oospores less likely to germinate)
- Chemical/physical properties of the sand/oat inoculum and sand used to grow carrot seedlings
- Temperature/moisture conditions used for inoculum production and seedling tests

Each of these are addressed below.

As stated above, direct comparison of the *P. violae* inoculum concentrations used here with previous research is difficult due to differences between the studies in the *Pythium* spp. being examined, how inoculum was quantified, and a lack of information on whether mycelium or oospores were responsible for infection. In this study, the concentrations of oospores in the seedling experiments were based on a preliminary experiment that caused mid to high seedling mortality, and suggested a dose response effect. *P. violae* oospores derived from V8 culture in this study were inoculated at a significantly lower level than that used by Pettitt *et al.* (2002). They applied 3 million oospores in approximately the same volume of substrate as was used in this study, where a maximum of 90,000 oospores were applied (max. 300 oospores g^{-1}). In another study, cultures of *P. myriotylum* were sonicated to ensure that oospores were the only viable propagule, and the density of *P. myriotylum* oospores required to infect rye was investigated (Mitchell, 1975). Seedling mortality ranged from 2-100 % for a density of 1-250 oospores g^{-1} soil (Mitchell, 1975). This is comparable with the oospore concentrations used in this study, meaning the

contrast in seedling mortality may be because different numbers of oospores are required for equivalent levels of infection in different *Pythium* spp..

As *P. violae* oospores were used as the primary inoculum source in this work (rather than mycelium), the viability and ability of these oospores to germinate is critical. Holmes *et al.* (1998) found that the ability of the biological control agent *P. oligandrum* to control damping-off in sugar beet by *P. ultimum* varied depending on inoculum type, concentration and the method of application. The addition of *P. oligandrum* mycelial suspensions were most effective, and a lower level of control (or no control) was achieved when oospores were applied (Holmes *et al*., 1998). They hypothesised that the failure of *P. oligandrum* oospore treatments may have been due to constitutive dormancy. After oospores mature, a period of dormancy is often required before germination can occur, and this is a common phenomenon across *Pythium* spp. (van der Plaats-Niterink, 1981; Higginbotham *et al.*, 2004). Poor germination of oospores has previously been reported for different *Pythium* spp.. For oospores derived from liquid V8 cultures, germination varied between 0-4% for *P. myriotylum*, 5-34% for *P. ultimum* and 89-90% for *P. aphanidermatum* (Ayers & Lumsden, 1975), demonstrating wide variation between species. Low germination levels were observed for *P. violae* oospores, ranging from 5-14% depending on age of V8 liquid culture, temperature and the agar medium on which germination was assessed (Pettitt *et al.*, 2002). However, it has been suggested that this variation is a normal aspect of *P. violae* oospore survival behaviour and is not an accurate reflection of viability (Pettit *et al*., 2002).

Several researchers have also reported on the dormancy of *Pythium* oospores. *P. aphanidermatum* oospores produced in 4-5 weeks old cultures did not germinate until they were exposed to the soil, and germination was enhanced the longer the oospores were left in soil, up to 70 days (Mondal *et al.*, 1996). It was also found that many *Pythium* spp. can germinate in response to root exudates (Hendrix & Campbell, 1973; Huisman, 1982). For example, percentage germination of zoospores of *P. aphanidermatum* increased in response to pea root exudates (Chang-Ho, 1970), and sowing seed of red pine into soil containing sporangia of *P. irregulare* allowed germination of the sporangia, which did not occur in control soil without red pine seedlings (Agnihotri & Vaartaja, 1970). However, for *P. violae* it has been shown that carrot root exudates did not enhance infection or mycelium growth (Suffert & Lucas, 2008).

Methods to break dormancy and increase germination of oospores have been widely studied across different oomycete species. El-Hamalawi and Erwin (1986) used a range of treatments to attempt to increase germination of oospores of *Phytophthora megasperma formae speciales* (f. sp.) *medicaginis* . They found a number of treatments, including treatment with $KMnO_4$ and heat shock, increased oospore germination. They also found that desiccation significantly decreased the viability and germination of oospores of *P. m.* f. sp. *medicaginis*. However, for *Pythium aphanidermatum,* it has been observed that oospore germination increased after desiccation of the cultures (Rubens & Halford, 1983), suggesting that oospores of different species of *Pythium* and *Phytophthora* may react differently to the same treatments. Similarly, Boutet *et al.* (2010) concluded that *Phytophthora* species react differently to methods for breaking dormancy. For instance *P. cactorum* oospore germination was increased following exposure to an oxidising agent (e.g. $KMnO_4$), but the same treatment had no effect on breaking dormancy of *P. ramorum* oospores. The use of a soil extract (made from commercial field soil incubated in SDW for 24 hours, then filtered through an 11 µm filter) also improved *P. cactorum* oospore germination, but not that of *P. ramorum.* For *Pythium* spp., cellulase has previously been demonstrated to enhance germination of oospores of *P. oligandrum*; a three-fold increase after 6 hours incubation (Holmes *et al.*, 1998) and *P. myriotylum* (Sauve & Mitchell, 1977). Enzyme treatment was also employed by Pettitt *et al.* (2002) to attempt to break dormancy of *P. violae* oospores grown in V8 liquid. Incubation for 24-48 hours with either cellulase or β-glucuronidase at 25˚C or 30˚C had no effect on oospore germination; however, incubation for 24 hours at 20˚C with cellulase doubled the percentage gemination compared to the untreated control (4.7% vs. 2.1% respectively). Oospores of a range of *Pythium* spp. have been shown to germinate in response to root exudates (Nelson, 1990) however no work has been conducted on how *P. violae* oospores respond to carrot root exudates. In all the experiments conducted during this PhD project, there was no treatment of *P. violae* oospores to artificially enhance germination. The transfer of oospores to non-sterile sand, alongside the presence of carrot seedling root exudates, was thought to be sufficient to stimulate germination. However, the variable results from these experiments suggest that these factors may not have been sufficient to reliably break *P. violae* oospore dormancy.

The age of *Pythium* inoculum can also affect the viability and/or germination ability of oospores produced, as well as the amount and viability of mycelium (Mondal *et al.*, 1996). Boutet *et al.* (2010) comments on how factors including age of oospores, and influence of soil or plant extract can affect oospore germination of *Phytophthora* species. Ayers

and Lumsden (1975) incubated oospores from *P. myriotylum*, *P. ultimum* and *P. aphanidermatum* on non-sterile soil agar (made from 10 g of fresh soil stirred into 1 L tap water, incubated for three days at 25˚C and filtered) for up to seven weeks. For *P. aphanidermatum,* germination increased after one week of incubation from 26.9% to 64.0%, and kept increasing up to seven weeks, resulting in over 90% germination. *P. ultimum* oospore germination also increased over time, with 0% pre-incubation rising to 34.4% after 14 days (for oospores dried prior to incubation). Oospores of *P. myriotylum* showed a reduction in germination, however: after 14 days germination was observed to be 4.5%, compared to 11.7% pre-incubation (Ayers & Lumsden, 1975). The researchers concluded that the conditions that govern oospore dormancy and germination vary markedly between different species. In this study, seedling Experiment 5 attempted to determine if *P. violae* oospore age contributed to the observed variation in infection but because the experiment had to be abandoned early, the results were inconclusive. Further work considering the effect of age on *P. violae* inoculum is therefore warranted.

As discussed above, oospore production, germination and subsequent infection can vary widely between different *Pythium* spp., and this may also be true for different isolates within a species. Few studies have addressed this variation for *P. violae*, and none have examined the effect of different *P. violae* isolates on seedling mortality. Previous results using laboratory-based pathogenicity tests have confirmed the variability between different isolates of the same *Pythium* spp. in their ability to cause cavity spot, with differences observed in the size of lesions produced (El-Tarabily *et al.*, 1996; El-Tarabily *et al.*, 2004; Suffert & Guibert, 2007). Studies on mature carrot roots with different *Pythium* spp. have also revealed a range of infection levels from different inoculum densities. El-Tarabily *et al.* (1996) tested different millet seed inoculum levels of *P. sulcatum* and *P. coloratum* isolates on their ability to induce cavity spot lesions with the former producing a few small lesions at both 8 and 10 g kg^{-1} soil and no lesions at 5 g kg^{-1} soil while the latter was able to produce a small number of lesions at 1 g kg^{-1}, and numerous large lesions at 5 g kg^{-1} soil. In the present study, *P. violae* isolate P4 caused less seedling mortality than isolate P10 and isolate P4 was also associated with smaller lesion sizes produced in previous laboratory pathogenicity experiments (Chapter 2, Section 2.3.3).

Other factors that may have affected the outcome of the seedling assays include the growth substrate (sand) in which the carrot seedlings were grown. When *P. violae* was applied to carrot seedlings in autoclaved sand, no infection was observed across any of

the treatments. Mondal *et al.* (1996) found that the germination of *P. aphanidermatum* oospores took longer in sterile soil extract compared to non-sterile soil extract (sterilised via autoclaving for 40 minutes at 121˚C each for two successive days before use). It has also been suggested that *P. violae* is an opportunistic pathogen that can only cause infection when certain other microorganisms are inhibited or removed (Barton, 1958; Hendrix & Campbell, 1973). This suggests that a sterile substrate might be more conducive to *P. violae* infection, but this was not observed in the experiments reported here, perhaps due to other factors preventing disease development.

The variation in success of pot and field experiments is challenging to decipher. Soil moisture and temperature are known to be important factors for *Pythium* disease development (Hendrix & Campbell, 1973). It is thought that while soil moisture and temperature are both important for infection, the optimum temperature for growth may not be the optimum temperature for infection (Hendrix & Campbell, 1973). *P. violae* generally favours lower temperatures (17-20˚C *in vitro*) and high soil moisture (Hiltunen & White, 2002). The seedling experiment conducted at two temperatures (14 and 10˚C; seedling Experiment 2) revealed no clear differences in infection rate. In addition, one major factor not controlled for was the influence of water. In the glasshouse experiments, pots were kept damp, but were not watered to weight, and there was no variation in watering regime to attempt to increase cavity spot incidence. This was due to both practical (restricted room and resources) and time constraints within the project. Research has shown that total water input, and the timing of water input, can influence cavity spot development (Hendrix & Campbell, 1973; Hiltunen & White, 2002) and so water regime is a fundamental factor that may be valuable to control in the future. Overall, although variable, the temperatures and moisture contents used across these experiments are very unlikely to have prevented infection.

The work presented in this chapter highlights the problems with using oospore-based inoculum. However, it is important to be able to identify a quantifiable relationship between oospores and disease level, as oospores are likely to be the only surviving propagule year on year in UK fields (Mitchell, 1978). Oospore production in solid media is beneficial for mass production, storage and handling, as well as being able to grow and apply in a more natural way. Overall, artificial inoculation with a solid substrate inoculum resulted in infection of carrots by *P. violae*. Infection was seen as increased damping-off, carrot root stunting and typical cavity spot lesions. The seedling experiments were extremely unpredictable, however the field inoculation proved more reliable. Variability

is common for many soil-borne pathogens (Higginbotham *et al.*, 2004), but more fundamental research into the factors that affect the viability and germination of *P. violae* oospores would be beneficial, as germination is likely to be the major source of variation in these experiments. The success of the field experiments, combined with the progress made under controlled conditions represents a significant first step in development of a reliable artificial inoculation method for cavity spot. This will be essential in understanding the mechanisms involved in cavity spot infection and for testing new control measures.

4 Development of quantitative methods for detection of *P. violae*

4.1 Introduction

4.1.1 Importance of quantitative detection of *P. violae*

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).

4.1.2 Traditional and serological detection methods of detection

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).

4.1.3 Molecular detection

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). Ensuring primers are species-specific enables correct identification (Schroeder *et al.*, 2006; Bilodeau *et al.*, 2007) but there are a number of challenges when using molecular approaches, 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). Methods to separate *Pythium* oospores from soil would therefore be beneficial (Wang & Chang, 2003). 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). 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). Furthermore, 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.

4.1.4 Detection of *Pythium* spp. causing cavity spot using PCR

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).

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. 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.

4.1.5 Aims

The aim of this work was to develop methods to enable the quantitative study of *P. violae* dynamics in soil. A protocol comprising oospore capture, DNA extraction and qPCR was developed to try and achieve accurate detection and quantification in soil from a commercial carrot field. The specific objectives were to:

1. Develop a capture method for extracting *P. violae* oospores from soil.
2. Develop a DNA extraction method for *P. violae* oospores.
3. Develop a robust, accurate and sensitive qPCR assay for *P. violae*.
4. Monitor a commercial carrot field for cavity spot development and assess the dynamics of *P. violae* in soil.

4.2 Materials and Methods

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

4.2.1.1 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.

4.2.1.2 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.

4.2.1.3 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.

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

4.2.2.1 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 4.1 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.

4.2.2.2 DNA extraction

DNA extraction was tested using extracted *P. violae* oospores (isolate P10; as washed from 5 µl filters in Section 4.2.1.1) 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 *P. violae* 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 ml screw-cap tubes.

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 in Section 4.2.2.1 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.

4.2.3 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. as described in Chapter 2, Section 2.3, 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); (Chapter 2, Section 2.2.2). 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.

4.2.3.1 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 4.2 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	TGGTGTTTTCGGACGCTGCGCT 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)	TGGTGTTTCGGACGCTGCGCTG TCCCGCACACACACATTGCTG (6FAM)CGGAGGAGGAACGAAG GTTGGTCTTGT(TAMRA)	A. Taylor, unpublished, WCC

4.2.3.2 Primers targeting other gene targets

Following initial problems with specificity of primers targeting the ITS regions (primer pairs 1-3), primer pairs 4 and 5 (Table 4.2) were designed to target a single-copy protein-coding flagella gene, *Ochromonas mastigoneme*-like protein (OCM1), which was previously identified as a useful tool for species identification in *Pythium* (Robideau *et al.*, 2014). A further primer pair (primer pair 6; Table 4.2) was then designed to target the cellulose synthase (CS) gene, which was previously used to distinguish different oomycete species including *P. violae* (Blum *et al*., 2012). All these primer pairs were tested using conventional PCR and the DNA extracted from the different *Pythium* spp., with annealing temperatures/times and cycle numbers adjusted to maximise specificity.

Following positive results using primers pairs 5 and 6, these were used to amplify DNA extracted from soil samples previously collected from fields with carrots affected by cavity spot in AHDB project FV405 (Clarkson, 2014). These had previously been shown to be positive or negative for *P. violae* using conventional PCR with primer pair 1 (Klemsdal *et al.*, 2008). The PCR was carried out as described above in Section 4.2.3. Soil collection, cavity spot scoring and PCR were carried out by Gill Prince (Warwick Crop Centre). Soil was collected from carrot fields and cavity spot incidence in the field recorded. *P. violae* in the soil samples was quantified using a scale of band brightness from 0-3, in which no detection (no band) was scored as 0, and full detection (a bright band) was scored as 3.

4.2.3.3 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 4.3 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.

4.2.4 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.

4.2.4.1 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.

4.2.4.2 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 4.4 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

4.2.4.3 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.

4.3 Results

4.3.1 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 4.5 Percentage oospore capture (compared with reference count) from five *P. violae* sand/oat inoculum samples[1].

Sucrose extraction		1	2	3	Total capture (%)
	1a	14.6	10.2	-	**24.7**
	1b	26.0	9.2	-	**35.2**
Sample no.	**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).

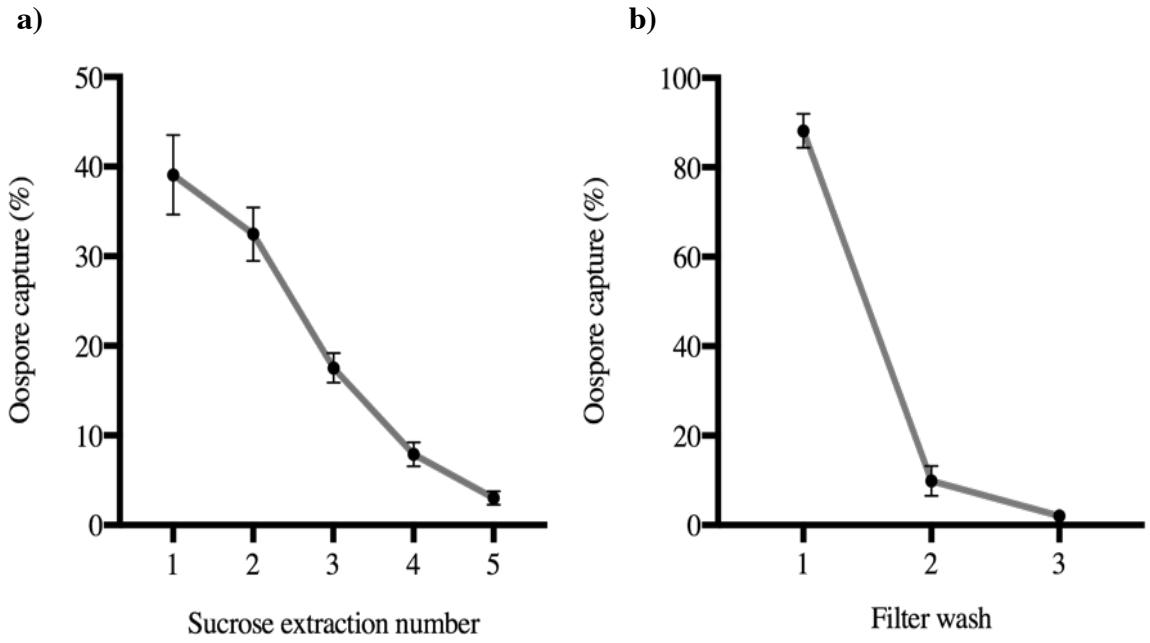


Figure 4.1 Mean percentage *P. violae* oospore capture from five extractions from the same sand sample (a); and mean percentage capture for each filter wash as a percentage of oospores captured in total in the sucrose extraction (mean of the five sucrose extractions) (b). Data represents mean ± SEM; N=5.

Table 4.6 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 4.7 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).

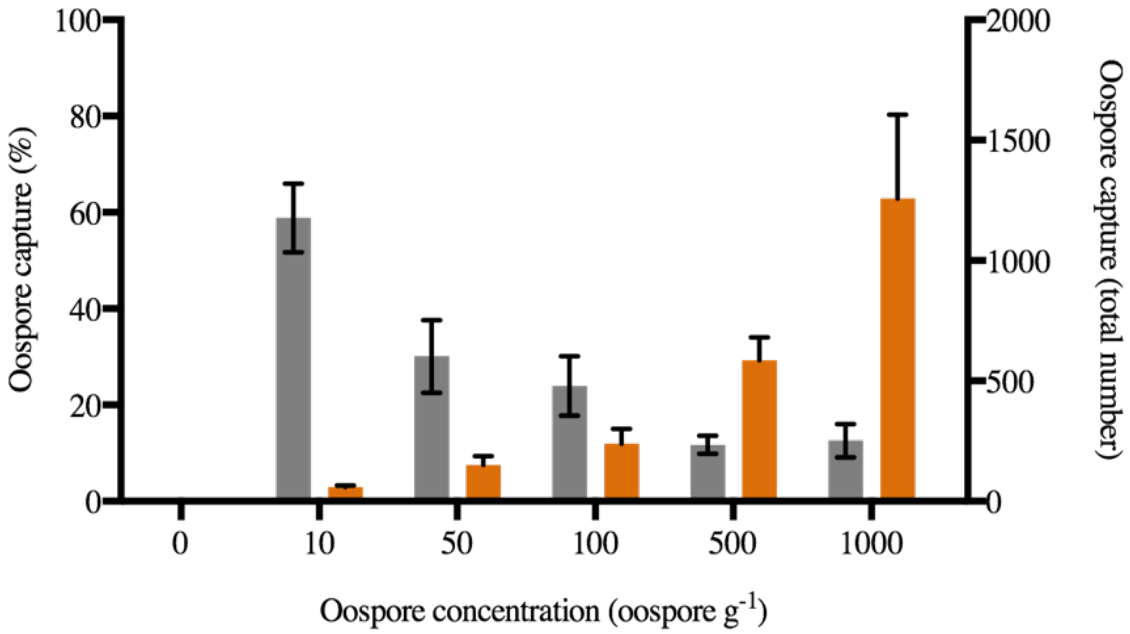


Figure 4.2 The percentage of *P. violae* oospores captured (grey bars, left y axis) and total number of *P. violae* oospores captured (orange bars, right y axis) from soil spiked with *P. violae* oospores at a range of concentrations. Data represents mean ± SEM.

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

4.3.2.1 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

Table 4.8 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

treatments compared to between 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.

4.3.2.2 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).

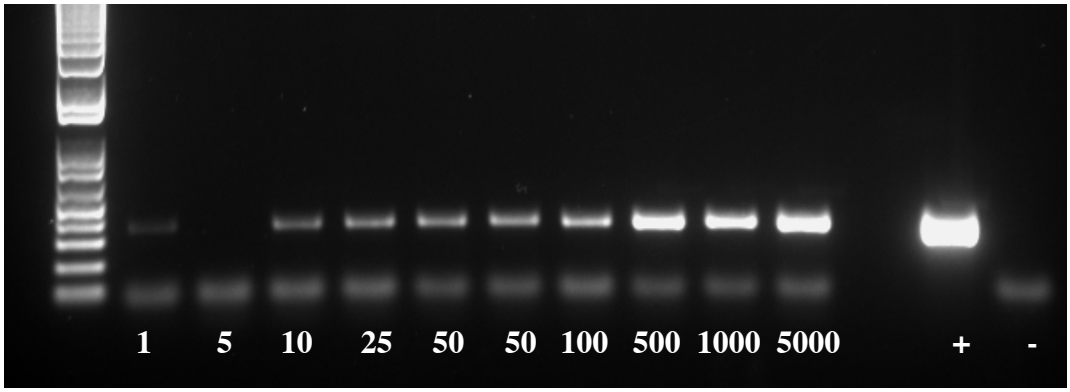


Figure 4.3 Representative electrophoresis gel showing sensitivity of *P. violae* DNA detection following optimised DNA extraction protocol using primer pair 1 (Klemsdal et al., 2008). Gel shows PCR amplicons following amplification of DNA extracted from 1-5000 *P. violae* oospores; + = positive control (*P. violae* DNA at 10ng μl^{-1}), - = negative control (water). A 1 Kb ladder was used as the molecular marker.

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

4.3.3.1 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*.

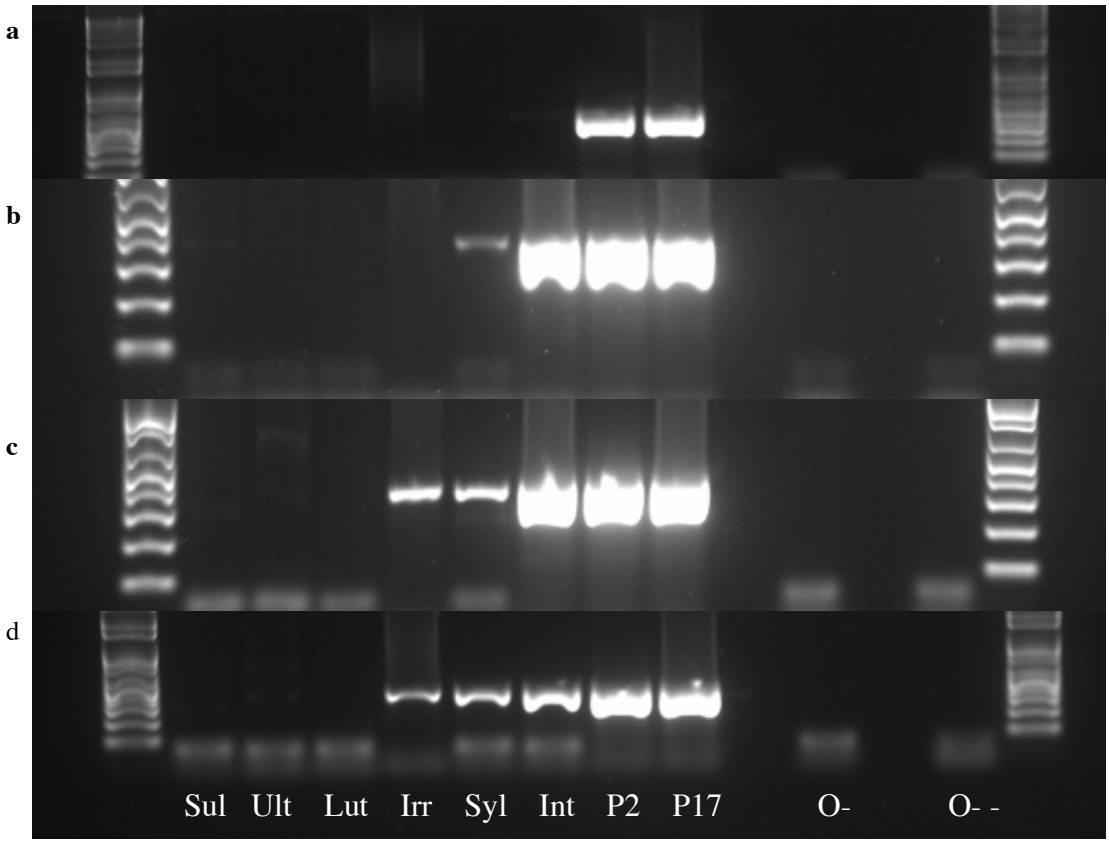


Figure 4.4 Electrophoresis gel showing amplification of *Pythium* spp. DNA following DNA extraction using primer pair 1 (Klemsdal *et al.*, 2008). Testing was carried out at 25 (a), 30 (b), 35 (c) and 40 (d) cycles. Sul = *P. sulcatum*, Ult = *P. ultimum,* Lut = *P. lutarium,* Irr = *P. irregulare,* 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).

4.3.3.2 Primers targeting other gene targets

Primer pairs 4 and 5 were designed based on the OCM1 gene, and DNA from both *P. violae* isolates P2 and P17 was successfully amplified using primer pair 4, but there was also weak amplification of DNA from the *P. intermedium* isolate which was not resolved through changes in cycle number or annealing temperature (Table 4.9). Use of primer pair 5 resulted in successful amplification of DNA from *P. violae*, but also resulted in some amplification of DNA from *P. intermedium* and *P. irregulare*. However, in this case, nearly all this non-target amplification was resolved when the annealing temperature was increased. With these conditions, *P. violae* amplification was not as efficient, but an intermediate annealing temperature with only weak amplification of the *P. intermedium* isolate and the *P. irregulare* isolate, and strong *P. violae* amplification was achieved (Table 4.9). Primer pair 6 was designed based on the cellulose synthase gene and DNA from both isolates of *P. violae* was successfully amplified by PCR with these primers. The PCR reaction was also very specific, with only very minor amplification of the *P. intermedium* isolate (Table 4.9). These primers showed the least non-target amplification of the non-ITS gene primers tested, and showed the strongest amplification of *P. violae*.

Once specificity had been established, the most promising primer pairs (5 and 6), were taken forward and tested for their ability to detect *P. violae* in DNA from soil samples from cavity spot infected fields in comparison to previously published primers (primer pair 1) (Klemsdal *et al*., 2008). The detection of *P. violae* in these naturally infested soil samples was much lower with the newly developed primer pairs (5 and 6) compared to primer pair 1. For DNA from soil samples where a strong band and clear amplification was observed with primer pair 1, no or very weak amplification was observed with both primer pair 5 (Figure 4.5) and primer pair 6 (data not shown). These primers were therefore not considered suitable for further development.

Table 4.9 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	■	■				▒	▒			▒	

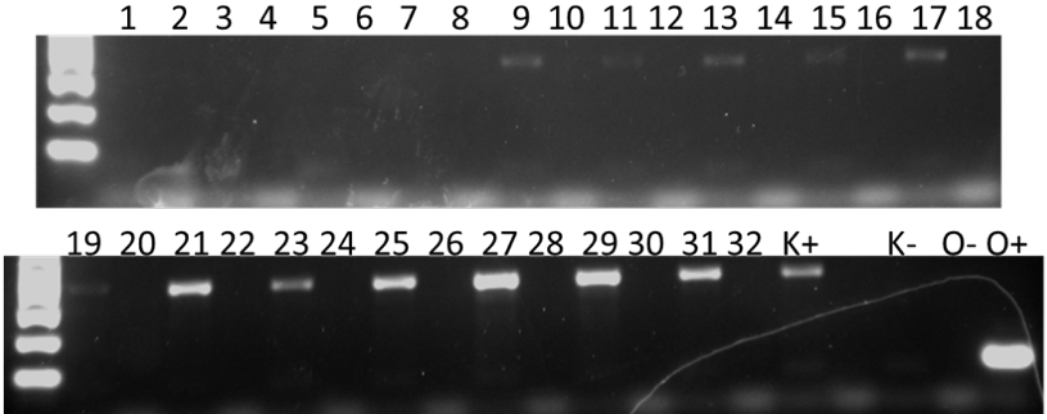


Figure 4.5 Comparison of primer pair 5 (OCM1 1128F/R) and primer pair 1 (PviolF/R) for detection of *P. violae* in DNA extracted from soil samples collected from fields with cavity spot affected carrots. PCR products from DNA extraction of each soil sample were run in adjacent wells numbered 1-32, e.g. 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.

4.3.3.3 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).

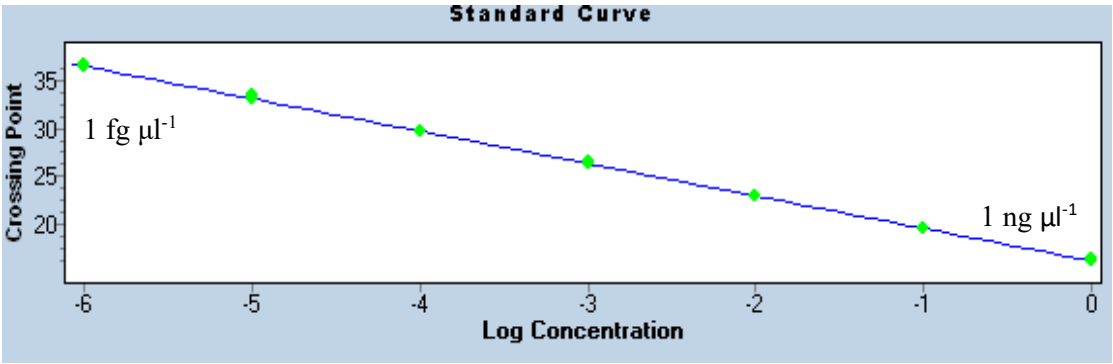


Figure 4.6 Standard curve calculated from serial dilutions of *P. violae* DNA from 1 ng μl^{-1}-1 fg μl^{-1} following a qPCR using a Taqman assay. Ct values shown are the mean values for triplicate reactions.

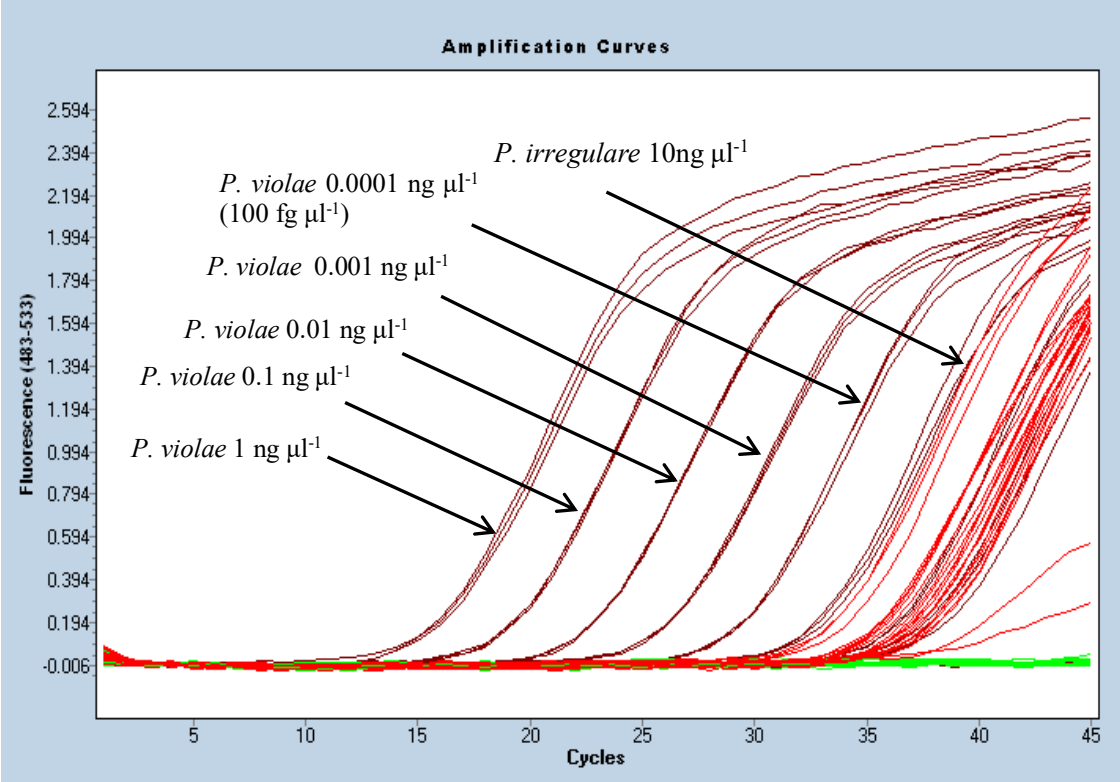


Figure 4.7 qPCR amplification curves for *P. violae* DNA at 1 ng ul^{-1}-10 fg µl^{-1} and other *Pythium* spp. (*P. sulcatum, P. ultimum, P. lutarium, P. irregulare, P. sylvaticum, P. intermedium*) DNA at 10 ng ul^{-1} concentration produced with Taqman assay.

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).

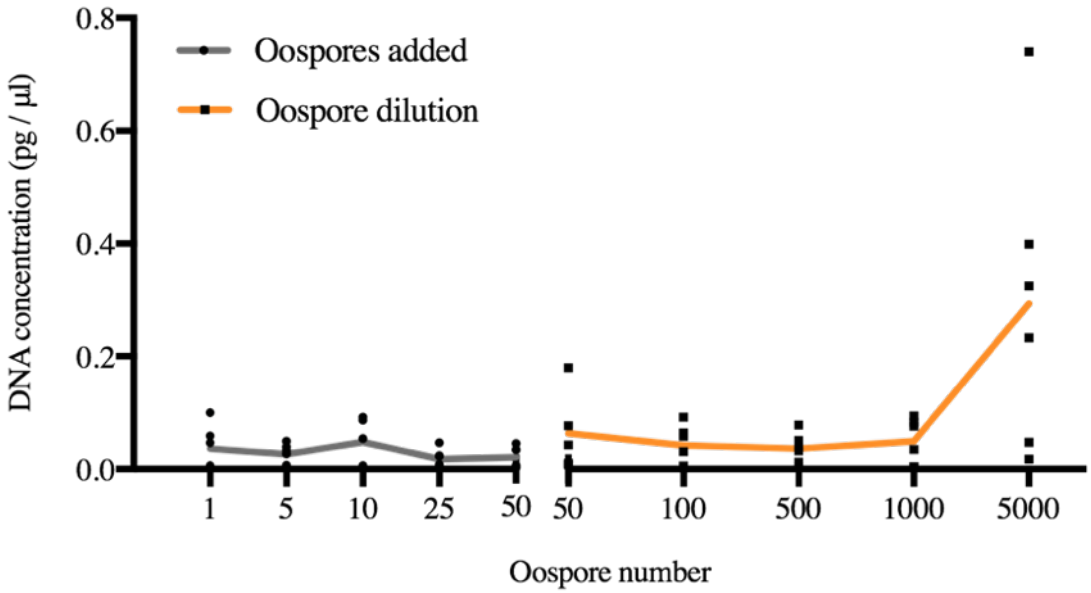


Figure 4.8 *P. violae* DNA concentration detected using the Taqman assay (primer pair 7) for different numbers of oospores. Oospores were individually counted out for samples containing between 1 and 50 oospores (grey line) while samples containing between 50 and 5000 oospores (orange line) were made 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, $R^2 = 0.007473$).

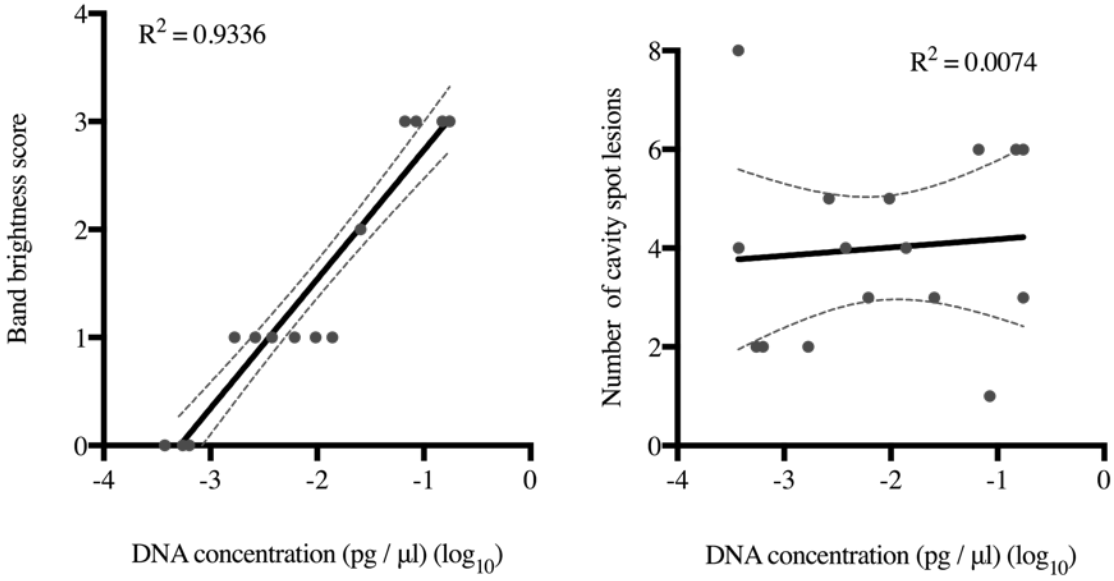


Figure 4.9 Relationship between *P. violae* DNA concentration in qPCR Taqman assay using primer pair 7 and band brightness score following conventional PCR using primer pair 1 (Klemsdal *et al.*, 2008) for soil samples collected from around cavity spot affected carrots (a) and cavity spot incidence recorded for carrots from which soil samples were taken (b).

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

4.3.4.1 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.

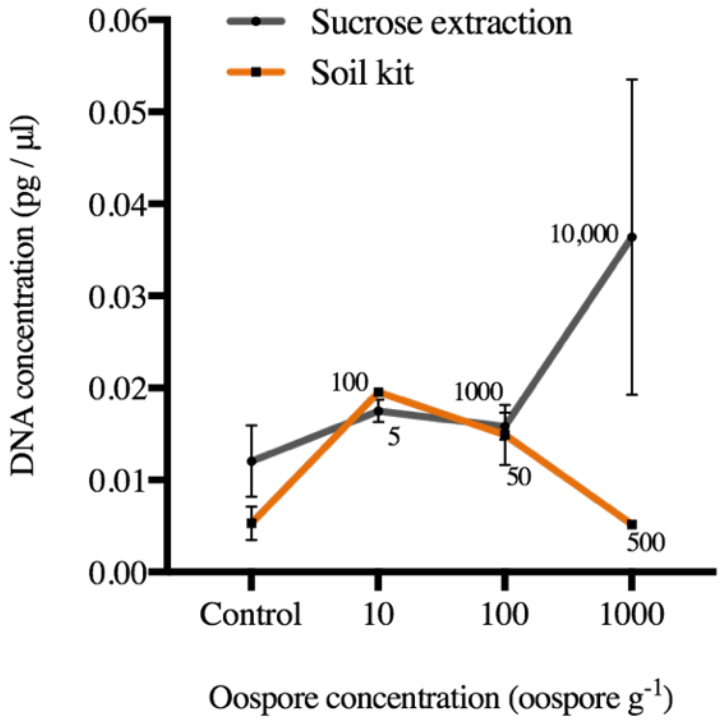


Figure 4.10 *P. violae* DNA concentration detected in soil samples spiked at a range of oospore concentrations. Orange line: oospores extracted using the GeneAll soil DNA extraction kit (0.5 g soil). Grey line: oospores extracted using oospore extraction method and DNAeasy kit (10 g soil). Numbers written 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.

4.3.4.2 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 4.11 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.

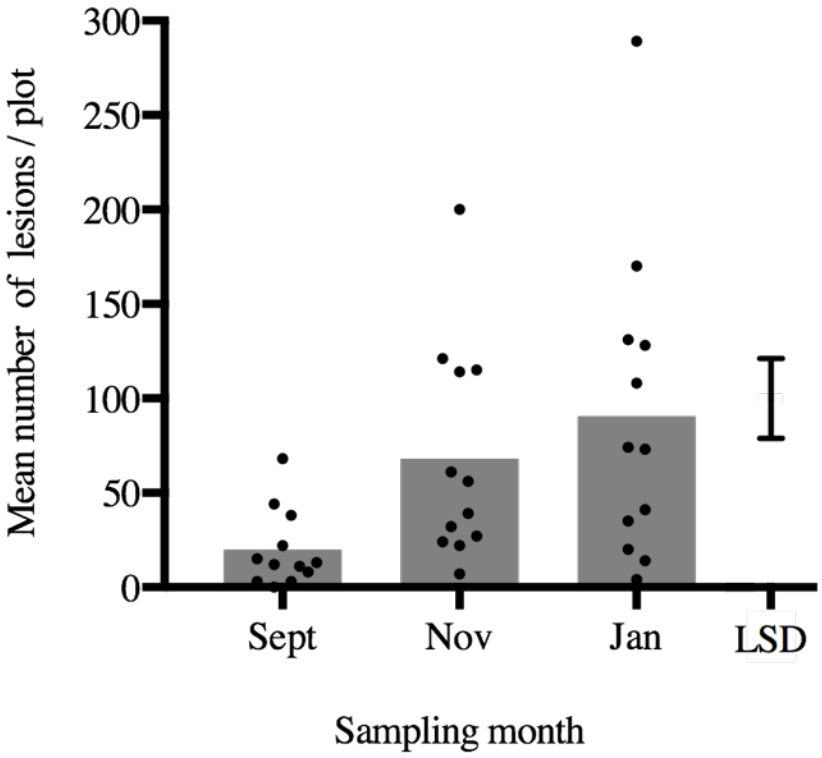


Figure 4.12 The mean total number of cavity spot lesions recorded for 20 carrots in each of 12 replicate plots from a commercial field site during the September, November and January sampling time points. Bars represent the mean number of lesions across all 12 plots, and dots represent the number of cavity spot lesions in each of the 12 plots. Error bar represents the least significant difference between treatments (LSD) at the 5% level.

Table 4.10 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

4.3.4.3 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).

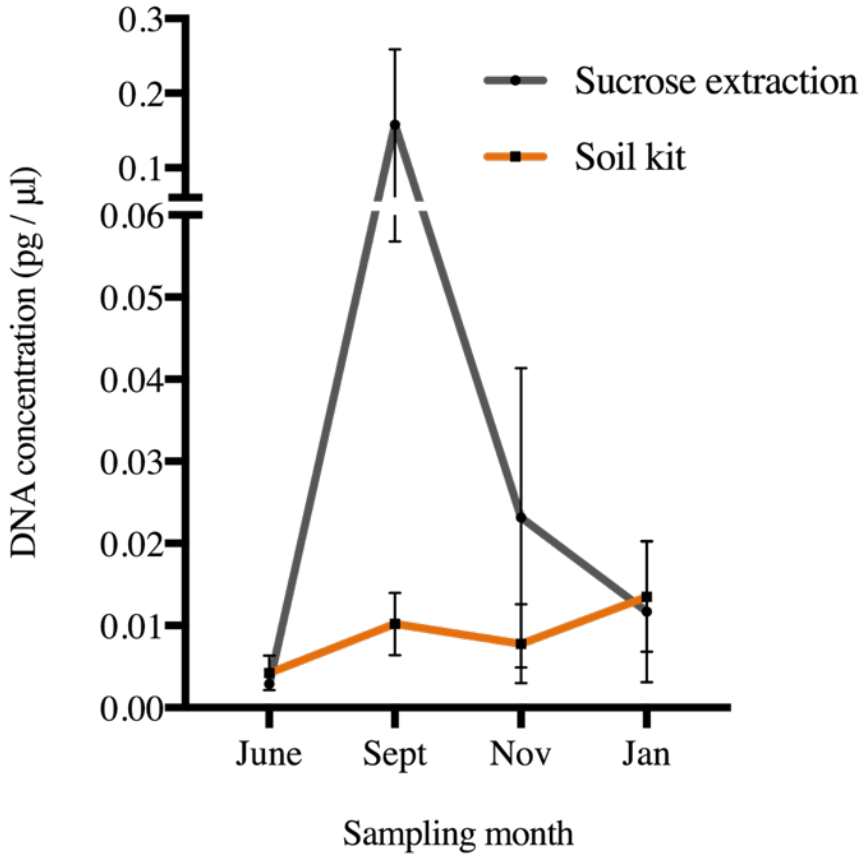


Figure 4.13 DNA concentration measured from extraction of DNA from *P. violae* oospores from soil samples taken from the commercial carrot field. Samples were from June, September, November and January time points. Orange line = oospores extracted via GeneAll soil DNA extraction kit without prior use 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.

4.4 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. These results are in contrast to a previous study where Pavon *et al.* (2007) used a similar sieving-centrifugation technique to quantify *Phytophthora capsici* oospores in spiked soil samples. Here, as oospore concentration increased (from 10^1-10^5 oospores per 10 g), percentage capture also increased (from 21.4-79.8% respectively). However, vanderGaag and Frinking (1997) used a similar method for extraction of *Peronospora viciae* spores and found that spore capture was independent of spore concentration, and was approximately 75% efficient.

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. However, the clay and silt were relatively small components of an overall sandy soil and previous studies have not reported similar problems. Pavon *et al.* (2007) spiked five different soils with *P. capsici* oospores, including soils with considerable ranges in organic matter, sand, silt and clay content, but found no significant differences in recovery rate. Similarly, vanderGaag and Frinking (1997) used both silty clay loam and loamy sand soil types and found that the percentage recovery of *P. viciae* spores was very similar across both. 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. In contrast, grinding the oospores in sodium hydroxide followed by a CTAB DNA extraction resulted in consistent detection. Similarly, Lees *et al.* (2012) found that despite numerous lysis steps, including sonication, grinding in liquid nitrogen, and freeze-thawing, *P. infestans* oospores in soil and water samples remained intact, and subsequent DNA extracts did not result in qPCR detection. 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. A number of studies have been conducted on the use of both mechanical and chemical methods of lysis, and bead-beating, as used in this PhD study, has been cited as the most effective method of disruption (Sagova-Mareckova *et al.*, 2008) particularly for spores (Robe *et al.*, 2003). 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. This could have been tested by running the DNA on a gel, although DNA concentrations were very small. In addition, given the very low amounts of DNA that would be expected from such a small number of oospores, losses during pipetting and transferring onto columns as part of the DNA extraction process may also have resulted in the loss of small DNA fragments and hence contributed to variability of the PCR detection for low numbers of *P. violae* oospores.

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). Multiple attempts to design primers and modify PCR conditions initially did not result in robust and specific amplification of *P. violae* with primers amplifying the ITS region. 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 (Bilodeau *et al.*, 2007; Tajadini *et al.*, 2014). For example, Bilodeau *et al.* (2007) were able to reliably distinguish between *Phytophthora ramorum* and *Phytophthora lateralis* using an ITS-based Taqman assay. These species are very closely related with only 1.4% divergence in the ITS region, but use of this assay resulted in additional specificity compared to molecular beacon and SYBR Green approaches (Bilodeau *et al*., 2007). This Taqman assay also provided the most sensitive assay (approximately 1 or 2 orders of magnitude more sensitive) of the gene targets tested (β-tubulin and elicitin genes) (Bilodeau *et al.*, 2007).

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. Bell *et al.* (2009) employed qPCR assays to quantify entomopathogenic fungal genomes of *Beauveria bassinana*, and concluded that although the use of a multi-copy rRNA gene for quantification allowed detection at very low levels, the counts of genomes were 'excessively influenced' by variation in pipetting. 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. In contrast, Pavon *et al.* (2007) reported a strong correlation between detected DNA

concentration and number of *P. capsici* oospores (R^2 = 0·93) where for 1000 oospores, mean detection was measured at 3.2 pg μl^{-1}. In this project, the mean DNA concentration measured for 1000 *P. violae* oospores was only 0.061 pg μl^{-1}. Other studies have also found a strong correlation between *Pythium* CFU counts and DNA concentration. For example, Li *et al.* (2010) studied the density of *P. intermedium* in forest soils, and found that the qPCR DNA concentration correlated well with the soil population density CFU count from soil dilution plating (R^2 = 0·97). Similarly, Schroeder *et al.* (2006) correlated DNA recovered from soil samples with populations of *P. irregulare* and *P. ultimum.* It was found that DNA concentrations ranged from approximately 10-100 pg μl^{-1} for both of these species and correlated well with pathogen densities obtained from dilution plating of 10-1000 propagules per gram (ppg) (R^2 = 0.91). It is possible that losses during the DNA extraction processes, as discussed above, may explain the lack of correlation between *P. violae* DNA and number of oospores in this study.

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.

A number of studies have attempted to correlate disease severity and pathogen DNA concentration for foliar diseases (Schena *et al.*, 2013), but a direct relationship between soil-borne pathogen DNA concentration and disease severity has been more difficult to establish. Kernaghan *et al.* (2007) reported a positive correlation between concentration of *Cylindrocarpon destructans* f. sp. *panacis* DNA, and disease severity in ginseng (R^2 =

0.765), and Huang *et al*. (2016) noted a positive correlation between disease severity of *Fusarium* wilt in tomato and inoculum concentration of *Fusarium oxysporum* f. sp. *lycopersici* in the soil (R^2 = 0.538). In contrast, Zhu *et al.* (2016) found no significant correlation between disease severity and *Fusarium commune* spore density in soil when studying *Fusarium* wilt in Chinese water chestnut.

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. DNA extraction efficacy is influenced both by soil chemistry and DNA binding capacities (Miller *et al.*, 1999; Martin-Laurent *et al.*, 2001). For instance, clay and organic matter particles are known to adsorb free DNA (Frostegard *et al.*, 1999; Kuczynska & Shelton, 1999; Sagova-Mareckova *et al.*, 2008) while the effect of PCR inhibitors extracted alongside the DNA from soil, (including humic acids which can bind to extracted DNA) can decrease DNA yields (Sagova-Mareckova *et al.*, 2008). As stated above, even small alterations in yields during extraction can result in extensive differences post-amplification (Li *et al.*, 2010).

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. It is still not known how *P. violae* grows both in and around the carrot root, and when oospores are produced. Both hyphae and oospores have been observed within the carrot tissue immediately after cavity spot lesions have formed (Groom & Perry, 1985; Benard & Punja, 1995), and in an initial experiment during this PhD project, the number of oospores captured in field soil taken from around carrots was considerably higher when diseased roots were still in the ground (280 oospore g^{-1}) compared to three months after diseased roots had been harvested (57 oospores g^{-1}), indicating that production of oospores may occur on the carrot root.

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.

5 General Discussion

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.

5.1 *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. Previous studies have examined the phylogenetic relationship between and within *Pythium* spp. (Martin, 2000; Levesque & de Cock, 2004; Villa *et al.*, 2006), but none have done so specifically for the complex of *Pythium* isolates associated with cavity spot. The findings from this project extended this knowledge extensively. This study was the first to characterise *Pythium* spp. using the NADH dehydrogenase subunit one gene which revealed a level of within-species diversity between isolates of *P. violae* that was not as apparent using other housekeeping genes. 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. Levesque and de Cock (2004) had previously found this was not possible using the ITS gene; however, their work was not focused on *Pythium* spp. involved in cavity spot. Understanding differences between and within *Pythium* spp. populations from carrot in different locations is important in understanding which predominate in different counties, and how control measures may need to be adapted to take account of these differences.

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. The use of such genome analysis would include determining the range of effector genes which could provide potential novel targets for future research on disease control methods, including plant resistance. Initial comparisons of effectors in *Pythium* and *Phytophthora* species has revealed differences in certain effector families. For example, RxLR effectors which are common in *Phytophthora* spp. have so far not been found in the current *Pythium* genomes sequenced. Identifying the differences in effectors between oomycetes, as well as more specifically between *Pythium* spp. associated with cavity spot, may help elucidate the virulence factors associated with pathogenicity.

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. Previous studies have proved inconclusive, with some researchers finding a correlation between root assays and field trials (Benard & Punja, 1995; Hiltunen & White, 2002), whereas others have found no relationship (Vivoda *et al.*, 1991; Hiltunen & White, 2002). 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.

5.2 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. Oospores of *Pythium* spp. are often dormant and require a period of time before germination can occur (van der Plaats-Niterink, 1981; Higginbotham *et al.*, 2004). However previous work with *Pythium* oospores has shown the requirements for induction of oospore germination vary between species (El-Hamalawi & Erwin, 1986). Any oospores produced via artificial inoculation need to be viable as well as 'active' (e.g. both alive, and able to germinate). Use of staining treatments may be useful in assessing viability and germination in oospores (Sutherland & Cohen, 1983; El-Hamalawi & Erwin, 1986) in combination with other methods such as plasmolysis and microscopic observation of the cytoplasm (Etxeberria *et al.*, 2011). 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.

The variation in results from the field and glasshouse inoculation experiments also imply that conditions required for *P. violae* infection and development of cavity spot disease are complex. Environmental factors have been researched extensively in the past (Hiltunen & White, 2002; Martin, 2014) but consistent conclusions have proved elusive. Unlike preceding studies, this project used genetically characterised *Pythium* isolates, although the majority of work focussed on one isolate of one *Pythium* species: *P. violae*. It is clear from the experiments conducted throughout this PhD project, as well as from previous research, that there is substantial variation in the growth and virulence of different *Pythium* spp., as well as of isolates within species (Hiltunen & White, 2002). It has previously been shown that *Pythium* spp. can form complexes in association with other pathogenic *Pythium* spp. (Suffert & Guibert, 2007), but this work did not investigate how mixtures of species interact during the infection process and what impact this has on cavity spot development. Given that the results from Chapter 2 identified multiple *Pythium* spp. can be isolated from the same field, future work should consider not only using more than one isolate of different species individually, but also combinations of species.

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 (Hendrix & Campbell, 1973). For example, Kerr (1963) reported that *P. ultimum* and *F. oxysporum* caused more severe damage to peas than either alone. It may be the case that certain soils have a more 'complimentary' microbe community that could aid *Pythium* infection, or support lesion development or expansion. In addition, fields treated with certain fungicides may diminish the incidence of species that may otherwise outcompete *Pythium*. Use of amplicon sequencing could help determine the complex of microorganisms associated with *P. violae* in lesions as well as the surrounding soil. 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.

5.3 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). Previous work had identified that detection of pathogens in small samples can be problematic and highly variable (Rodriguez-Molina *et al.*, 2000; Schena *et al.*, 2013). Use of larger soil samples means detection of *Pythium* is more likely to be accurate over larger spatial scales. 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.

Despite some success with the development of a molecular detection method, creation of research tools that are reliable enough for quantifying disease dynamics proved to be a problem that was not overcome within this PhD project. The combination of the capture and DNA detection methods in a field setting did not allow reliable quantification of *P. violae* in soil unless levels were very high. This means the molecular tools are not yet useful to carrot growers or researchers, as disease levels are likely to be high and cavities visible before the threshold for detection achieved here is reached. Although time constraints limited any further evaluation of the oospore capture method developed, there is a clear effect of substrate on capture rate, and there are a number of potential variations on capture method that could be attempted to improve this. These include variations in sonication technique (varying probe types, power and time), altering centrifugation technique and adjusting how oospores and soil particles are captured on, and separated from, the filters.

Even considering these limitations, 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.

Concluding remarks

The work in this PhD project has been an important step in building understanding of the biology of *Pythium* spp. associated with cavity spot, and has used novel methods to develop techniques to induce and monitor cavity spot development. If future research can be progressed to combine use of the research tools developed in this PhD project, *P. violae* dynamics could be monitored alongside the artificial inoculation assay to help elucidate the relationship between inoculum in soil and subsequent disease development, leading to better understanding of *P. violae* epidemiology and identification of new control approaches.

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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

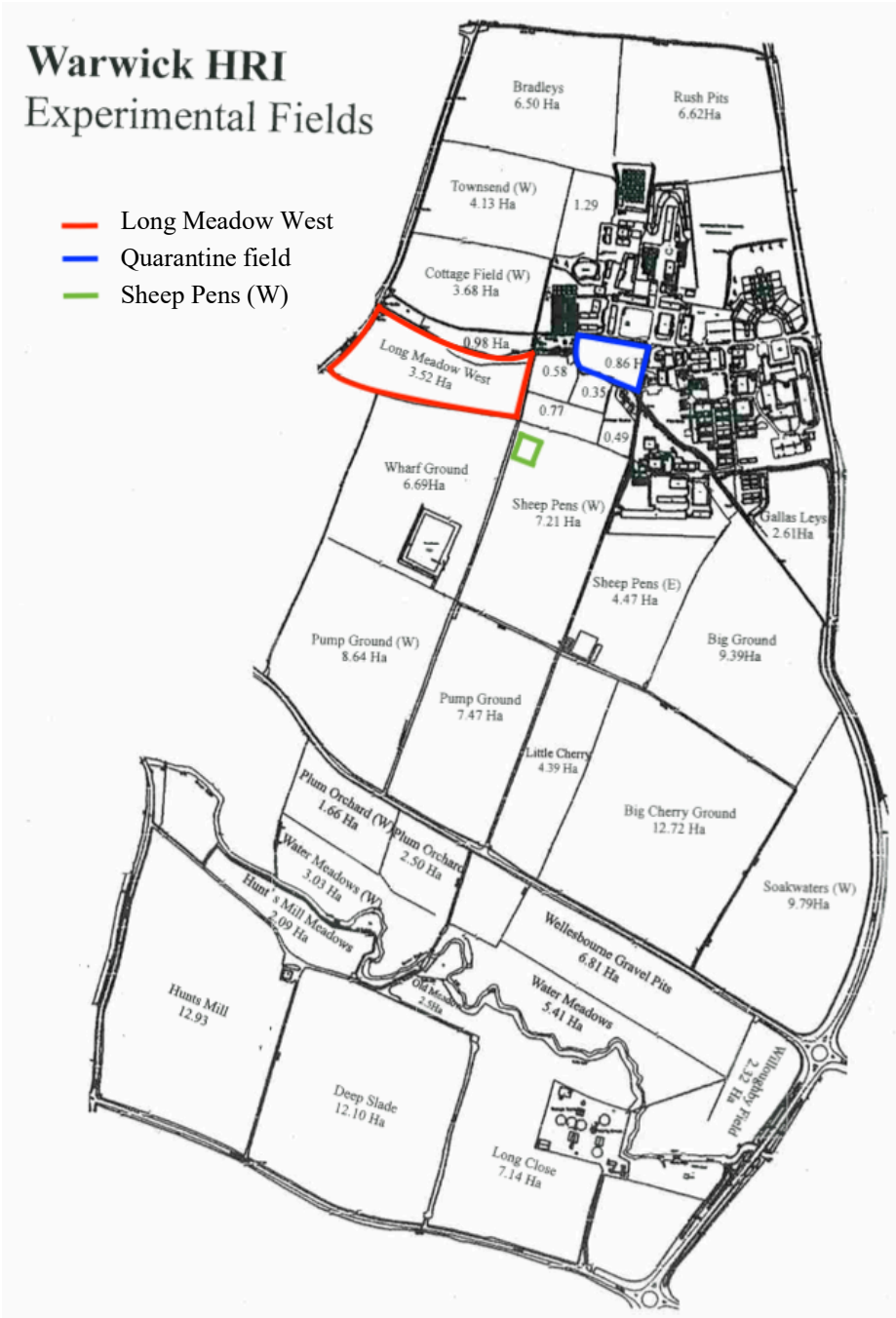

Figure B.1 Map of Warwick Crop Centre, Wellesbourne Campus (Horticultural Services, 2007). Long Meadow West Highlighted in red, quarantine field highlighted in blue, Sheep Pens (W) highlighted in green.

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.

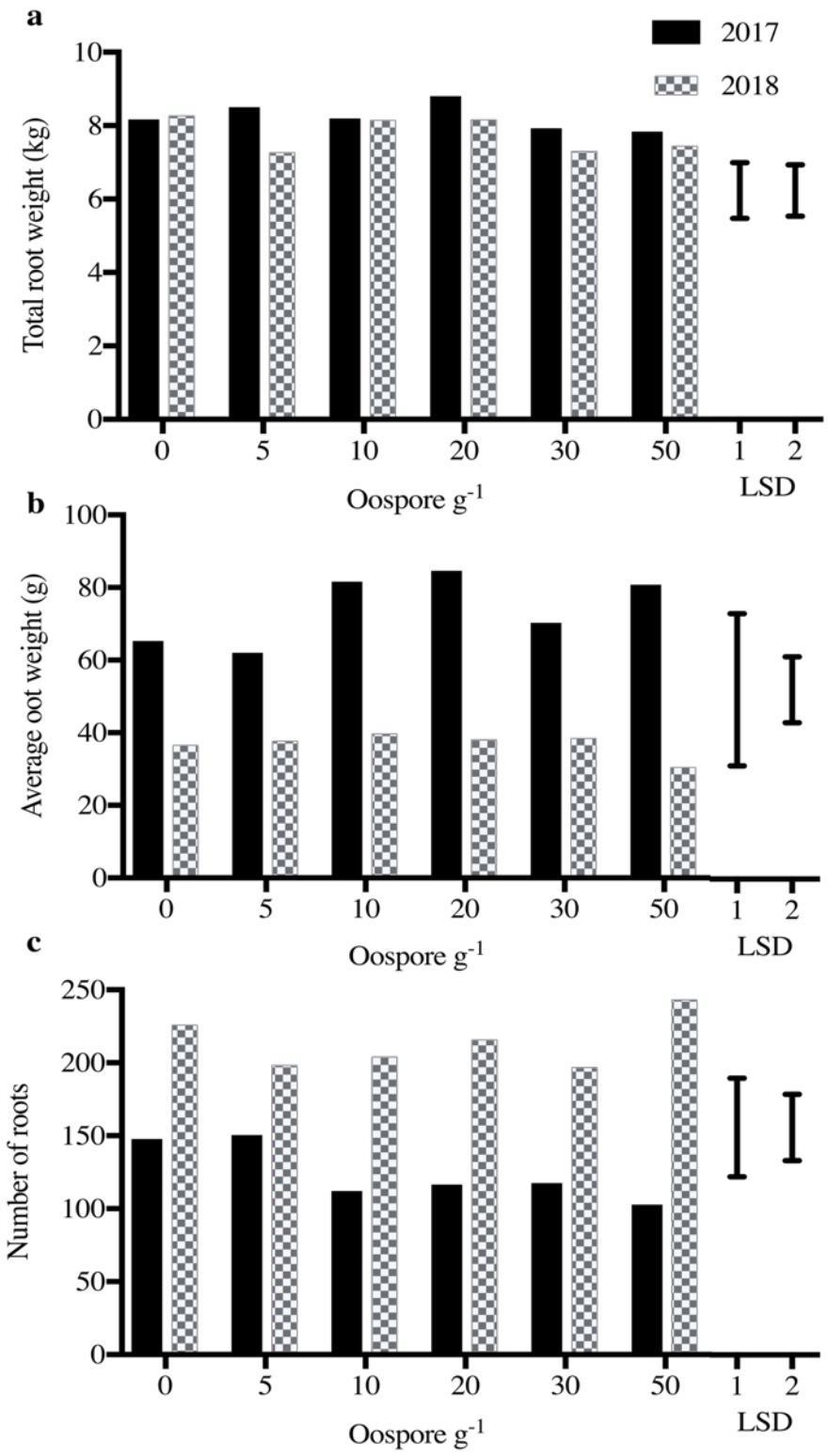

Figure D.1 Effect of different concentrations of *P. violae* oospores produced in solid sand/oat medium on carrot weight and number in field macrocosms: total root weight per macrocosm (a) and mean root weight per carrot (b) and total number of roots per macrocosm (c) for 2017 (black solid bars) and 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.

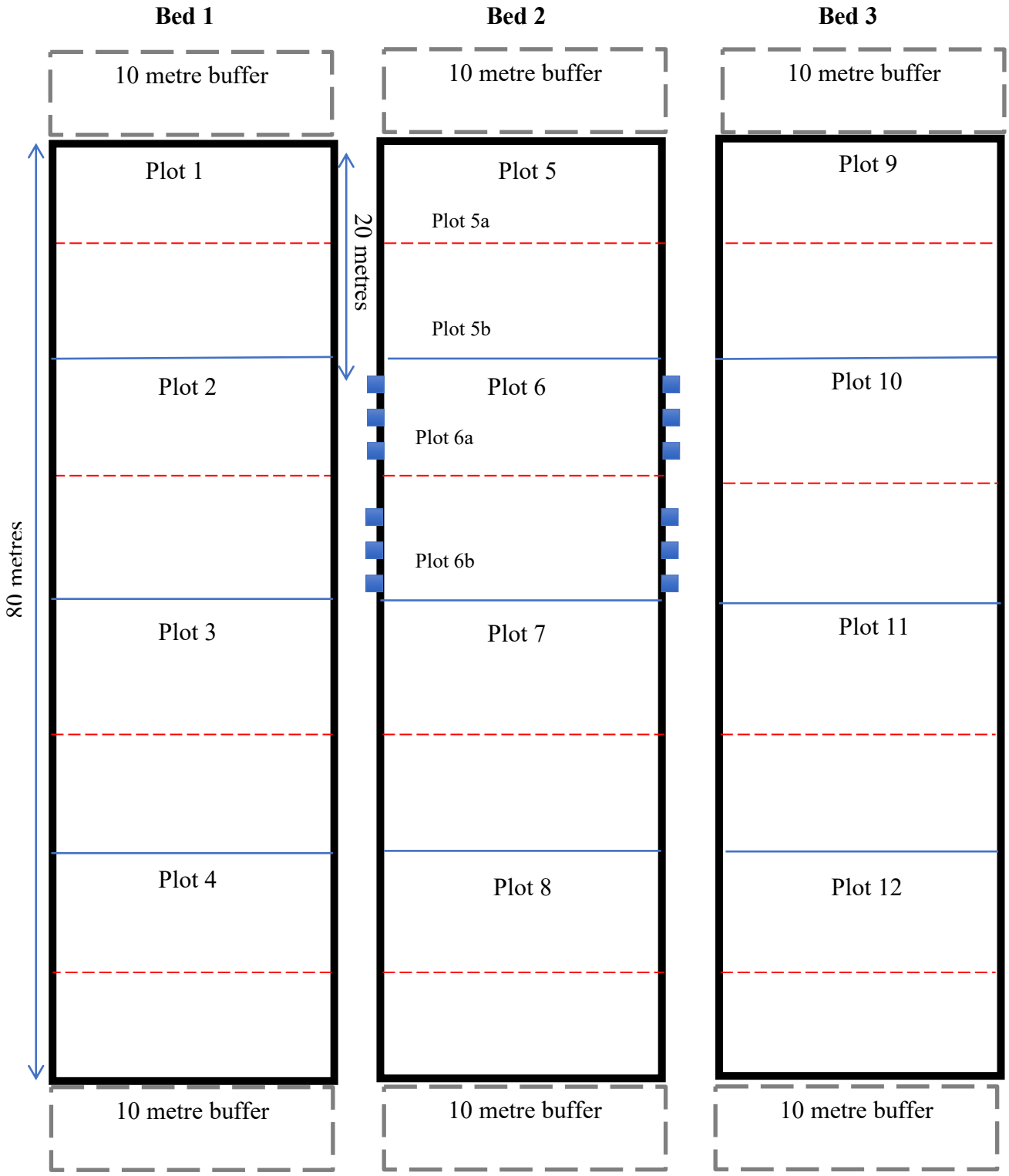

Figure E.1 Commercial carrot monitoring field site trial plan. Solid black lines indicate the three beds, blue lines within each bed indicate the 12 plots, orange dashed lines indicate the 24 sub-plots. Blue squares in plot 6 indicate how soil was samples prior to the June sampling time points (when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