Project title:	Carrots: Further development of artificial inoculation techniques for cavity spot caused by *Pythium violae*
Project number:	FV 391b
Project leader:	John Clarkson, Warwick Crop Centre, University of Warwick
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

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

Dr John Clarkson

Reader

University of Warwick

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GROWER SUMMARY

Headline

Inoculation of pot-grown carrot plants in the glasshouse with a sand/oat *Pythium violae* oospore inoculum resulted in stunted carrots and cavity spot lesions but levels were variable. A millet seed mycelial inoculum resulted in much higher disease incidence. In field macrocosms, inoculation of carrots with the oospore inoculum resulted in high incidence of cavity spot.

Background

Cavity spot of carrots in the UK is principally caused by the soilborne oomycete pathogen *Pythium violae* and continues to be the most economically important disease for UK carrot growers with losses of at least £3-5 million per season. Management of the disease relies heavily on metalaxyl-M fungicide but its efficacy is variable, possibly due to enhanced degradation in some fields. The reliance on this single fungicide, its efficacy in controlling the disease and the potential withdrawal of approval in the future are major concerns for the industry and hence there is an urgent need to identify new actives or approaches for control. To address this, AHDB Horticulture projects have been commissioned to improve the management and control of cavity spot including testing of potential new fungicides, biological control treatments, pre-planting calcium applications and biofumigation (FV 391, FV 405). These projects were field based but despite best efforts results have not been forthcoming due to no, or low levels of cavity spot development in many of the trials. This has resulted in a failure so far to reliably identify any new actives or approaches to control of *P. violae*.

One solution to the problem of low cavity spot levels is to artificially inoculate carrots with *P. violae* in pots or the field to ensure high enough levels of disease development such that activity of control treatments can be assessed reliably. In a previous project (FV 391a) we developed methods to produce large numbers of *P. violae* oospores in both a liquid medium and a sand/oat-based solid substrate culture. However, initial investigations indicated that oospore germination on agar was negligible as has been observed previously by other researchers. Nonetheless, in two glasshouse experiments, artificial inoculation of the growing media using the *P. violae* solid substrate at different concentrations resulted in some seedling death, reduced seedling size and a decrease in growth of carrot foliage. However, at harvest, the principal effect of *P. violae* inoculation was the formation of small, stubby and stunted carrots with a much-reduced weight compared to the uninoculated control plants. Typical cavity spot lesions were also observed in a large proportion of these stubby carrot roots.

These initial artificial inoculation experiments with *P. violae* in FV 391a were quite successful compared to previous attempts; however, results between experiments were variable. In this project we build on this initial work to address this variation and try and increase the number of cavity spot lesions observed on carrots artificially inoculated with *P. violae* both in the glasshouse and field. We hypothesised that differences in cavity spot disease levels or impact on carrot growth could be due to variation in oospore viability and their ability to germinate (in the presence of carrots) between different batches of *P. violae* inoculum or between different isolates. Hence lab-based experiments were designed to test this for *P. violae* and also *P. sulcatum*. A liquid *P. violae* inoculum was also tested in an initial pot-based glasshouse experiment but due to lack of carrot infection using this approach, further work investigated the ability of different *P. violae* and *P. sulcatum* isolates to induce cavity spot using the sand/oat solid inoculum employed successfully in FV 391a and also tested a millet-based inoculum as previously employed for *P. sulcatum*. Finally, the feasibility of producing solid inoculum of *P. violae* on a large scale in solid state fermentation for potential use in field experiments was assessed.

The overall aim of this project was therefore to further develop methods for producing *P. violae* inoculum and determine the potential to cause cavity spot disease. The specific objectives were:

1) Test vital stains and different chemical treatments to assess *P. violae* oospore viability and increase germination.
2) Test the efficacy of different rates of *P. violae* liquid and solid inoculum in producing cavity spot symptoms in pot grown carrots in the glasshouse.
3) Test the efficacy of different rates of solid *P. violae* inoculum in producing cavity spot symptoms in field grown carrots.
4) Examine the feasibility of large-scale *P. violae* inoculum production in solid state fermentation.

Summary

Methods to assess *Pythium* oospore viability and induce germination

Two methods were investigated to assess *P. violae* oospore viability as a way of checking the quality of inoculum, as this was hypothesised to be a potential source of the variation in cavity spot disease levels and effects on carrot growth observed in experiments in FV 391a. A vital stain method tested showed that live (viable) oospores from both *P. violae* and *P. sulcatum* stained purple in contrast to dead (non-viable) oospores which were stained black or colourless. However, there was some difficulty distinguishing between very dark purple (viable) and black (non-viable) oospores. A plasmolysis method where oospores were incubated in a saline solution showed that the cytoplasm of live (viable) oospores contracted into a ball-like structure due to the resulting loss of water; in contrast, dead (non-viable) spores did not display this plasmolysis due to the lack of functional cell membranes. It was concluded therefore that the plasmolysis method was the most reliable for assessing *P. violae* oospore viability.

To test approaches for inducing *P. violae* oospore germination we first had to develop an effective method for producing *Pythium* oospore preparations free of mycelial fragments as live, residual mycelium hampers microscopic assessment of germination as it quickly overgrows oospores. This involved treatment with Glucanex, a mixture of lysing enzymes from *Trichoderma harzianum*, which effectively digested and deactivated mycelial fragments in *P. violae* and *P. sulcatum* oospore preparations whilst the walls of oospores were left intact. The survival of oospores during this process was confirmed as there was little or no loss of viability as assessed by the plasmolysis assay. It has also been suggested previously that lysing enzymes may also break oospore dormancy and enable germination but neither Glucanex treatment alone nor the addition of potassium permanganate (also reported to promote oospore germination in some *Phytophthora* and *Pythium* spp.) induced germination in *P. violae* oospores. In contrast, a high proportion of Glucanex-treated oospores of one *P. sulcatum* isolate germinated within 2 to 3 days. Only one concentration of $KMnO_4$ and one incubation period was tested in this study, and other factors such as desiccation, heat shock, exposure to soil and plant exudates may be important for *Pythium* oospore germination. The outcomes of this series of experiments highlighted that the requirements for oospore germination potentially vary considerably between *P. violae* and *P. sulcatum* isolates. Hence, more extensive research is required to investigate potential triggers of oospore germination in multiple isolates of *P. violae* and *P. sulcatum*.

Development of inoculation methods to produce cavity spot symptoms in pot grown carrots in the glasshouse

Experiments were carried out to continue the initial work in FV 391a to develop an artificial inoculation system for *P. violae* for pot-grown carrots in the glasshouse. An initial experiment using a liquid oospore inoculum resulted in no cavity spot symptoms and was also difficult to apply consistently. Further experiments therefore focussed on developing the use of a solid *P. violae* inoculum. Initially, an experiment was set up using a solid sand/oat inoculum to test the effect of metalaxyl seed treatment, firstly to investigate whether it would control cavity spot and secondly to determine if it could delay early infection of *P. violae* yet allow symptoms to develop later on in mature carrot roots. A follow up experiment using the same type of inoculum then evaluated the ability of different isolates of both *P. violae* and *P. sulcatum* to induce cavity spot symptoms to examine if there were more virulent isolates that could induce higher levels of cavity spot than observed in previous tests.

Overall, the major finding from these first two glasshouse experiments was that artificial inoculation of carrots with the sand/oat *P. violae* oospore inoculum resulted in a reduction in seed germination and foliage development as well as the formation of short stubby carrots with a reduced root weight. Typical cavity spot lesions were also formed while tap roots were misshapen and brown. The pathogen was consistently isolated both from lesions and taproots, confirming that infection was successful. These observations were consistent with the results from FV 391a where the same range of symptoms was also evident. In addition, results from this project and FV 391a also indicated that increasing the concentration of *P. violae* oospores did not result in a corresponding increase in subsequent disease metrics across the range of 10-100 oospores g^{-1} soil. Significantly, the extent of disease indicators between experiments both in this project and FV 391a was variable despite careful quantification of *P. violae* inoculum. For instance, reduction in carrot weight for *P. violae* inoculated treatments was significant in both the experiments in FV 391a but not in the first two experiments in this project. Cavity spot incidence for *P. violae* was 0.75-23% and 5-20% in this project and FV 391a respectively, with corresponding disease severity of 1-3 and 1-2 lesions per carrot for those roots affected. Overall therefore, while inoculation of carrots with the *P. violae* sand/oat inoculum always results in some cavity spot disease, the level at which this occurs is unpredictable. In this project, similar growth effects on carrot and cavity spot symptoms were observed following inoculation with one isolate of *P. sulcatum* while another two failed to cause any infection.

The reasons for this variability in cavity spot disease are unclear and may be related to oospore viability, the ability of oospores to germinate or environmental factors (e.g. water availability) in the glasshouse. Viability and germinability of oospores used in these

experiments was not directly assessed as the methods described above to achieve this were only developed in the latter stages of the project. However, viability of *P. violae* and *P. sulcatum* oospores is unlikely to have been a significant factor given subsequent results of the plasmolysis assay. Previous studies have also reported variation within-species variation in virulence for *P. violae* and *P. sulcatum*.

Finally, results from the experiment testing the effect of metalaxyl treated seed suggested that this had little overall benefit in reducing cavity spot which is in contrast to previously published work. However, there was some protective effect of metalaxyl at a *P. violae* inoculum level 1 oospore g^{-1} only, which may suggest some benefit at low disease pressure. There was also some evidence that in the absence of the pathogen, metalaxyl reduced carrot seed germination which has also been noted previously.

Given the variability in cavity spot disease development and plant growth effects in experiments using the sand/oat oospore inoculum, a new approach was tested in a third glasshouse experiment where a millet seed mycelium-based inoculum of *P. violae* and *P. sulcatum* was tested at two different concentrations. Although there was a significant carrot growth promoting effect of the millet itself at the high concentration of 50 mg g^{-1} soil, which was not evident at the low concentration of 5 mg g^{-1} soil, both levels of inoculum resulted in very high incidence of cavity spot for *P. violae* isolate HL with 49% and 82% carrots affected for low and high inoculum levels respectively. Cavity spot incidence was lower for *P. sulcatum* isolate P67 at the lower inoculum concentration (17%) while no carrots survived to maturity at the higher inoculum level due to extensive damping off. Overall therefore, the millet inoculum resulted in much higher cavity spot disease incidence and severity than observed in any of the experiments using the sand/oat inoculum either in this study or the previous project FV 391a. This suggests that the millet-based *Pythium* inoculum may be a better approach for inoculation of carrots in the glasshouse although further testing is required to confirm this.

Development of an inoculation method to produce cavity spot symptoms in field grown carrots

Experiments were set up over two years to test the effect of different concentrations of *P. violae* isolate HL sand/oat oospore inoculum on development of cavity spot disease in field macrocosms. Overall, this approach was extremely successful and in contrast to the pot-based experiments resulted in high cavity spot incidence ranging from 30.4-35.7% roots affected in the second year (2018) with similar levels in year 1. Again however, mean disease severity was low with a mean of 2-3 lesions per carrot. Nonetheless this is the first report of artificial inoculation with *P. violae* resulting in significant cavity spot disease under field conditions. These results may suggest that *P. violae* oospores germinate more readily in field

soil than in the growing medium employed in the pot tests, perhaps due to exposure to certain soil factors or environmental conditions that are more conducive to activation of oospores and infection of carrot roots.

Conclusions

- High numbers of *P. violae* and *P. sulcatum* oospores can be produced in a liquid medium and solid substrate.
- A plasmolysis assay was developed to determine viability of *P. violae* and *P. sulcatum* oospores.
- Treatment with a Glucanex enzyme mix or potassium permanganate did not promote germination of *P. violae* oospores.
- Oospores of a *P. sulcatum* isolate germinated readily on agar in contrast to an isolate of *P. violae*
- Artificial inoculation of a compost / sand growing medium with a *P. violae* solid substrate sand/oat oospore inoculum resulted in successful infection of pot grown carrots. Symptoms included some seedling death, reduced seedling size, a decrease in growth of foliage and the formation of small, stubby and stunted carrots with brown tap roots and typical cavity spot lesions. *P. violae* was consistently isolated from tap roots and cavities.
- Metalaxyl seed treatment had little effect or no effect on cavity spot levels or on reducing effects on carrot plant growth in pot tests following artificial inoculation with a *P. violae* sand/oat oospore inoculum.
- Different isolates of *P. violae* and *P. sulcatum* varied in their ability to cause cavity spot symptoms or reduce carrot plant growth in pot tests following inoculation with sand/oat oospore inoculum.
- A solid substrate millet seed mycelial inoculum of *P. violae* resulted in high incidence of cavity spot level in pot tests.
- Artificial inoculation of soil contained in field macrocosms with the *P. violae* solid substrate sand/oat oospore inoculum resulted in a very high incidence of cavity spot.

Financial Benefits

Artificial inoculation in filed or glasshouse may now allow more reliable testing of new control products, hence resulting in considerable financial benefits associated with a reduction in the number of failed trials.

Action Points

None at this time.

SCIENCE SECTION

Introduction

Cavity spot of carrots in the UK is principally caused by the soilborne oomycete pathogen *Pythium violae* although other species such as *P. sulcatum* and *P. intermedium* have also been associated with the disease (Hiltunen and White, 2002). Cavity spot continues to be the most economically important disease for UK carrot growers with losses of at least £3-5 million per season (Martin, 2013). Management of the disease relies heavily on metalaxyl-M fungicide but its efficacy is variable, possibly due to enhanced degradation in some fields (Davison & McKay, 1999). The reliance on this single fungicide, its efficacy in controlling the disease and the potential withdrawal of approval in the future are major concerns for the industry and hence there is an urgent need to identify new actives or approaches for control. To address this, AHDB Horticulture projects have been commissioned to improve the management and control of cavity spot. Recently, AHDB FV 391 (Gladders, 2014) tested a range of potential new fungicides, biological control treatments and pre-planting calcium applications while FV 405 (Clarkson, 2016) investigated the potential of biofumigation. These projects were field based and relied on high disease levels occurring naturally in order for treatment efficacy to be evaluated effectively and with statistical significance. However, despite best efforts to identify high risk carrot growing sites for these experiments, or to encourage disease by irrigation, results have not been forthcoming due to no, or low levels of cavity spot development in many of the trials. This has resulted in a failure so far to reliably identify any new actives or approaches to control of *P. violae*.

One solution to the problem of low cavity spot levels is to artificially inoculate carrots with *P. violae* in pots or the field to ensure high enough levels of disease development such that activity of control treatments can be assessed reliably. Field scale inoculation is challenging for any soilborne plant pathogen but a variety of standard methods exist for pot-scale inoculation for a wide range of plant pathogens. Significantly however, no standard inoculation method has been developed for *P. violae* and results using different techniques have been variable. Several researchers have inoculated freshly harvested carrots roots with agar plugs of mycelium from *P. violae* or other *Pythium* species associated with cavity spot, but although this may give an indication of the pathogenicity of different isolates, it does not always result in typical cavity spot lesions, nor is it appropriate or practical for testing control treatments (e.g. White, 1986; Allain-Boule *et al*., 2004). A more realistic approach is to artificially inoculate a

growing medium to try and induce disease in carrot seedlings or mature plants. Pettit (2002) used an oospore inoculum produced in a V8 liquid medium in a mixture of cornmeal / sand to infect carrot seedlings and showed that this resulted in reduced emergence and colonisation of roots by *P. violae*. However, the inoculum concentrations required to achieve different levels of disease in seedlings were not defined. In the same study, soils from infested field sites were also used to induce cavity spot lesions in pot-grown carrots, which was also an approach used by White (1986). Although successful in some cases, the results were unpredictable and again, levels of *Pythium* inoculum were not defined. In another approach, Suffert & Guibert (2007) used barley grains inoculated with *P. violae* for field inoculation with some success although the inoculum potential was demonstrated to decline over time in parallel pot tests. Barbara (2010) also investigated a variation of the above method using wheat grain, for pot experiments but results were disappointing, with the number of roots with lesions and the number of lesions per root only slightly higher in inoculated pots compared to controls.

Overall there is therefore a clear need to develop artificial inoculation systems for *P. violae* in order to test potential new products and approaches to cavity spot control. In a previous project (FV 391a) we developed methods to produce large numbers of *P. violae* oospores in both a liquid medium and a sand/oat-based solid substrate culture. However, initial investigations indicated that oospore germination on agar was negligible as has been observed previously by other researchers. Nonetheless, in two glasshouse experiments, artificial inoculation of the growing media using the *P. violae* solid substrate at different concentrations resulted in some seedling death, reduced seedling size and a decrease in growth of carrot foliage. However, at harvest, the principal effect of *P. violae* inoculation was the formation of small, stubby and stunted carrots with a much-reduced weight compared to the uninoculated control plants. These infected carrots were also characterised by a long hairy brown tap root with increased lateral root formation, many of which were collapsed. Typical cavity spot lesions were also observed in a large proportion of these stubby carrot roots in the first experiment (up to 26%) but disease incidence was less in experiment 2. *P. violae* could also be consistently isolated from the infected tap roots and cavity spot lesions, confirming that these symptoms were due to the inoculation. Generally, there was no clear effect of oospore concentration on the severity of any of these symptoms associated with *P. violae* inoculation.

These initial artificial inoculation experiments with *P. violae* in FV 391a were therefore quite successful compared to previous attempts; however, results between the two experiments were variable. In this project we build on this initial work to address this variation and try and increase the number of cavity spot lesions observed on carrots artificially inoculated with *P. violae* both in the glasshouse and field. We hypothesised that differences in cavity spot disease levels or impact on carrot growth could be due to variation in oospore viability and

their ability to germinate (in the presence of carrots) between different batches of *P. violae* inoculum or between different isolates. Hence lab-based experiments were designed to test this for *P. violae* and also *P. sulcatum*. A liquid *P. violae* inoculum was also tested in an initial pot-based glasshouse experiment but due to lack of carrot infection using this approach, further work investigated the ability of different *P. violae* and *P. sulcatum* isolates to induce cavity spot using the sand/oat solid inoculum employed successfully in FV 391a and also tested a millet-based inoculum as previously employed for *P. sulcatum* (El-Tarabily, 2004). Finally, the feasibility of producing solid inoculum of *P. violae* on a large scale in solid state fermentation for potential use in field experiments was assessed.

The overall aim of this project was therefore to further develop methods for producing *P. violae* inoculum and determine the potential to cause cavity spot disease. The specific objectives were:

1) Test vital stains and different chemical treatments to assess *P. violae* oospore viability and increase germination.
2) Test the efficacy of different rates of *P. violae* liquid and solid inoculum in producing cavity spot symptoms in pot grown carrots in the glasshouse.
3) Test the efficacy of different rates of solid *P. violae* inoculum in producing cavity spot symptoms in field grown carrots.
4) Examine the feasibility of large-scale *P. violae* inoculum production in solid state fermentation.

Materials and methods

General procedures

Culturing and maintenance of *Pythium violae* and *Pythium sulcatum*

P. violae isolate HL derived from infected carrots (cv. Nairobi) collected in Holton, Lincolnshire was included in all experiments as previously. It i) consistently produced high numbers of oospores (on agar and in sand/oat cultures), ii) caused damping off in carrot seedlings (Hales & Clarkson, 2016), iii) caused lesions on carrot roots (when an agar plug of mycelium was inoculated onto carrot roots) and iv) resulted in some cavity spot lesions in the field in a preliminary un-replicated trial.

All further isolates of *P. violae* and *P. sulcatum* used in this project were selected from a Warwick Crop Centre culture collection (Hales, 2018) (Table 1).

Table 1: Origin of *P. violae* and *P sulcatum* isolates used in this project

Isolate	Species	Origin	Year isolated
HL	*P. violae*	Holton, Lincolnshire	2013
P34	*P. violae*	Elveden, South Yorkshire	2014
P59	*P. violae*	Raskelf, North Yorkshire	2014
P138	*P. violae*	Raskelf, North Yorkshire	2015
P67	*P. sulcatum*	Carlton in Lindrick, Nottinghamshire	2014
P91	*P. sulcatum*	Farnsfield, Nottinghamshire	2014
P127	*P. sulcatum*	Unknown, Shropshire	2014

Identity of the *Pythium* isolates was determined by sequencing of the ITS regions of the rDNA gene (Hales & Clarkson, 2016). All isolates were routinely cultured on cornmeal agar (CMA; Sigma-Aldrich, UK) or CMA with rifampicin (30 mg L^{-1}; CMA/Rif) at 17°C and plugs of mycelium were excised from the growing edge of colonies and submerged in sterile water at 4°C for long-term storage.

Preparation of liquid and solid media for *P. violae* inoculum production

V8 Juice broth (V8B) has previously been reported to support vigorous growth of *Pythium* and other oomycete species (Sutherland & Cohen, 1983; Pettit, 2002) and was selected as a liquid growth medium for oospore production. V8B was prepared with incorporation of cholesterol, as this has been reported to improve oospore production and maturation (Ayers & Lumsden, 1975). V8 Juice (1 L) was stirred with 20 g $CaCO_3$ for 45 min and the mixture centrifuged at

9000 rpm for 30 min at room temperature.) For experiments, a 10% (v/v) V8B was prepared by combining 100 ml of the clarified supernatant with 900 ml distilled water and autoclaving at 121°C for 15 min. Cholesterol (Sigma-Aldrich, UK) from a stock solution (15 mg ml^{-1} in 95% ethanol) was added to V8B to achieve a final concentration of 30 mg L^{-1} (V8B+C).

A horticultural sand (J. Arthur Bower's, UK or Westland Horticulture, UK) supplemented with oats was chosen as a solid substrate medium for producing *Pythium* inoculum for glasshouse and field experiments. Oospore production by *P. violae* has been shown to be profuse on this type of medium and was previously used by Pettit (2002) and Hales & Clarkson (2016) for inoculum production. The solid substrate was prepared by mixing dry sand (425 g) with oats (5 g; porridge oats ground into a fine powder) in 1 L conical flasks and adding 75 ml sterile distilled water (SDW) to achieve a final moisture content of 15% w/w. Flasks were autoclaved twice for 15 min at 121°C with an interval of 24 h in-between each cycle. Flasks were inoculated with 15 agar plugs from actively growing cultures of *P. violae* or *P. sulcatum* isolates and incubated at 20°C in the dark.

A millet seed, mycelium-based inoculum was also included in one glasshouse experiment as this type of inoculum has been found to induce cavity spot disease in artificial inoculation experiments with *P. coloratum*, *P. sulcatum* and *P. ultimum* (El-Tarabily *et al*. 1996, 2004). The substrate was prepared by wetting 70 g white millet seed (Cotswolds Seeds Ltd.) in 500 ml conical flasks with 112 ml SDW to give a final moisture content of 61.5% w/w. Flasks were autoclaved three times at 121°C for 30 min with 24 h in-between each cycle. After each cycle, the flasks were shaken well to avoid clumping of the moist grain. Flasks were inoculated with 14 agar plugs from actively growing cultures of *P. violae* isolate HL or *P. sulcatum* isolate P67 and incubated at 20°C in the dark.

Objective 1: Test vital stains and different chemical treatments to assess *P. violae* oospore viability and increase germination

A series of experiments was designed to i) compare the use of a tetrazolium bromide (MTT) vital stain and a plasmolysis method for determining oospore viability for *P. violae* and *P. sulcatum* isolates and ii) assess the effects of potassium permanganate on *P. violae* oospore germination. Additionally, the oospore extraction protocol from liquid culture described in FV 391a was further developed to obtain an oospore extract free of live mycelial debris which could be used in further viability and germination tests.

1.1. Experiment to determine oospore viability in two *P. violae* and two *P. sulcatum* isolates using MTT staining and plasmolysis

Agar plugs of mycelium (5 x 5 mm) from actively growing cultures of *P. violae* HL and P34 and *P. sulcatum* P67 and P127 on CMA were used to inoculate 20 ml aliquots of V8B+C in flat cell culture flasks (50 ml capacity; VWR, UK) with vented lids. Cultures were incubated in the dark at 15°C. After four months, two mycelial mats of each isolate were removed with sterile forceps to a Petri dish and washed three times in 10 ml SDW by gentle pipetting.

Mycelial mats were transferred to a 50 ml Falcon tube containing 10 ml SDW. The probe of a Silverson Laboratory Mixer was sterilised by flaming in 100% ethanol and mycelial mats homogenised on a medium setting for four 30 s bursts with 30 s intervals on ice in-between to avoid overheating the extract. The probe was washed with off with 5 ml SDW and this combined with the homogenate to give a final volume of approximately 20 ml. The homogenate was successively filtered by vacuum through pre-sterilised 250 & 80 µm nylon meshes to remove most mycelial fragments (Pettit, 2002), each time washing through with 5 ml SDW. The filtrate was centrifuged at 1300 *x g* (2500 rpm, Sorvall, UK) for 10 min to concentrate the oospores and the supernatant removed leave a volume of approximately 2 to 3 ml. The pellet was resuspended and a 1ml aliquot autoclaved at 121°C, 15 min to kill the oospores. Non-autoclaved and autoclaved (dead) oospores were kept at 4°C until ready for staining and plasmolysis.

MTT staining

Three subsamples of autoclaved and non-autoclaved *P. violae* and *P. sulcatum* oospore suspensions (150 µl) were mixed with 0.1% MTT ((4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, VWR) in a 1:1 v/v ratio according to the method of Sutherland & Cohen (1983). Suspensions were incubated for 24 h in darkness at 35°C and examined

microscopically (Olympus CHA, x200). Oospores that stained purple were classed as viable but dormant, whilst those that were blue, black or colourless were considered non-viable.

Plasmolysis method

Three subsamples of autoclaved and non-autoclaved *P. violae* and *P. sulcatum* oospore suspensions (150 µl) were centrifuged at 1000 *x g*, 2 min and the supernatant removed. Oospores were incubated in hypertonic solution (4 M NaCl, 150 µl) at RT for 45 min and microscopically assessed (Jiang and Erwin, 1990). Those displaying a centralised ball-like structure due to contraction of the oospore membrane were considered viable, whereas non-plasmolysed oospores in which the membrane had not pulled away from the cell wall were classed as non-viable (Fig. 9).

In both methods, 100 oospores were assessed for viability from three replicate tubes for each of the autoclaved and non-autoclaved *P. violae* and *P. sulcatum* oospore suspensions. Only intact oospores with a double wall were considered and care was taken not to include oogonia in the counts. Photomicrographs were taken using a Leica DMi 1 inverted microscope fitted with an M170 digital camera and images captured using Leica LAS software.

1.2. Experiment to determine the effect of Glucanex treatment on oospore viability and germination in isolates of *P. violae* and *P. sulcatum*

A mixture of lysing enzymes from *Trichoderma harzianum* (Glucanex; Sigma-Aldrich) was tested for efficacy in producing oospore extracts free of mycelial debris and for an effect on oospore viability. Glucanex has previously been employed to digest mycelial fragments and asexual spores in oospore preparations of *Phytophthora ramorum* (Boutet *et al*., 2010) and *Phytophthora capsici* (Hurtardo-Gonzales, PhD. thesis, 2008).

Concentrated oospore suspensions of *P. violae* HL and *P. sulcatum* P67 were prepared as in 1.1 and the number estimated by counting in a Modified Fuchs-Rosenthal chamber. Subsamples of autoclaved and non-autoclaved oospore suspensions from each isolate were then centrifuged at 1000 *x g*, 2 min and the supernatant removed. Approximately 1 x 10^4 to 1 x 10^5 oospores were then resuspended in 700 µl filter-sterilised Glucanex solution (5 mg ml^{-1}) and incubated for 24 h in darkness at 20°C. Oospores were washed three times by centrifugation (1000 *x g*, 2 min, MSE) with 1 ml SDW. Viability of oospores pre- and post-Glucanex treatment was estimated by the plasmolysis method described above.

To assess germination, Glucanex-treated *P. violae* and *P. sulcatum* oospores were plated (approximately 500 oospores / plate) onto CMA/Rif, incubated at 20°C and observed periodically over two weeks using a Leica DMi 1 inverted microscope (x50). Three replicate

plates were prepared for each species and 100 oospores were examined for germination per plate.

1.3. Experiment to examine the effect of potassium permanganate treatment on oospore germination in four *P. violae* isolates

Concentrated oospore suspensions of *P. violae* HL, P34, P59 and P138 were prepared as in 1.1, with the exception that three mycelial mats of each isolate instead of two were used as the starting material (to allow for loss of oospores during the extended extraction procedure). Oospore preparations were treated with Glucanex and washed as described in 1.2. A portion of Glucanex-treated oospores was retained for germination and viability testing, while the remaining oospores were subjected to potassium permanganate treatment according to the methods of Guo & Ko (1994) and Ruben *et al*. (1980). Subsamples of Glucanex-treated oospore suspensions (750 µl) were mixed with 0.2% $KMnO_4$ (Honeywell Speciality Chemicals, Germany) in a 1:1 v/v ratio. Oospores were washed three times by centrifugation (3500 *x g*, 2 min) with 1.5 ml SDW. Glucanex-treated *P. violae* oospores with/without $KMnO_4$ treatment were then plated (approximately 50 to 100 oospores / plate) onto CMA/Rif and 5% V8 Agar plus rifampicin (30 mg L^{-1}), incubated at 20°C and observed over two weeks using a Leica DMi 1 inverted microscope (x50) to assess germination. Three replicate plates of each media type were prepared for each isolate and 50 oospores were assessed per plate.

Objective 2: Test the efficacy of different rates of *P. violae* liquid and solid inoculum in producing cavity spot symptoms in pot grown carrots in the glasshouse

2.1. Inoculation of pot-grown carrots with liquid cultures of *P. violae* and *P. sulcatum*

A 50:50 v/v mix of compost (John Innes No. 3, Erin, UK) and a horticultural grade sharp sand (Westland, UK) was selected as the growing medium in all glasshouse experiments as this substrate drains well, is free of large particulates, but still retains adequate water and is rich in nutrients due to the high compost content (soil analysis, Appendix 1). Plastic pots (5.5 L capacity, 20 cm diameter, 27.5 cm tall) were filled with growing medium (5.65 kg per pot), placed in deep saucers and watered to ensure a high moisture content. Pots were sown with untreated carrot seed (10 seeds per pot, cv. Nairobi, Elsoms Seeds Ltd, UK) and the growing

medium kept damp with overhead watering while carrot seedlings emerged, with additional weekly watering in the saucers. The glasshouse compartment was maintained at maximum temperature of 18°C and minimum of 16°C with supplementary lighting used from 5:00 to 20:00 h. After 7 weeks, carrot seedlings were thinned out to five per pot and plants grown on until inoculation 4 months later. To produce the liquid inoculum, mycelial plugs from the growing edge of *P. violae* isolates HL, P34 and P59 and *P. sulcatum* isolates P67 and P127 were used to inoculate 100 ml aliquots of V8B+C in flat cell culture flasks (400 ml capacity; VWR, UK) with vented lids. Cultures were incubated in the dark at 15°C for 5 weeks (Fig. 1) and periodically checked under a stereo microscope for oospore formation.

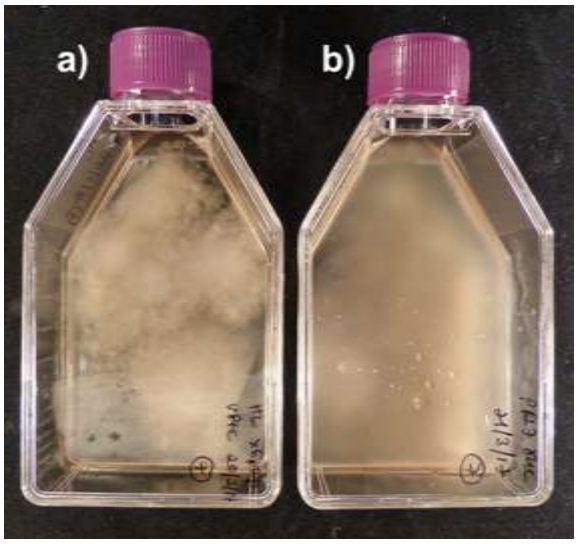

Figure 1: Mycelial mats of (a) *P. violae* HL and (b) *P. sulcatum* P127 grown in V8B+C for 5 weeks at 15°C.

Mycelial mats of each *P. violae* and *P. sulcatum* isolate were removed from two flasks with sterile forceps and each washed twice with 40 ml SDW by gentle pipetting. Mats were transferred to a sterile bulbous flask (MSE, UK) and 45 ml SDW added. The blade of a motorised MSE homogeniser was sterilised by flaming in 100% ethanol and mycelial mats homogenised on a low setting for 8 min. The homogenate was transferred to a sterile 50 ml Falcon tube, measured and decanted into a sterile 500 ml Duran flask. The blade of the homogeniser and flask were washed twice with 20 ml SDW and the process was repeated for two further mycelial mats of the same isolate. The homogenates were then combined and the total volume made up to 450 ml with SDW. The blade of the homogeniser was re-sterilised in between handling each isolate. Aliquots of each homogenate were retained for counting to determine oospore concentration.

Homogenates containing mycelial fragments and oospores of each *P. violae* and *P. sulcatum* isolate were used to inoculate 4 month-old carrots. A total of 100 ml homogenate was applied as a drench to the surface of each pot using a 10 ml pipette (i.e. almost one full mycelial mat

was applied to five carrots). Four replicate pots were prepared for each isolate and four control pots received 100 ml SDW only. Gentle overhead watering was applied after two days and plants subsequently watered via the saucers and overhead for a further 10 weeks to maintain a high moisture content. At harvest, carrot root and shoot weight were measured and roots examined for presence of cavity spot lesions and tap root browning. However, despite the concentrated oospore and mycelial inoculum applied, there was little or no evidence of cavity spot disease and no reduction in root or shoot weight compared to the control plants for any of the *P. violae* and *P. sulcatum* isolates.

Three additional pot-based glasshouse experiments were therefore set up to further investigate solid substrate inoculum of *P. violae* and *P. sulcatum*. The first of these (2.2) further investigated the use of the sand/oat oospore-based *P. violae* inoculum which induced cavity spot symptoms in FV 391 but also tested the effect of metalaxyl seed treatment. The hypothesis was that the metalaxyl might prevent early infection of carrots by *P. violae* and hence promote normal carrot development (as opposed to the stunting observed in FV 391) but also allow later infection by the pathogen resulting in cavity spot lesions on mature carrots. The second glasshouse experiment (2.3) investigated the efficacy of different *P. violae* and *P. sulcatum* isolates in inducing cavity spot symptoms with the hypothesis that different isolates have different levels of virulence which would result in different levels of disease. A third experiment (2.4) investigated the efficacy of a mycelium-based millet seed inoculum in inducing cavity spot symptoms for different isolates of *P. violae* and *P. sulcatum*. This method was reported to successfully induce cavity spot symptoms for *P. sulcatum* (El-Tarabily *et al*., 1996, 2004). The hypothesis was that this approach would result in more consistent cavity spot disease development as it does not rely on oospores being viable or able to germinate. This mycelium-based inoculum is also much quicker to produce than oospore-based sand/oat oospore inoculum.

2.2. Determine the effect of metalaxyl seed treatment on cavity spot development in pot grown carrots in the glasshouse using *P. violae* sand/oat inoculum

Inoculation of growing medium, experimental set-up and maintenance

A 50:50 v/v mix of compost (John Innes No. 3, Erin, UK) and a horticultural grade sharp sand (Westland, UK) was again used as the growing medium in this experiment. Solid substrate sand/oat inoculum of *P. violae* isolate HL (4 months old) was prepared as outlined above

(general procedures) using the J Arthur Bower's sand in 1 L flasks. The contents of four flasks were decanted into a large grip-seal bag to give approximately 2 kg of starting material and thoroughly shaken by hand for 10 min to dislodge oospores and ensure homogeneity. *P. violae* oospore concentration was then initially estimated by vortexing 1 g of this inoculum in 10 ml SDW for 1 min and counting in a Sedgewick-Rafter 1 ml counting chamber (two replicate counts for each of four 1 g samples). 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 approx. 5000 oospores g^{-1} (checked by a further spore count) after which a final dilution was carried out to obtain another bulk 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 mixed with the compost/sand growing medium in a cement mixer to obtain growing medium infested with final *P. violae* oospore concentrations of 1, 10, 50 and 100 oospores g^{-1} (1.1, 10.8, 54 & 108 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 tall) were filled with the infested growing medium (5.65 kg per pot) at each of the five *P. violae* oospore concentrations. Control pots received a mixture of 50:50 v/v compost/sand only. Pots were placed in deep saucers within a glasshouse compartment (max 18°C, min 16°C; supplementary lighting used from 5:00 to 20:00 h when day length was reduced) and watered to ensure a high moisture content before sowing with metalaxyl-treated or untreated carrot seed (10 seeds per pot, cv. Nairobi, Elsoms Seeds Ltd, UK). Initially, the growing medium was kept damp in the top zone by gentle overhead watering while carrot seedlings emerged, with additional weekly watering in the saucers. After 7 weeks, seedlings were thinned out to five per pot. Once plants had established, watering was increased from both above and below via the saucers to keep the growing medium damp, and was adjusted appropriately for the prevailing conditions. Carrot plants also received watering with a nutrient solution via the saucers from nine weeks after sowing alternating with 2N:1P:4K (Vitax Vitafeed) and 0N:1P:3K (Solufeed) feeds. Aphiline, Amblyline, Encarline, Exhibitline & Hypoline biocontrol agents were routinely used for controlling aphids, thrips, whitefly and sciarid flies. In total, 12 replicate pots were prepared for each oospore concentration for both untreated and metalaxyl-treated seed in a randomised block design consisting of 12 blocks arranged over two benches (Fig. 2). The experiment was set up on 16/12/16 and ran for 26 weeks.

Figure 2: Pot-grown carrots inoculated with *P. violae* HL (March 2017 at 12 weeks after sowing).

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

Carrot seedling emergence and any damping off symptoms were recorded weekly for the first 7 weeks. At harvest after 26 weeks, the foliage from the five carrots in each pot was removed and the total fresh and dry weight recorded. Carrots were then gently removed from the soil to reduce damage to the long tap roots, washed and total weight recorded. Each carrot was then carefully assessed for presence / absence of cavity spot (incidence) and also the number of lesions present (severity).

To confirm infection by *P. violae*, one carrot was selected from 8 out of 12 replicate pots from each seed treatment and each oospore concentration (total of 80 roots). The long thin tap root from the selected roots was excised and stored in sterile water. Roots were surface sterilised in 70% ethanol for 1 min and washed twice in sterile water after which they were cut into three sections of equal length (top - nearest the base of the carrot, middle & bottom). A 1 - 2 cm length of each section was then plated out onto CMA/Rif, incubated in the dark at 17°C and the number with *Pythium* growing out recorded after 5 - 10 days. In addition, small pieces of

carrot tissue excised from selected cavity spot lesions in each treatment were plated directly onto CMA amended with rifampicin and monitored for *P. violae* growth.

All data recorded were subjected to statistical analysis using ANOVA with angular transformation of % seed germination and cavity spot incidence and $\log_{10}$ transformation of number of cavity spot lesions per carrot (severity).

2.3. Determine the efficacy of different *P. violae* and *P. sulcatum* isolates in inducing cavity spot symptoms in pot-grown carrots in the glasshouse using sand/oat inoculum

Inoculation of growing medium, experimental set-up and maintenance

This experiment was set up according to the method described for 2.2 with the following modifications. Solid substrate sand/oatmeal inoculum of *P. violae* isolates HL, P34 and *P. sulcatum* isolates P67, P91, P127 (5 months old) were prepared as outlined above (general procedures) using Westland Horticultural grade sharp sand in 1 L flasks. Compost/sand growing medium was infested to give final *Pythium* oospore concentrations of 10 and 100 oospores g^{-1} (1.1 and 108 oospores/cm^3) and the cement mixer disinfected with Jet 5 (Certis, UK) and washed with water in between preparing batches of growing medium infested with each *Pythium* isolate. Eight replicate pots of each oospore concentration for each *Pythium* isolate and 16 untreated control pots were prepared and arranged in 8 blocks each consisting of 12 pots in a randomised block design. Pots were sown with untreated carrot seed (cv. Nairobi) on 26/09/17 and ran for 22 weeks.

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

As before, carrot seedling emergence and any damping off symptoms were recorded weekly for the first 7 weeks. Plants were harvested when carrots reached maturity at 22 weeks and carrot foliage weight and root weight for each pot determined. Each carrot was carefully assessed for presence / absence of cavity spot (incidence) and also the number of lesions present (severity). To confirm infection by *Pythium*, one carrot was selected from 7 out of 8 replicate pots from each isolate and oospore concentration treatment (total of 105 roots) and selected roots excised and plated onto CMA/Rif to assess *Pythium* infection as described in 2.2. In addition, small pieces of carrot tissue excised from selected cavity spot lesions in each treatment and plated directly onto CMA amended with rifampicin and monitored for *P. violae* growth.

As before, all data recorded were subjected to statistical analysis using ANOVA with angular transformation of % seed germination and cavity spot incidence and log_{10} transformation of number of cavity spot lesions per carrot (severity). Due to the difference in the number of replicates for the untreated controls (16 replicates) compared the number for the inoculated treatments (8 replicates), two LSD values were generated. LSD 1 allows comparison of the untreated control means (the maximum number of replicates) with those for the inoculated treatments (the minimum amount of replicates). LSD 2 allows comparison just between two inoculated treatments (Fig. 19).

2.4. Determine the efficacy of different *P. violae* and *P. sulcatum* isolates in inducing cavity spot symptoms in pot-grown carrots in the glasshouse using millet inoculum

Inoculation of growing medium, experimental set-up and maintenance

A 50:50 v/v mix of compost (John Innes No. 3, Erin, UK) and a horticultural grade sharp sand (Westland, UK) was again used as the growing medium in this experiment. The set up of this third glasshouse was modified from the methods above to test a mycelium-based millet seed inoculum of *P. violae* isolate HL and *P. sulcatum* isolate P67 prepared as described above (general procedures) and incubated for 3 weeks at 20°C in the dark. Appropriate amounts of millet seed inoculum of each isolate were mixed with compost/sand growing medium in a cement mixer to obtain final concentrations of 5 and 50 mg colonised millet seed g^{-1} of growing medium. Two sets of 'dead inoculum' control treatments also received the same quantities of colonised millet seed for each isolate (5 and 50 mg g^{-1} *P. violae* HL and *P. sulcatum* P67) that had been autoclaved twice for 1 h. This was to allow for the potential effect of the millet seed itself on carrot growth. At experimental set up, a few grains of live and dead millet inocula were plated onto CMA and incubated for 1 week at 20°C to confirm that *P. violae* and *P. sulcatum* had successfully colonised the grain (Fig. 3 a, b) and that no live *Pythium* remained following autoclaving inoculum for the dead inoculum controls (Fig. 3 c).

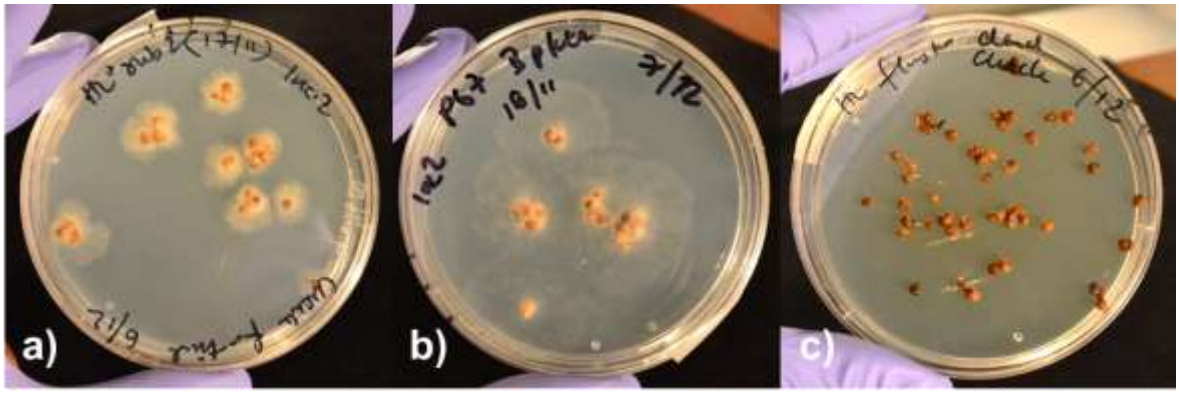

Figure 3: Growth a) *P. violae* HL and b) *P. sulcatum* P67 from colonised millet grain after incubation on CMA for 1 week at 20°C compared to c) no growth from colonised millet grain that had been autoclaved twice for 1 h for the dead inoculum controls.

Eight replicate pots for both *P. violae* HL and *P. sulcatum* P67 isolates were prepared for each millet concentration (5 and 50 mg g^{-1}) as well as eight replicates for dead inoculum controls at each millet concentration. In addition, 16 untreated control pots were set up with unamended compost/sand growing medium. Pots were arranged on one bench in a randomised block design consisting of eight blocks, each with eight pots. Ten untreated carrot seeds were sown per pot (08/12/17) and the plants maintained and thinned in the same way as for the experiments above.

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

As in experiments described above, carrot seedling emergence and any damping off symptoms were recorded weekly for the first 7 weeks. Plants were harvested when carrots reached maturity at 22 weeks and carrot foliage weight and root weight for each pot determined. Each carrot was carefully assessed for presence / absence of cavity spot (incidence) and also the number of lesions present (severity). To confirm infection by *Pythium*, one carrot was selected from all eight replicate pots from each isolate and inoculum concentration treatment in each block (total of 64 roots) and selected roots excised and plated onto CMA/Rif to assess *Pythium* infection as described in 2.2. In addition, small pieces of carrot tissue excised from selected cavity spot lesions in each treatment were plated directly onto CMA amended with rifampicin and monitored for *Pythium* growth.

As before, all data recorded were subjected to statistical analysis using ANOVA with angular transformation of % seed germination and cavity spot incidence and $\log_{10}$ transformation of number of cavity spot lesions per carrot (severity). Due to the difference in the number of replicates for the untreated controls (16 replicates) compared the number for the inoculated

treatments, including the dead inoculum controls, (8 replicates), two LSD values were generated. LSD 1 allows comparison of the untreated control means (the maximum number of replicates) with those for the inoculated treatments (the minimum amount of replicates). LSD 2 allows comparison just between two inoculated treatments (Fig. 25).

Objective 3: Test the efficacy of different rates of solid *P. violae* inoculum in producing cavity spot symptoms in field grown carrots

Experiments were carried out as part this project and PhD project FV 432 over two years from May 2016 to February 2017 (Year 1) and May 2017 to February 2018 (Year 2) to test the efficacy of different rates of solid *P. violae* inoculum in producing cavity spot symptoms in field grown carrots in 24 'macrocosm' plots located in the Wellesbourne quarantine field (Fig. 4). The macrocosms comprised sunken concrete tubes 1 m in diameter and 60 cm deep. Each macrocosm was initially filled with a gravel layer, followed by a bottom 20 cm layer of sandy silt loam ('Wick' series, Wellesbourne, UK; 225 kg) mixed with 40 kg horticultural sand (Westland, UK). A 30 cm top layer consisting of 328 kg sieved soil and 60 kg horticultural sand (soil analysis, Appendix 1) was then added to provide a suitable sandy loam substrate for carrot growing and inoculation.

P. violae isolate HL sand/oat inoculum was produced in 1 L flasks as outlined previously and decanted into a large grip seal 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}. These stocks were further diluted in sand to provide 5 kg batches of inoculum, which were raked into the top 10 cm of the macrocosms on 26/05/16 to give final oospore concentrations of 5, 10, 20, 30 and 50 oospores g^{-1}. Uninoculated control plots received 5 kg sand alone and each treatment was replicated across four macrocosms in a randomised block design. Each macrocosm was sown with approximately 280 untreated carrot seeds (cv. Nairobi) and seeds covered with a 1 to 2 cm layer of sieved soil. In Year 1, two weeks after sowing, excessive rainfall led to flooding in the macrocosms and at four weeks after sowing, plots showed poor seedling establishment and hence were re-sown with approximately 100 carrot seeds. Macrocosms were re-inoculated with two-month-old *P. violae* HL inoculum in Year 2 on 24/05/17 to achieve the same oospore concentrations and each macrocosm re-sown with approximately 280 untreated carrot seeds (cv. Nairobi). Macrocosms were watered regularly throughout the growing season and sprayed periodically with Hallmark (lambda-cyhalothrin) to reduce damage from carrot root fly. A thick layer of straw was applied to the

surface of each macrocosm in November 2016 (Year 1) to prevent winter frost damage. In early December 2017 (Year 2), macrocosms were covered with a layer of fleece and straw (Fig. 4 b).

Carrots were harvested on 23/02/17 in Year 1 and 12/03/18 in Year 2. Carrot yield (total carrot weight per plot and total number of carrots per plot) was recorded and all roots were carefully assessed for presence / absence of cavity spot (incidence) and the number of lesions present (severity). Cavities were only scored as typical lesions if they were sunken lesions or full cavities, elliptical to round in shape and > 2mm in diameter on the root surface. In 2018, some 'atypical' lesions were more apparent, also on carrots from control macrocosms. These were very dark, superficial lesions rather than full cavities on the carrot surface and were not scored as cavity spot lesions. Isolations from a small subset of atypical and typical lesions were made by plating out carrot tissue on CMA/Rif. DNA extraction followed by PCR and sequencing of the ITS region of the rRNA gene was carried out for purposes of molecular identification as described in project FV 432 (Hales & Clarkson, 2016). All harvest data recorded were subjected to statistical analysis using ANOVA with angular transformation of cavity spot incidence and $\log_{10}$ transformation of severity across all roots and only those roots infected.

Figure 4: Field macrocosms used for *P. violae* inoculation of carrots (a) in July 2016 and (b) in December 2017 insulated with fleece and straw for the winter to prevent frost damage.

Objective 4. Examine the feasibility of large-scale *P. violae* inoculum production in solid state fermentation

Initial tests were set up to examine the feasibility of employing a microbial fermenter for large-scale production of *P. violae* sand/oat inoculum. A BioFlo PRO Industrial Fermenter (300 L model; Fig. 5 a), located in a Warwick Manufacturing Group laboratory (WMG, Warwick University Main Campus), was internally modified to hold a custom-made basket for inoculum. This was designed to fit on to a central spindle (Fig. 5 b), which can be rotated to allow for introduction of inoculum at multiple points on the substrate via a port on the top of the reactor. The basket was specially manufactured from perforated aluminium and stainless steel (Fig. 5 c) and lined with 125 µM nylon mesh to accommodate up to 20-30 kg of sand/oatmeal

substrate. The time taken to have this specially manufactured has therefore led to considerable delay to this objective.

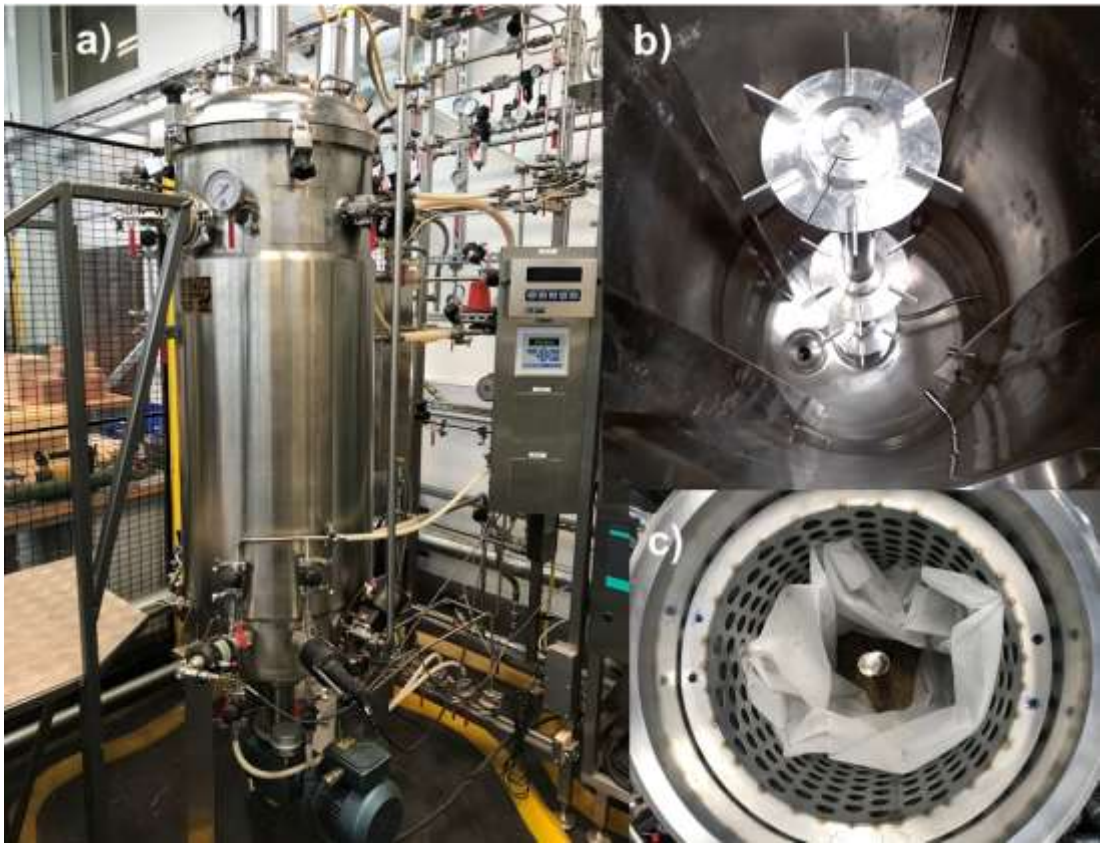

Figure 5: a) BioFlo PRO Industrial Fermenter for large-scale production of a solid sand/oat *P. violae* inoculum. The internal bioreactor spindle structure (b) has been adapted to hold a metal basket for the sand/oatmeal substrate (c).

A pilot test was set up in September 2018 with a batch of moist Arthur J. Bower's sand to test the bioreactor sterilisation cycles only. Dry sand (4.3 kg) was mixed with SDW (760 ml) to achieve a final moisture content of 15% w/w and the substrate transferred to the mesh basket. Two sterilisation cycles each of 42 min were run (max temperature 123°C) with 24 h in-between each cycle. The sand retained moisture after this process. Inoculation of a 15 kg batch of sand/oatmeal substrate is planned using a mycelial 'slurry' prepared by homogenising mycelial mats of *P. violae* isolate HL in SDW. Mycelial mats were produced from mycelial plugs from the growing edge of *P. violae* isolate HL, which were used to inoculate 20 ml aliquots of V8B in flat cell culture flasks (50 ml capacity; VWR, UK) with vented lids and incubated in the dark at 15°C. Inoculation of the solid substrate sand/oat inoculum has yet to be carried out via a port on top of the reactor.

Results

Objective 1: Test vital stains and different chemical treatments to assess *P. violae* oospore viability and increase germination

1.1. Experiment to determine oospore viability in two *P. violae* and two *P. sulcatum* isolates using MTT staining and plasmolysis

P. violae (isolates HL, P34) and *P. sulcatum* (isolates P67, P127) oospores were produced in liquid culture and subjected to MTT staining for viability after 4 months. Those that stained purple were classed as viable, whilst those that stained blue, black or colourless were considered non-viable. The proportion of oospores in the different staining classes for each *Pythium* isolate is shown in Fig. 6. Purple colouration ranged from lavender to dark magenta with some oospores exhibiting partial or uneven staining for both *P. violae* and *P. sulcatum* isolates (Fig. 7 b, c; Fig. 8 b, c). Black and colourless oospores were also evident in both *P. violae* and *P. sulcatum* preparations (Fig. 7 d, e; Fig 8 c, e), but no blue-stained oospores were observed for *P. violae* HL or P34 after MTT treatment. A very low proportion of *P. sulcatum* oospores stained blue (3.0 ± 0.5% and 0.6 ± 0.2% for P67 and P127, respectively; Fig. 6), but these displayed uneven staining and distorted cytoplasm (Fig. 8 d), so were considered non-viable.

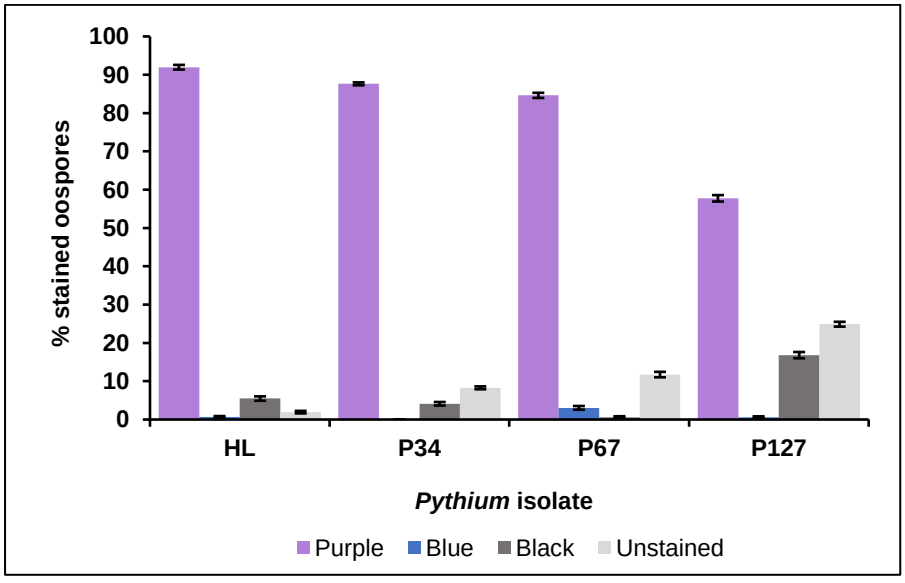

Figure 6: Percentage of *P. violae* (HL and P34) and *P. sulcatum* (P67 and P127) oospores in different staining classes after treatment with 0.1% MTT (24 h, 35°C). Purple: viable; blue, black and unstained:

non-viable. The data, presented as a percentage, represents the mean of three counts of 100 oospores, Error bars are standard error of the mean.

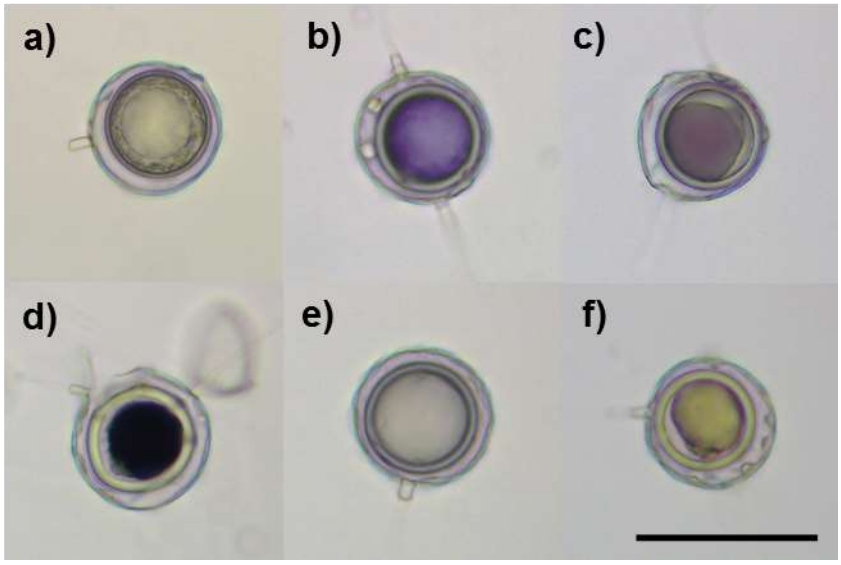

Figure 7: Oospores of *P. violae* isolates HL and P34 before (a) and after (b-f) MTT staining. a) mature HL oospore before staining; b) purple stained viable HL oospore; c) purple partially stained P34 oospore; d) black stained non-viable HL oospore; e) colourless non-viable HL oospore; f) autoclaved colourless HL oospore showing distorted cytoplasm. Bar = 50 µm.

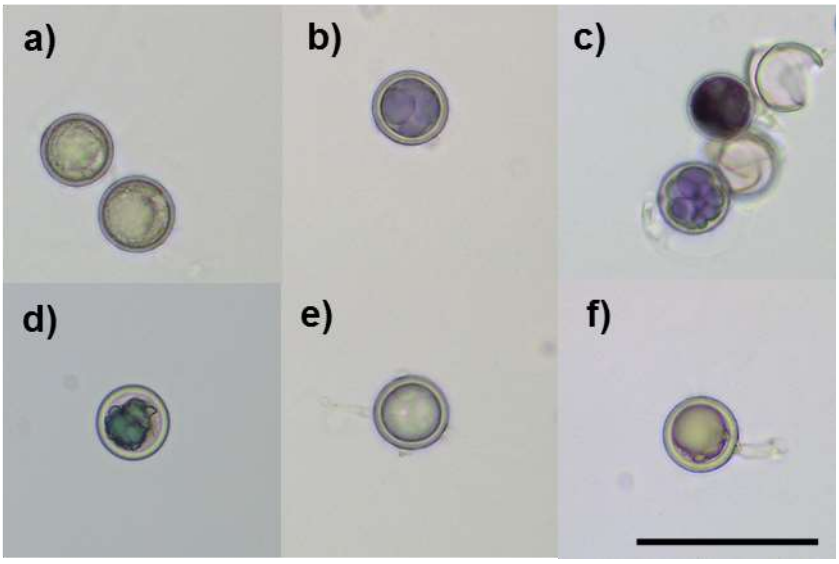

Figure 8: Oospores of *P. sulcatum* isolates before (a) and after (b-f) MTT staining; a) P67 oospores before staining; b) purple stained viable P67 oospore; c) P127 oospores showing variable staining; d) blue non-viable P67 oospore; e) colourless non-viable P67 oospore f) autoclaved colourless P67 oospore showing distorted cytoplasm. Bar = 50 µm.

The *P. violae* and *P. sulcatum* oospores produced in liquid culture were also subjected to a plasmolysis test in NaCl for viability after 4 months. Plasmolysis was clearly visible in viable *P. violae* oospores as demonstrated by obvious detachment of the cytoplasm from the oospore wall (Fig. 9 a). Non-viable oospores showed no such contraction of the cytoplasm (Fig. 9 b) while autoclaved (dead, non-viable) oospores as well as displaying no contraction of the cytoplasm also had distorted, clumped cytoplasm (Fig. 9 c). The same morphology was observed in non-autoclaved and autoclaved *P. sulcatum* spores (Fig. 9 d-f), but owing to the comparatively small size of these oospores, more careful visual assessment was required.

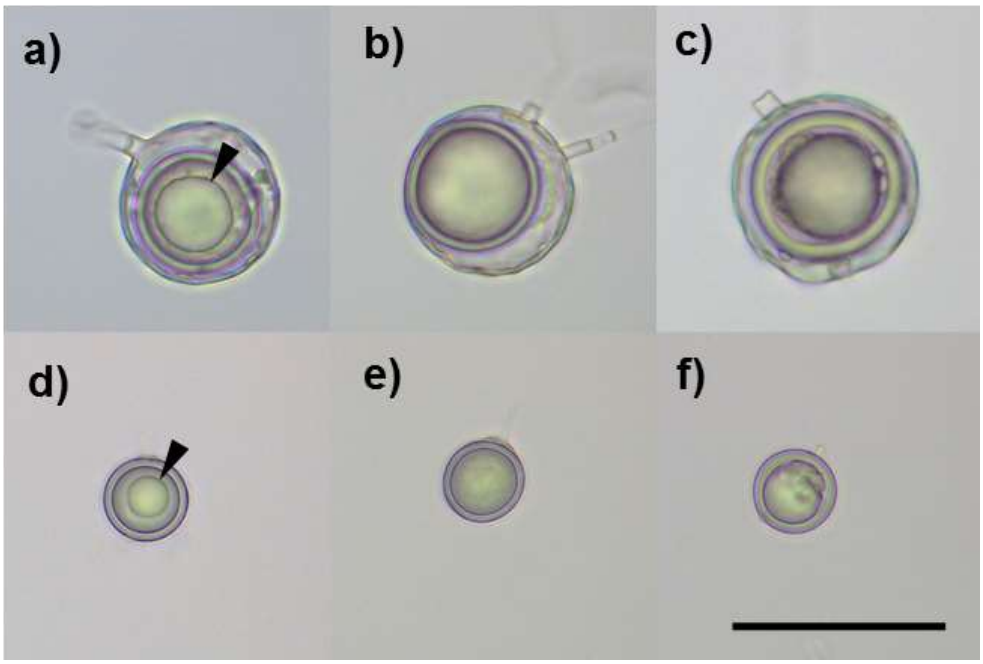

Figure 9: Oospores of *P. violae* HL (a-c) and *P. sulcatum* P67 (d-e) following plasmolysis in 4 M sodium chloride solution for 45 min; a) and d) viable, plasmolysed oospores with black arrow showing contraction of the plasma membrane; b) and e) non-viable, non-plasmolysed oospores showing no shrinkage of the plasma membrane; c) and f) autoclaved, non-viable oospores showing no plasmolysis with distortion of the cytoplasm. Bar = 50 µm.

Comparison of viability assessments using MTT staining and plasmolysis

A high percentage of non-autoclaved *P. violae* HL and P34 oospores stained purple and were deemed viable (92.0 ± 0.6% for HL and 87.6 ± 0.4% for P34; Fig. 6, Table 2) and these results were consistent with those obtained using the plasmolysis method (90.3 ± 1.1% for HL and 85.6 ± 0.3% for P34). However, the percentage oospore viability obtained using MTT for the two *P. sulcatum* isolates was higher (84.6 ± 0.7% for P67 and 57.7 ± 0.4% for P127) than those obtained using plasmolysis (69.5 ± 2.4% for P67 and 2.9 ± 1.1% for P127). Hence, more potential false positives were observed in the MTT test, particularly for *P. sulcatum* P127.

Table 2: Comparison of percentage viability for live (non-autoclaved) and dead (autoclaved) oospores for *P. violae* (isolates HL, P34) and *P. sulcatum* (P67, P127) using MTT staining and plasmolysis methods. Data represent mean percentage spore viability from three counts of 100 oospores with corresponding standard errors.

	% Viability (MTT test)[a]		% Viability (Plasmolysis test)[b]	
Isolate	**Non-autoclaved oospores**	**Autoclaved oospores**	**Non-autoclaved oospores**	**Autoclaved oospores**
***P. violae* HL**	92.0 ± 0.6	0.0 ± 0	90.3 ± 1.1	0.3 ± 0.3
***P. violae* P34**	87.6 ± 0.4	2.6 ± 0.3	85.6 ± 0.3	0.0 ± 0
***P. sulcatum* P67**	84.6 ± 0.7	0.3 ± 0.2	69.5 ± 2.4	0.0 ± 0
***P. sulcatum* P127**	57.7 ± 0.4	16.2 ± 0.3	2.9 ± 1.1	0.0 ± 0

[a] Stained with 0.1% tetrazolium bromide (MTT) solution for 24h at 35°C in darkness

[b] Plasmolysis in 4 M NaCl for 45 min, RT

1.2. Experiment to determine the effect of Glucanex treatment on oospore viability and germination in isolates of *P. violae* and *P. sulcatum*

Microscopic observation of *P. violae* HL and *P. sulcatum* P67 oospores before and after treatment with the Glucanex revealed that mycelial fragments had largely been digested with the enzyme treatment (Fig. 10). Some degraded mycelial debris and oogonia remained despite the washing steps involved in the method (Fig. 10 d). However, residual mycelial fragments were not viable, as none of these gave rise to fresh mycelial growth following plating onto CMA/Rif.

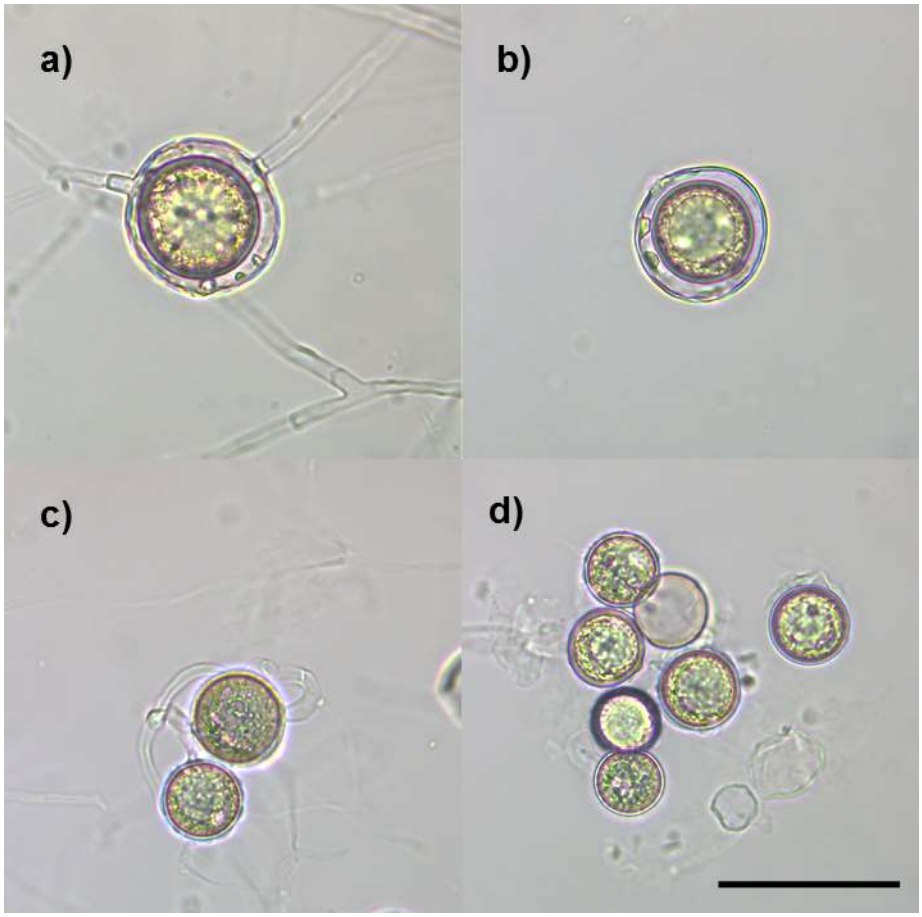

Figure 10: Oospores of *P. violae* HL (a, b) and *P. sulcatum* P67 (c, d) showing attached intact mycelium (a, c) before, and digested / degraded mycelium (d) after treatment with Glucanex (5 mg ml^{-1}, 24 h, 20°C). Bar = 50 µm.

Oospore viability assessment using the plasmolysis method showed that incubation in Glucanex for 24 h at 20°C had no effect on percentage viability of *P. violae* HL oospores with 90% plasmolysis recorded for oospore preparations both pre- and post-Glucanex treatment (Table 3). In contrast, a small reduction in viability was observed for Glucanex-treated *P. sulcatum* P67 oospores, in which percentage viability was 44.8 ± 4.8% compared to 54.8 ± 3.7% for the untreated oospores. None of the autoclaved oospores demonstrated plasmolysis indicating they were all dead and non-viable.

Table 3: Percentage viability of non-autoclaved (live) and autoclaved (dead) *P. violae* HL and *P. sulcatum* P67 oospores pre- and post-Glucanex[a] treatment as determined via plasmolysis in 4 M sodium chloride[b]. Data, presented as percentage, represent the mean of three counts of 100 oospores from replicate plasmolysis reactions with corresponding standard errors.

		% Oospores viable (plasmolysed)[b]	
Isolate	**Treatment[a]**	**Non-autoclaved live oospores**	**Autoclaved dead oospores**
***P. violae* HL**	Pre-Glucanex	89.9 ± 0.8	0 ± 0
***P. violae* HL**	Post-Glucanex	89.7 ± 1.7	0 ± 0
***P. sulcatum* P67**	Pre-Glucanex	54.8 ± 3.7	0 ± 0
***P. sulcatum* P67**	Post-Glucanex	44.8 ± 4.8	0 ± 0

[a] Oospores incubated with Glucanex (5 mg ml^{-1}), 24 h, 20°C

[b] Plasmolysis in 4 M NaCl for 45 min, RT

P. sulcatum P67 oospores germinated readily at 20°C in darkness on CMA/Rif, with dense mycelium quickly developing (Fig. 11) and reached a level of 43.5% germination after 3 days (Table 4). In contrast, no germination was observed in *P. violae* HL oospores, even after periodic assessment for 2 months after plating. No growth from mycelial fragments of either isolate was recorded on the plates.

Table 4: Germination of *P. violae* HL and *P. sulcatum* P67 oospores following Glucanex[a] treatment after incubation for 3 days at 20°C on CMA/Rif.

	***P. violae* HL**		***P. sulcatum* P67**	
Replicate CMA/Rif plate	**No. germinated oospores**	**No. ungerminated oospores**	**No. germinating oospores**	**No. ungerminated oospores**
1	0	109	42	69
2	0	102	52	59
3	0	104	50	59
Total oospores	**0**	**315**	**144**	**187**
% germination	**0**		**43.5**	

[a] Oospores incubated with Glucanex (5 mg ml^{-1}), 24 h, 20°C

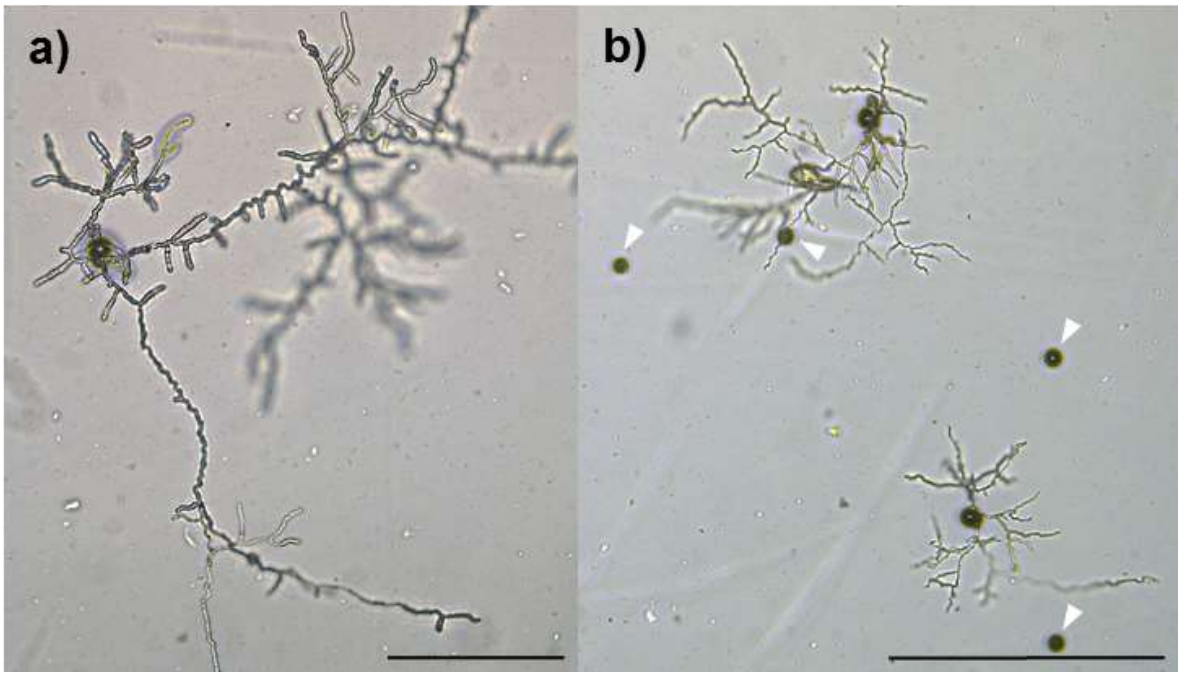

Figure 11: Germination of *P. sulcatum* P67 oospores observed after incubation at 20°C for 3 days on CMA/Rif (a, b). White arrows (b) indicate non-germinating oospores. Bars = 200 µm (a) and 500 µm (b).

1.3. Experiment to examine the effect of potassium permanganate treatment on oospore germination in four *P. violae* isolates

Application of $KMnO_4$ (0.1% w/v, 15 min) to Glucanex-treated *P. violae* oospores did not induce oospore germination. A crude viability assessment of the four *P. violae* isolates demonstrated that oospores were viable following the Glucanex treatment (Table 5) as the percentage oospores plasmolysed in all isolates was found to be similar range (76.9 - 85.8%). Despite this, oospores with and without $KMnO_4$ treatment almost all failed to germinate on 5% V8A/Rif or CMA/Rif. Only 1 out of 150 *P. violae* P138 Glucanex-treated oospores (without $KMnO_4$ treatment) germinated on CMA/Rif. Viability was not fully determined for $KMnO_4$-treated oospores as very limited amounts remained after plating.

Table 5: Crude estimation of viability via the plasmolysis method in Glucanex-treated oospores of four *P. violae* isolates. One plasmolysis reaction carried out in 4 M NaCl for 45 min, RT.

***P. violae* isolate**	**No. oospores assessed**	**% Oospores Plasmolysed**
HL	155	80.6
P34	130	76.9
P59	127	85.8
P138	120	80.8

Objective 2: Test the efficacy of different rates of *P. violae* liquid and solid inoculum in producing cavity spot symptoms in pot grown carrots in the glasshouse

2.1. Inoculation of pot-grown carrots with liquid cultures of *P. violae* and *P. sulcatum*

There was no evidence of cavity spot disease or effect on root or foliage growth for the carrots inoculated with liquid cultures of *P. violae* isolates HL, P34 and P59 and *P. sulcatum* isolates P67 and P127. For this reason as described above, further experiments tested the effect of solid inoculum on cavity spot development for different *P. violae* and *P. sulcatum* isolates.

2.2. Determine the effect of metalaxyl seed treatment on cavity spot development in pot grown carrots in the glasshouse using *P. violae* sand/oatmeal inoculum

This experiment further investigated the ability of the sand/oat oospore-based *P. violae* inoculum to induce cavity spot symptoms, but also tested the effect of metalaxyl seed treatment. Statistical analyses however, revealed that metalaxyl seed treatment had no overall significant effect on carrot seed germination, foliage or root dry weight at harvest, or on cavity spot incidence and severity.

Effects on seed germination and carrot growth

In pots sown with metalaxyl treated-seed and inoculated with solid sand/oat inoculum of *P. violae* HL, carrot seed germination ranged from 77-94% (Fig. 12) compared with 74% for the uninoculated control. Germination from untreated seed ranged from 74-93% in the *P. violae* inoculated treatments. In the presence of *P. violae*, germination from metalaxyl-treated carrot seed was significantly higher at 1, 50 & 100 oospores g^{-1} relative to the appropriate uninoculated control. Germination was only significantly reduced compared to the uninoculated control for untreated seed inoculated at 1 and 10 oospores g^{-1}, but not for 50 and 100 oospores g^{-1}. A seed treatment x oospore concentration interaction was observed for germination (P = 0.009; Appendix 2, Table a), indicating that carrot seed germination from metalaxyl-treated and untreated seed differs in the presence of varying concentrations of *P. violae* oospores. However, the effect of the metalaxyl seed treatment was inconsistent for

different oospore concentrations (Fig. 12). No or little post-emergence seedling damping off was observed with < 1% occurring across all treatments (data not shown).

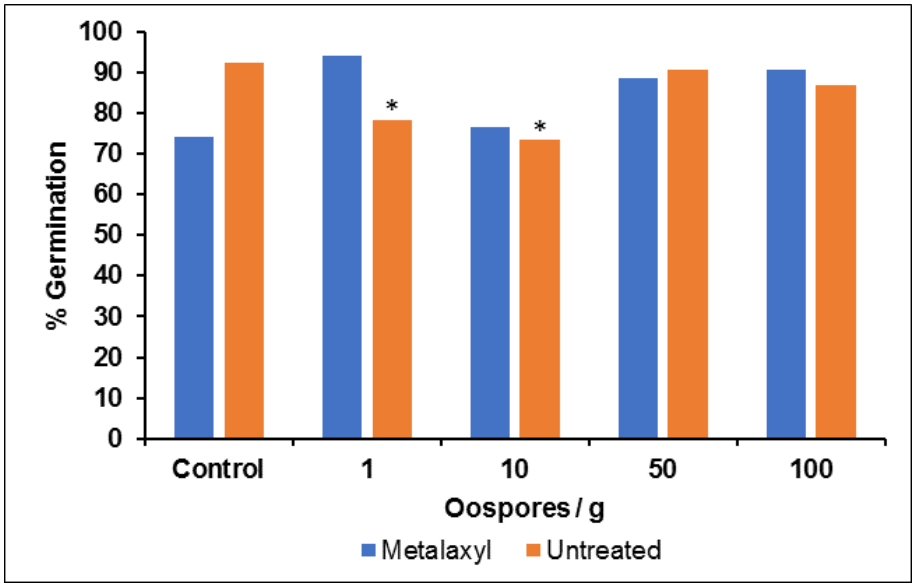

Figure 12: Effect of *P. violae* HL inoculation with sand/oatmeal inoculum at different concentrations on mean percentage seed germination for metalaxyl-treated and untreated carrot seed (back transformed data). Asterisks indicate significant reduction compared to the appropriate uninoculated control.

Inoculation with the solid sand/oat inoculum of *P. violae* HL led to a significant reduction in mean carrot foliage dry weight at harvest compared to the uninoculated control when combining data from metalaxyl-treated and untreated seed (P = 0.002, data not shown). In *P. violae* treatments, the mean foliage dry weight per plant ranged from 2.9-3.2 g and 3.0-3.2 g for metalaxyl treated and untreated seed treatments respectively (Fig. 13 a; Appendix 2, Table a). The mean foliage dry weights for the respective controls were 3.4 and 3.3 g per plant. No seed treatment x oospore concentration interaction was evident (*P* = 0.12), indicating that overall, the response to different concentrations of *P. violae* for foliage weight did not vary with seed treatment. *P. violae* inoculation resulted in stunted, stubby carrots (Fig. 14), although this effect was less pronounced than observed previously in the glasshouse experiments reported in FV 391a. Again as observed in FV 391a, inoculated carrots (from both metalaxyl-treated and untreated seed) displayed hairy brown tap roots with profuse collapsed lateral roots (Fig. 15). Combining data from metalaxyl and untreated seed, inoculation with *P. violae* had a significant effect overall on carrot weight (*P* = 0.013, data not shown), but there was no effect of oospore concentration. In the metalaxyl seed-treated carrots, the mean weight per carrot in the *P. violae* treatments ranged between 100.2-108.7 g, compared to 115.5 g for the corresponding control (Fig 13 b; Appendix 2, Table a).

These results indicate a trend for a reduction in carrot weight in *P. violae* inoculated pots, but this was found only to be significant at 100 oospores g^{-1}. There was no consistent reduction in mean carrot weight across the different oospore concentrations for untreated seed with mean values ranging from 99.7-112.1 g compared to 110.8 g for the appropriate control. No seed treatment x oospore concentration interaction was observed (*P* = 0.697; Appendix 2, Table a).

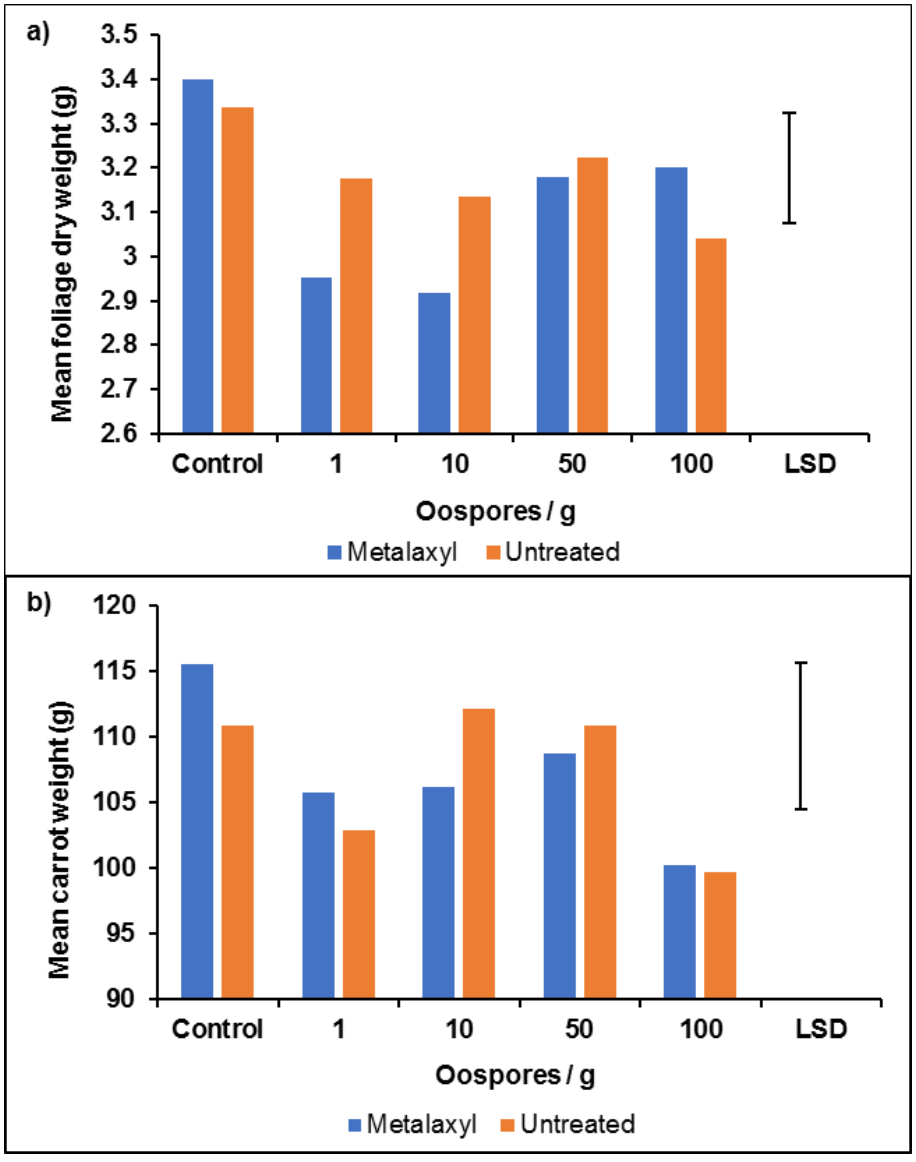

Figure 13: Effect of *P. violae* HL inoculation with sand/oat inoculum at different concentrations on a) mean foliage dry weight per plant and b) root weight per carrot for carrots grown from metalaxyl-treated and untreated carrot seed. Bars represent the least significant difference (LSD) between treatments at the 5% level.

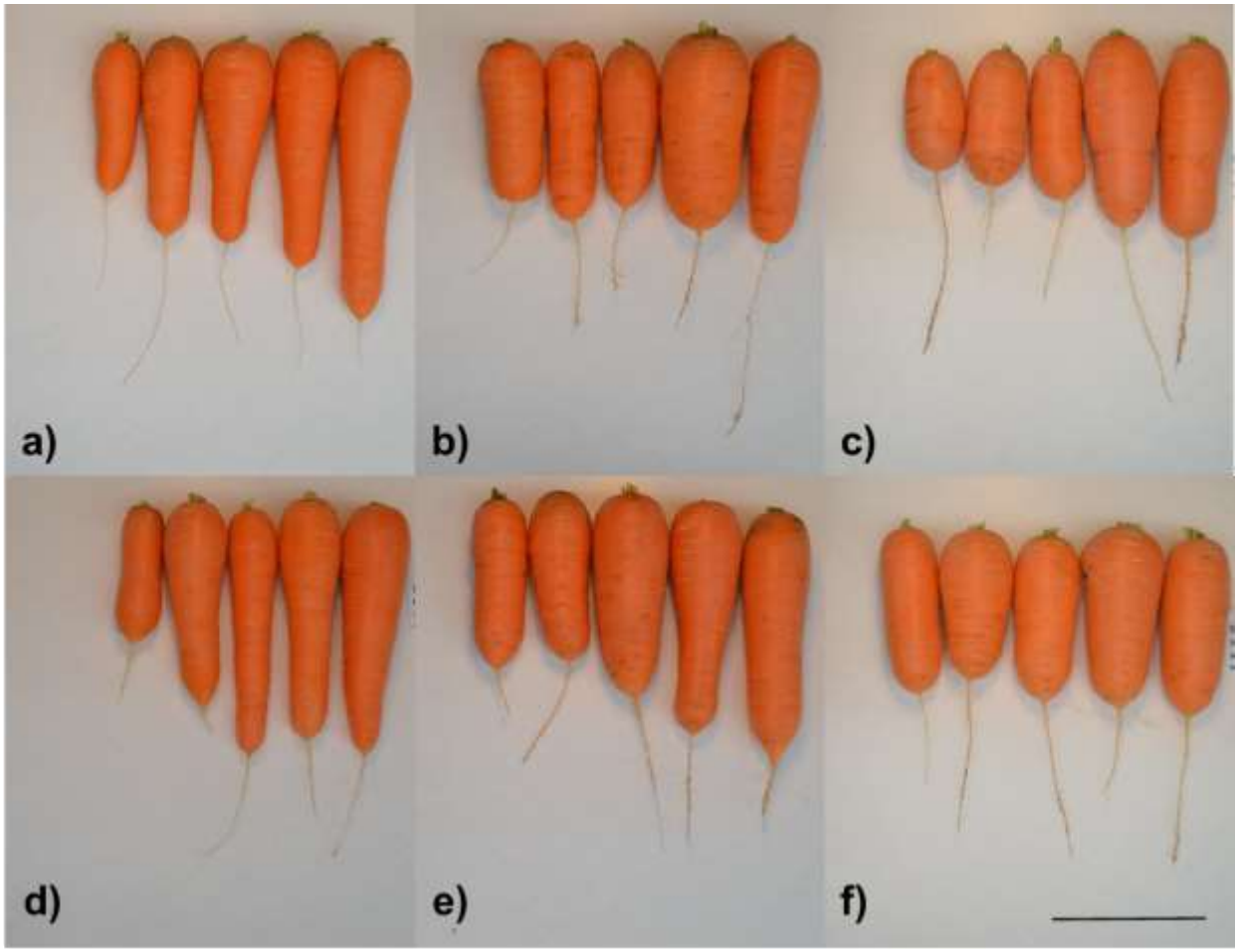

Figure 14: Effect of *P. violae* HL inoculation with sand/oat inoculum at different concentrations on carrot size for metalaxyl-treated (a - c) and untreated seed (d - f); a, d; uninoculated controls: b, e; 10 oospores g^{-1}: c, f; 100 oospores g^{-1}. Bar = 10 cm.

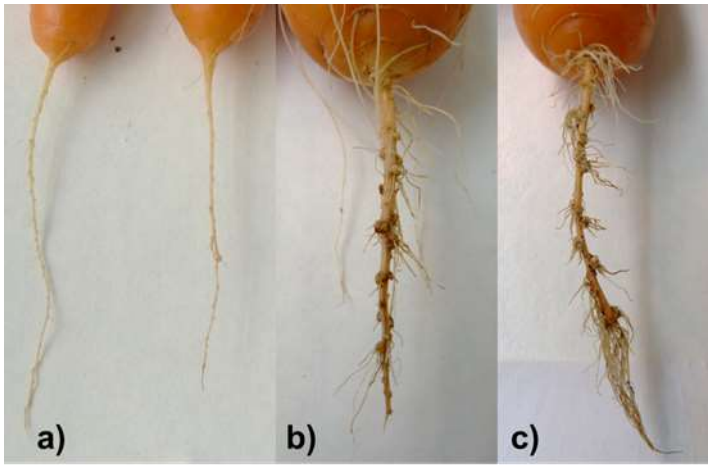

Figure 15: Carrot tap roots for a) uninoculated control treatment displaying normal morphology and for b, c) carrots inoculated with *P. violae* HL sand/oat inoculum showing brown colouration and increased lateral root formation.

Effects on cavity spot incidence and severity

Cavity spot symptoms were observed in carrots from all the *P. violae* inoculated treatments as shown by the presence of typical sunken elliptical lesions and expanded cavities where the carrot periderm had disintegrated (Fig. 16). Cavity spot incidence (percentage of carrots displaying one or more lesions) varied between 3 and 23% across the inoculated treatments (Fig. 17 a). However, a low level of incidence (3%) was also observed in some of the uninoculated control carrots (metalaxyl-treated seed), presumably resulting from cross-contamination. Combining the data from both metalaxyl and untreated seed treatments, *P. violae* inoculation significantly increased cavity spot incidence (P = 0.002, data not shown). However, no seed treatment x oospore concentration interaction was observed (P = 0.158; Appendix 2 Table a), suggesting that overall, the response to different concentrations of *P. violae* for cavity spot incidence did not vary with seed treatment. However, cavity spot incidence was significantly higher in untreated seed treatments inoculated at 1 and 100 oospores g^{-1} compared to the untreated control (Fig 17 a). For the metalaxyl seed treatments, there was a trend towards increased cavity spot incidence at 1, 10 & 100 oospore g^{-1} compared to the corresponding control, but this was not statistically significant, most likely due to the contamination found in two control pots.

Mean severity of cavity spot (no. of cavities / total no. of carrots scored) was low, ranging from 0.05-0.20 and 0.02-0.40 cavities per carrot in metalaxyl-treated seed and untreated seed carrots respectively, compared to 0.06 and 0 in the corresponding controls (Fig. 17 b). Nevertheless, a seed treatment x oospore concentration interaction was observed (P = 0.03; Appendix 2, Table a). Cavity spot severity, as with incidence, was significantly higher for *P. violae* treatments with untreated seed inoculated at 1 and 100 oospores g^{-1} compared to the uninoculated control, but there was no difference in disease severity in carrots from metalaxyl-treated seed at the different oospore concentrations (Fig 17 b; Appendix 2, Table a). Across infected carrots only, disease severity ranged from 1.3-2.5 and 1.5-2.3 lesions per carrot for metalaxyl-treated seed and untreated seed, respectively (Appendix 3, statistical analysis not possible due to the large number of missing values i.e. unaffected roots). These severity values were in a similar range to those reported for the two glasshouse experiments reported in FV 391a.

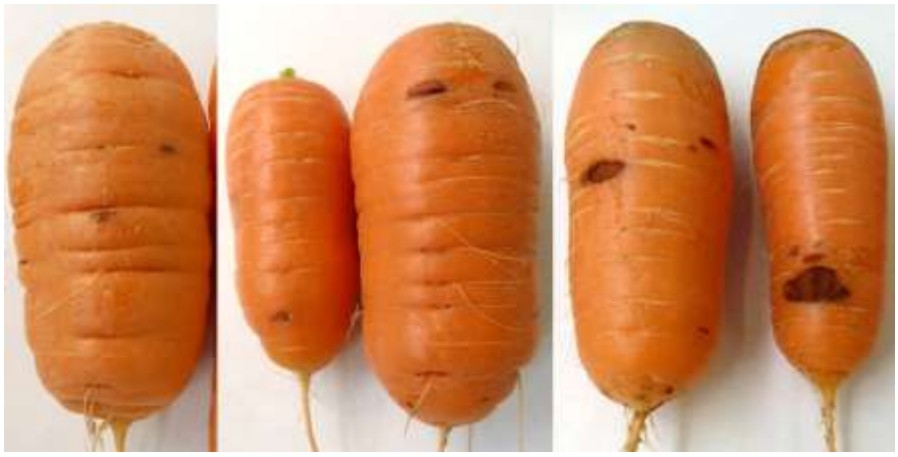

Figure 16: Cavity spot symptoms following inoculation with *P. violae* HL

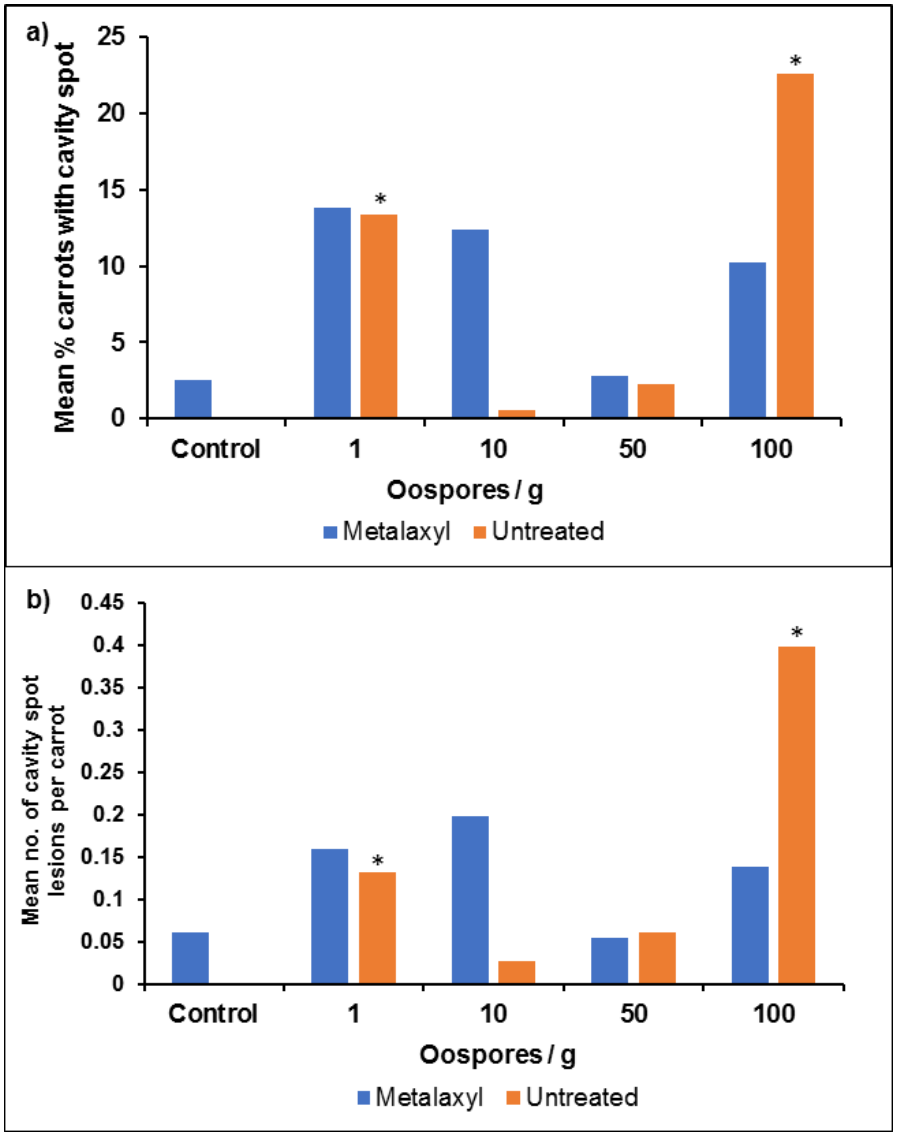

Figure 17: Effect of *P. violae* HL inoculation with sand/oatmeal inoculum at different concentrations on a) cavity spot incidence (% carrots affected) and b) severity (no. lesions across all carrots) for carrots grown from metalaxyl and untreated seed. Data presented are back transformed mean values and asterisks indicate a significant increase compared to appropriate control.

Isolation of *P. violae* from roots

A high proportion of carrot tap root pieces from inoculated treatments pots resulted in *P. violae* colonies following plating onto CMA/Rif. The percentage of root pieces showing *P. violae* infection ranged from 4.2-62.5% and 8.3-62.5% for carrots from metalaxyl-treated seed and untreated seed respectively, compared to 4.2 and 0% for the corresponding uninoculated control treatments (Appendix 3). There was no clear difference between the metalaxyl-treated and the untreated seed nor amongst the different oospore concentration, but a χ^2 analysis revealed that infection between the uninoculated controls and *P. violae*-inoculated treatments across all root pieces tested was statistically different (χ^2, $p < 0.001$). *P. violae* colonies also developed from selected cavity spot lesions when plated onto CMA/Rif, again clearly demonstrating successful infection by the pathogen.

2.3. Determine the efficacy of different *P. violae* and *P. sulcatum* isolates in inducing cavity spot symptoms in pot-grown carrots in the glasshouse using sand/oat inoculum

This experiment investigated the ability of different two *P. violae* and three *P. sulcatum* isolates to induce cavity spot disease. There was no overall effect of oospore concentration in this experiment, with no significant differences between the two oospore concentrations tested (10 and 100 oospores g^{-1}) in terms of effects on carrot seed germination, foliage or root dry weight at harvest, or cavity spot incidence and severity.

Effects on seed germination and carrot growth

Across all *Pythium* isolate treatments and oospore concentrations, percentage carrot seed germination ranged from 62.9 - 98.6% compared to 88.1% in the uninoculated control (Fig. 18). When considering specific treatments, a significant reduction in carrot seed germination was found for carrots inoculated with *P. violae* isolates HL and P34 and *P. sulcatum* P91 for the 10 oospores g^{-1} concentration relative to the uninoculated control (Fig. 18). Reduced germination was also observed for these isolates at 100 oospores g^{-1}, but this was only statistically significant for *P. violae* HL and *P. sulcatum* P91. Conversely, germination of carrot seed for *P. sulcatum* P67 and P127 treatments at 10 oospores g^{-1} was significantly increased compared to the uninoculated control and to the other isolates (Appendix 2, Table b). However, no differences in germination were observed for these isolates for the 100 oospores g^{-1} concentration. An isolate x oospore concentration interaction was observed for germination

(P = 0.004; Appendix 2 Table b), demonstrating that seed germination differed across the isolate treatments depending on oospore concentration.

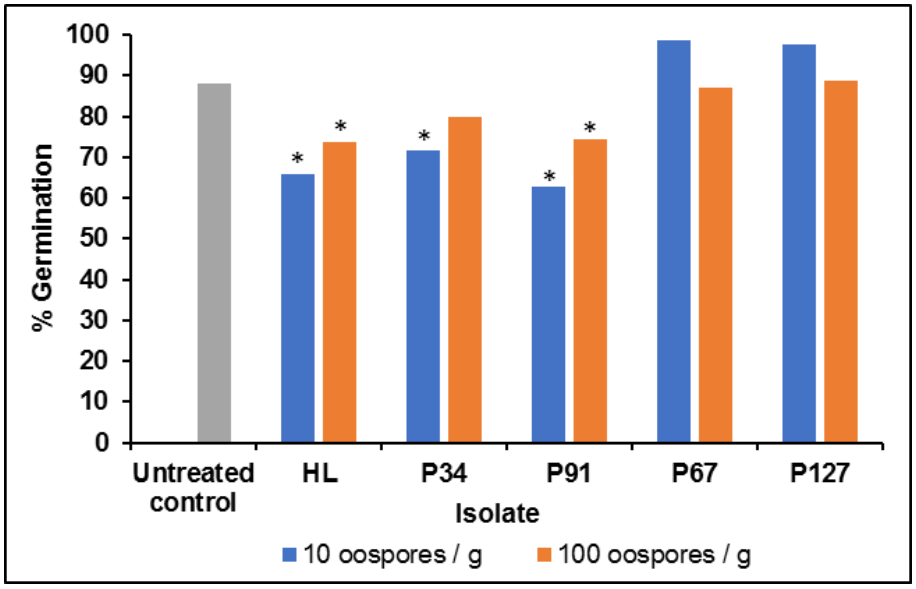

Figure 18: Effect of *P. violae* (HL and P34) and *P. sulcatum* (P91, P67 and P127) inoculation with sand/oat inoculum at two concentrations on mean percentage carrot seed germination (back transformed means). Asterisk indicates significant reduction compared to uninoculated control.

At harvest, mean carrot foliage dry weight per carrot across all *Pythium* isolates ranged from 1.4-2.0 g and 1.5-2.0 g at 10 and 100 oospores g^{-1}, respectively, compared to 1.7 g in the uninoculated control (Fig 19 a; Appendix 2, Table b). Overall there was a significant difference in foliage weight across the different *Pythium* isolate treatments (P < 0.001) largely due to the *P. sulcatum* P67 treatments where the mean foliage weights at 10 and 100 oospores g^{-1} were significantly lower than for the uninoculated control and all other isolate treatments. However, inoculation with the other *Pythium* isolates gave an inconsistent response; for example, *P. violae* HL and *P. sulcatum* P91 at 10 oospores g^{-1} resulted in an increase in foliage dry weight. No isolate x oospore concentration interaction was observed for foliage dry weight at harvest (P = 0.359; Appendix 2, Table b).

The effects of stunting and root browning with *Pythium* inoculation were far less obvious in this experiment compared with those observed in 2.2 and experiments reported in FV 391a. As with foliage dry weight at harvest, a significant difference in mean carrot root weight was observed overall across the different *Pythium* isolate treatments (P < 0.018; data not shown). Across inoculated treatments, mean carrot root weight ranged from 40.8-53.7 g compared with 48.6 g in the uninoculated control (Fig. 19 b). Inoculation with *P. sulcatum* P67 induced some stunting symptoms compared with the other isolate treatments (Fig. 20) and this effect was particularly apparent in the 100 oospore g^{-1} treatment, where there was a significant reduction

in mean carrot root weight compared to the uninoculated control and all the other treatments (Fig 19 b; Appendix 2, Table b). However, no consistent reductions in mean carrot root weight compared to the uninoculated control were observed with *P. violae* HL or P34, or *P. sulcatum* P91 or P127. Conversely, a significant increase in mean root weight was observed for *P. sulcatum* P91 at 10 oospores g^{-1}. However, it should be noted that one replicate pot in this treatment (10 oospores g^{-1}) only had two very large carrots surviving, which increased the overall mean for this measurement. There was also no significant isolate x oospore concentration interaction (*P* = 0.698; Appendix 2, Table b).

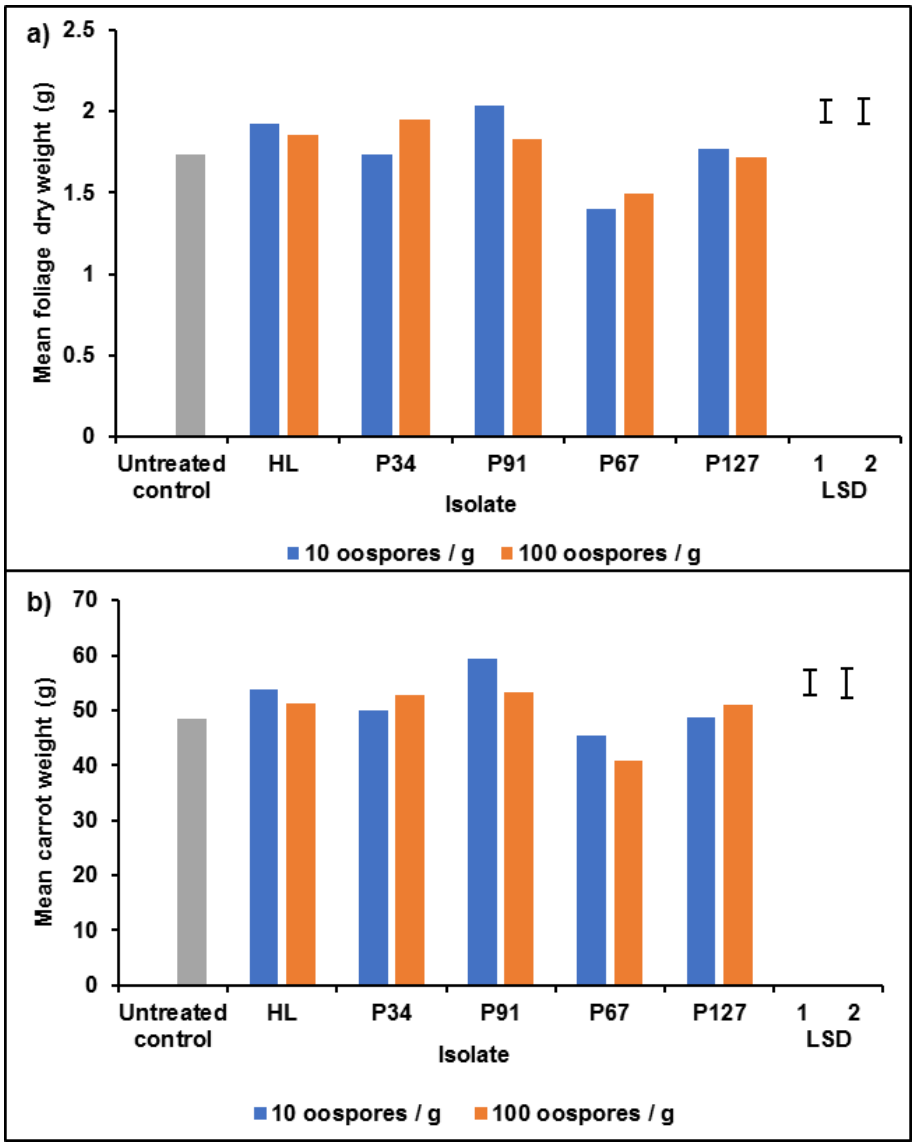

Figure 19: Effect of *P. violae* (HL and P34) and *P. sulcatum* (P91, P67 and P127) inoculation with sand/oat inoculum at two concentrations on a) mean carrot foliage dry weight per plant and b) root weight per carrot in pot-grown carrots. Bars represent the least significant difference (LSD) between treatments at the 5% level; LSD 1 allows comparison between the untreated control (16 replicates) and inoculated treatments (8 replicates) and LSD 2 allows comparison between inoculated treatments only (8 replicates).

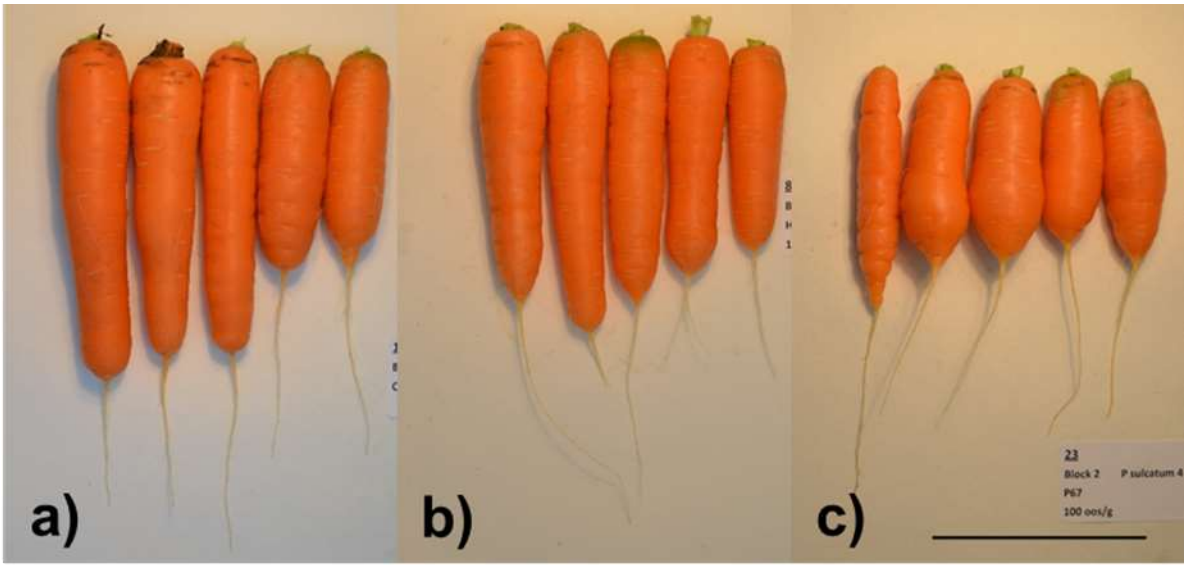

Figure 20: Stunting symptoms observed in carrots inoculated with *P. sulcatum* P67 at 100 oospores g^{-1} (c) compared with uninoculated control carrots showing normal morphology (a) and carrots inoculated with *P. violae* HL at 100 oospores g^{-1} showing no stunting (b). Bar = 10 cm.

Effects on cavity spot incidence and severity

Typical cavity spot lesions were observed only for *P. violae* HL and P34 and *P. sulcatum* P67 treatments although cavity spot incidence (percentage of carrots displaying one or more lesions) was very low, ranging from 0-3.8% (Fig. 21 a). Consequently, there was no significant difference in cavity spot incidence between the different *Pythium* isolate treatments ($P = 0.437$, data not shown) and no isolate x oospore concentration interaction was observed ($P = 0.608$; Appendix 2, Table b). The highest incidence of cavity spot was recorded for *P. violae* P34 at 100 oospores g^{-1} (3.8%) largely owing to unusually heavy infection observed in one replicate pot (Fig. 22). No cavity spot symptoms were recorded for *P. sulcatum* P91 and P127 or in carrots from the uninoculated control.

As for cavity spot incidence, mean severity of cavity spot across all *Pythium* treatments (no. of cavities / total no. of carrots scored) was low ranging from 0-0.09 (Fig. 21 b) and across infected carrots only, ranged from 1.0-10.4 (Appendix 3). There was no therefore no significant difference in disease severity across the different *Pythium* isolates ($P = 0.516$, data not shown) and no isolate × oospore concentration interaction was observed ($P = 0.572$, Appendix 2, Table b). As for cavity spot disease incidence, the highest disease severity score was observed for *P. violae* P34 at 100 oospores g^{-1} owing to the abnormally high level of cavities scored in one replicate pot (Fig. 22).

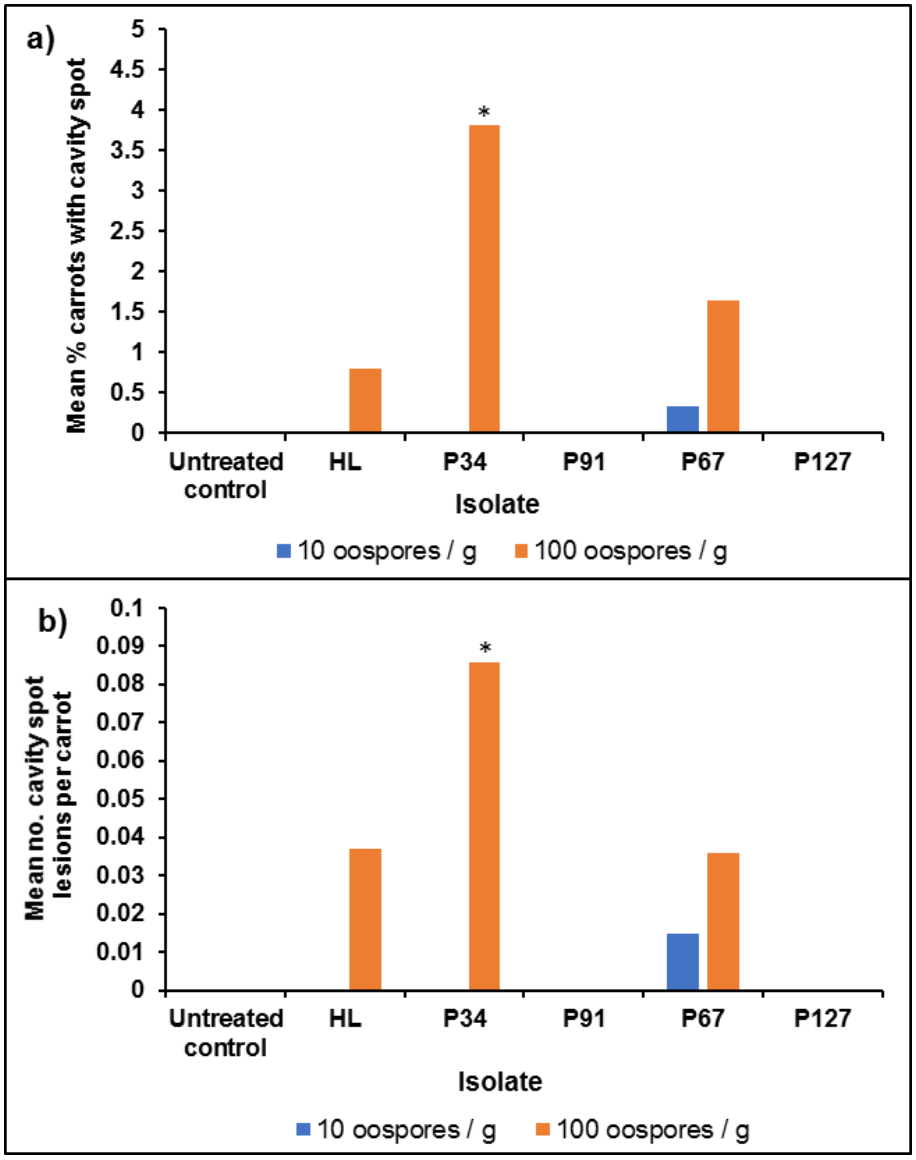

Figure 21: Effect of *P. violae* (HL and P34) and *P. sulcatum* (P91, P67 and P127) inoculation with sand/oat inoculum at two concentrations on a) cavity spot incidence (% carrots affected) and b) severity (no. lesions across all carrots) in carrots inoculated with *P. violae* isolates HL and P34 and *P. sulcatum* isolates P91, P67 and P127. Data presented are back transformed mean values and asterisk indicates a significant increase compared to appropriate control.

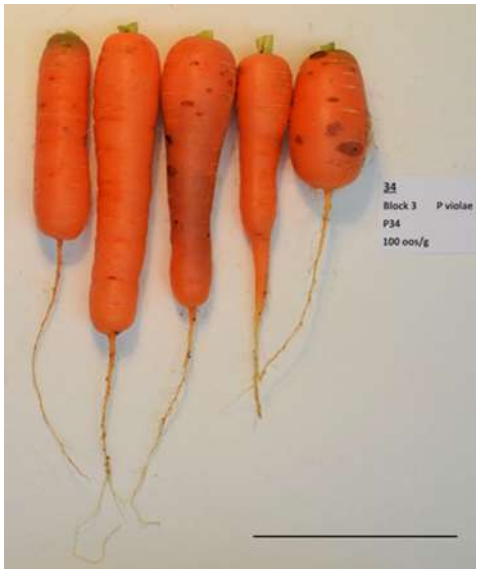

Figure 22: Cavity spot symptoms in one replicate pot following inoculation with *P. violae* P34 at 100 oospores g^{-1}. Bar = 10 cm.

Isolation of *P. violae* from roots

Tap root pieces from carrots inoculated with *P.violae* HL and P34 and *P. sulcatum* P67 resulted in *Pythium* colonies when plated onto CMA/Rif (Fig. 23). The percentage of root pieces showing *Pythium* infection ranged from 4.8 to 52.4% (Appendix 3). These results reflected those obtained for cavity spot incidence and severity, which show that the same three isolates caused typical cavity spot symptoms. Although there was no effect of varying oospore concentration, a χ^2 analysis revealed that infection between the uninoculated controls and *Pythium*-inoculated treatments across all root pieces tested was statistically different (χ^2, $P < 0.001$). The *Pythium* isolate contributing most to this result was *P. sulcatum* P67, where the percentage of root pieces with *Pythium* infection was 52.4 and 28.6% at concentrations of 10 and 100 oospores g^{-1}, respectively. After plating a small selection of cavities onto CMA/Rif, *P. violae* and *P. sulcatum* colonies developed, which also confirmed successful infection by these pathogens.

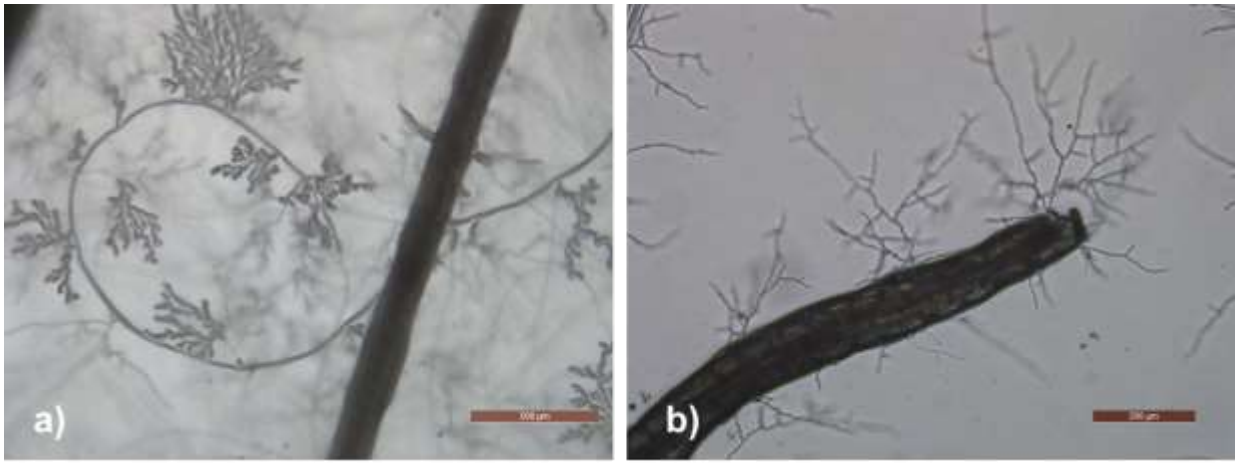

Figure 23: Diseased carrot tap root pieces plated on CMA/Rif showing growth of a) *P. violae* P34 with characteristic hyphal coiling and b) *P. sulcatum* P67 showing highly branched mycelium. Bars = 500 µm (a) and 200 µm (b).

2.4. Determine the efficacy of different *P. violae* and *P. sulcatum* isolates in inducing cavity spot symptoms in pot-grown carrots in the glasshouse using millet inoculum

This experiment investigated the efficacy of a mycelium-based millet seed inoculum in inducing cavity spot symptoms for different isolates of *P. violae* and *P. sulcatum*.

Effects on seed germination and carrot growth

The results of this final glasshouse trial using the mycelium-based millet seed inoculum had to be considered carefully due to the inclusion of the 'dead inoculum controls'. The highest concentration of 50 mg g^{-1} soil dead inoculum significantly affected carrot seed germination, foliage weight and carrot root weight, demonstrating that incorporation of the actual millet seed affected these plant growth variables. Therefore, when considering the results, responses to the addition of the live *Pythium* inocula should be compared to the relevant dead inoculum control to take account of the effect of the millet seed.

Incorporation of the dead inoculum at 5 mg g^{-1} had no effect on carrot seed germination relative to the uninoculated control, but at 50 mg g^{-1} germination was significantly reduced from 90% (control) to 46% (Fig. 24). Addition of the live *P. violae* HL and *P. sulcatum* P67 millet inocula at 5 mg g^{-1} had no effect on germination relative to the appropriate dead inoculum control at 5 mg g^{-1} as shown by comparison of the blue bars in Fig. 24. Whilst *P. violae* HL at 50 mg g^{-1} had no effect on germination compared to the dead inoculum control at

this concentration, *P. sulcatum* P67 at 50 mg g^{-1} completely inhibited germination, indicating that this isolate caused pre-emergence damping off. This was a therefore a contributory factor to the highly significant isolate x inoculum concentration interaction observed for carrot seed germination ($P < 0.001$; Appendix 2, Table c). Due to the complete pre-emergence damping off in the *P. sulcatum* P67-inoculated pots at 50 mg g^{-1}, this treatment is absent in subsequent figures for carrot foliage dry weight, root weight and cavity spot incidence and severity.

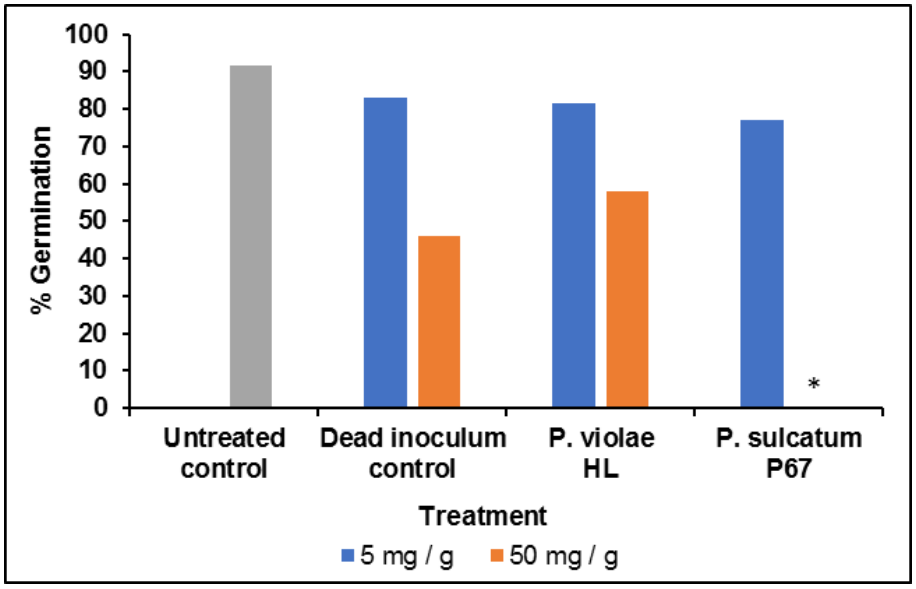

Figure 24: Effect of *P. violae* isolate HL and *P. sulcatum* isolate P67 inoculation with millet seed inoculum at two concentrations (5 mg g^{-1} and 50 mg g^{-1}) on mean percentage carrot germination (back transformed means). Asterisk indicates significant reduction compared to appropriate dead inoculum control.

Incorporation of the dead millet inoculum at 50 mg g^{-1} resulted in a significant increase in carrot foliage dry weight (3.96 g per carrot) compared to the uninoculated control (no millet, 2.39 g per carrot; Fig 25 a, Appendix 2, Table c). By comparison, the mean foliage dry weight from plants inoculated with *P. violae* HL at 50 mg g^{-1} was 2.88 g per carrot, which was significantly lower than the corresponding dead inoculum (50 mg g^{-1}) control at 3.96 g per carrot. This therefore indicated that the addition of the live pathogen reduced carrot foliage weight. However, both these values were significantly higher than the 2.39 g per carrot for the uninoculated control (no millet seed). The dead inoculum treatment at 5 mg g^{-1} resulted in a mean carrot foliage dry weight of 2.35 g per carrot, while the addition of the live inocula of *P. violae* HL and *P. sulcatum* P67 at this concentration reduced foliage weight to 1.87 and 1.85 g per carrot respectively (the latter being significant). An isolate x inoculum concentration interaction was only just non-significant ($P = 0.085$; Appendix 2, Table c).

Inoculation with live millet inoculum of *P. violae* HL at 5 and 50 mg g^{-1} and *P. sulcatum* P67 at 5 mg g^{-1} resulted in stunted and less tapered carrots (Fig. 26 d, e, f) compared to those from the uninoculated control (Fig. 26 a). The tap roots of *Pythium*-inoculated carrots also exhibited clear root browning and an increase in lateral root formation. The dead inoculum at 5 mg g^{-1} had little effect on carrot shape (Fig. 26 b), whilst at 50 mg g^{-1} a high proportion of the resulting carrots were misshapen and abnormally large (Fig. 26 c) compared to the untreated controls.

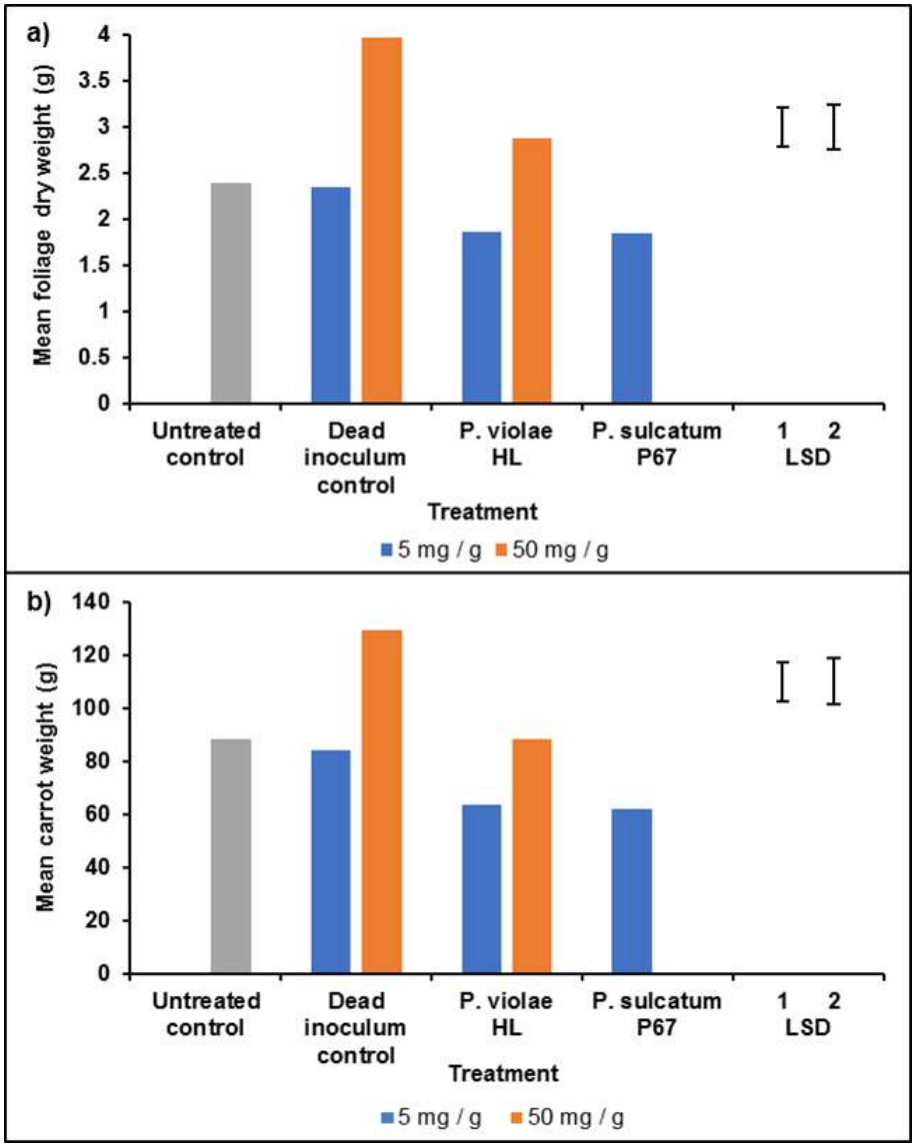

Figure 25: Effect of *P. violae* isolate HL and *P. sulcatum* isolate P67 inoculation with millet seed inoculum at two concentrations (5 mg g^{-1} and 50 mg g^{-1}) on a) mean foliage dry weight per carrot and b) root weight in pot-grown carrots. Bars represent the least significant difference (LSD) between treatments at the 5% level. LSD 1 allows comparison between the untreated control (16 replicates) and inoculated treatments (8 replicates) and LSD 2 allows comparison between inoculated treatments only (8 replicates).

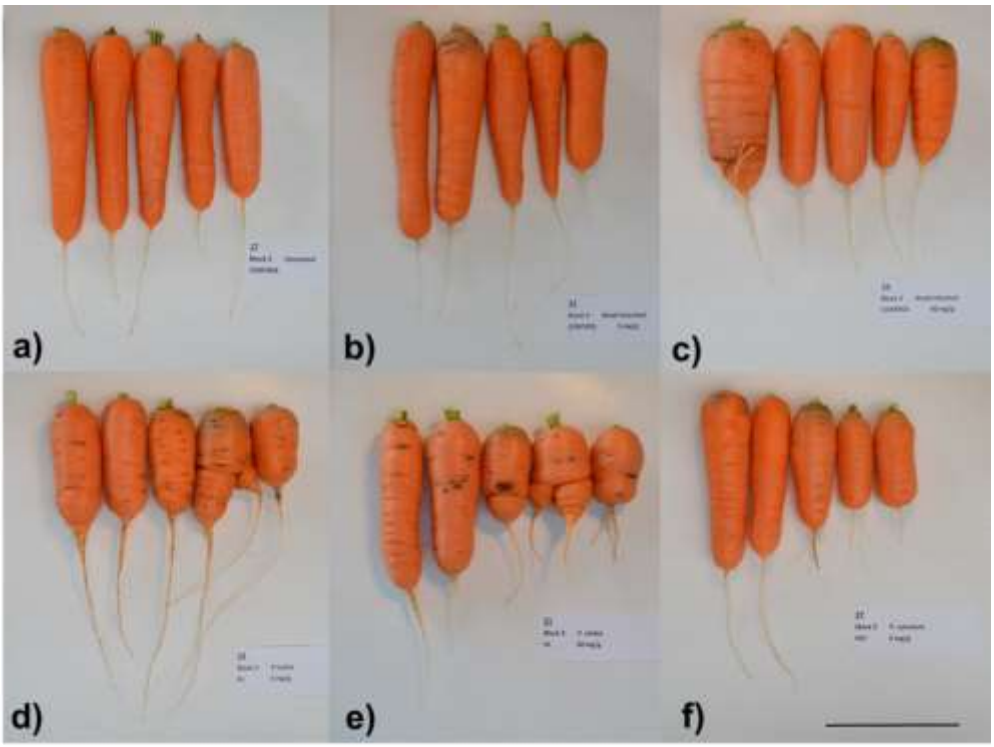

Figure 26: Cavity spot symptoms and stunting observed in carrots inoculated with millet inocula of *P. violae* HL at 5 mg g^{-1} (d) and 50 mg g^{-1} (e) and *P. sulcatum* P67 at 5 mg g^{-1} (f) compared with uninoculated control carrots showing normal morphology (a). Carrots inoculated with dead inoculum at 5 mg g^{-1} (b) showing no difference in morphology compared to the uninoculated control (a) and at 50 mg g^{-1} (c) showing increased growth. Bar = 10 cm.

Treatments induced similar responses in terms of mean carrot root weight as to those observed for foliage dry weight. Carrots from the dead inoculum treatment at 50 mg g^{-1} had a mean root weight of 129.1 g, which was significantly greater than that of the untreated control plants at 88.5 g (Fig. 25 b; Appendix 2, Table c). Carrots inoculated with *P. violae* at 50 mg g^{-1} had a mean carrot root weight of 88.2 g which was very similar to that of the untreated control, but significantly lower than 129.1 g which was measured for dead inoculum at 50 mg g^{-1}. The mean root weight in the dead inoculum control at 5 mg g^{-1} was 84.4 g, while *P. violae* HL and *P. sulcatum* P67 at the same concentration significantly reduced mean root weight to 63.4 and 62.0 g, respectively. Although the results show an overall significant difference between treatments ($P > 0.001$, data not shown), there was no isolate x inoculum concentration interaction ($P = 0.101$; Appendix 2, Table c).

Effects on cavity spot incidence and severity

Typical cavity spot lesions were observed in carrots receiving millet inoculum of *P. violae* HL and *P. sulcatum* P67 (Fig. 27) and these generally appeared wider and deeper than those seen in the other glasshouse experiments reported above. High cavity spot incidence of 49.5 and 82% carrots affected was observed for the *P. violae* HL treatments at 5 and 50 mg g^{-1} respectively, compared with 17.4% in the *P. sulcatum* P67 treatment at 5 mg g^{-1} (Fig 28 a). No incidence of cavity spot was recorded in control carrots or carrots treated with dead inoculum. Overall, these results show statistically significant differences in terms of cavity spot incidence between *P. violae* HL and *P. sulcatum* P67 ($P < 0.001$) and different inoculum concentrations ($P < 0.001$). An isolate x inoculum concentration interaction was also evident ($P = 0.021$; Appendix 2, Table c) indicating that disease incidence varied depending on isolate and inoculum concentration. However some caution must be exercised in interpreting results from the interaction data, due to the fact that values for the *P. sulcatum* P67 treatment at 50 mg g^{-1} are missing.

As for cavity spot incidence, severity of cavity spot was higher than for the cavity spot glasshouse trials reported above and in FV 391a. Mean severity values were 0.80 and 1.85 cavities per carrot (across all carrots) for *P. violae* HL treatments at 5 and 50 mg g^{-1}, respectively, and 0.22 cavities per carrot for the *P. sulcatum* P67 treatment at 5 mg g^{-1} (Fig. 28 b). Statistical analyses revealed significant differences in cavity spot severity between isolates ($P <0.001$) and inoculum concentrations ($P < 0.017$) and an isolate x inoculum concentration interaction was also evident ($P = 0.042$; Appendix 2; Table c). Cavity spot severity across only infected carrots, were 2.10 and 2.75 cavities per carrot in *P. violae* HL treatments at 5 and 50 mg g^{-1}, respectively, and 1.33 cavities per carrot in the *P. sulcatum* P67 treatment at 5 mg g^{-1} (Appendix 3; statistical analysis not possible due to the large amount of missing values i.e. unaffected carrots).

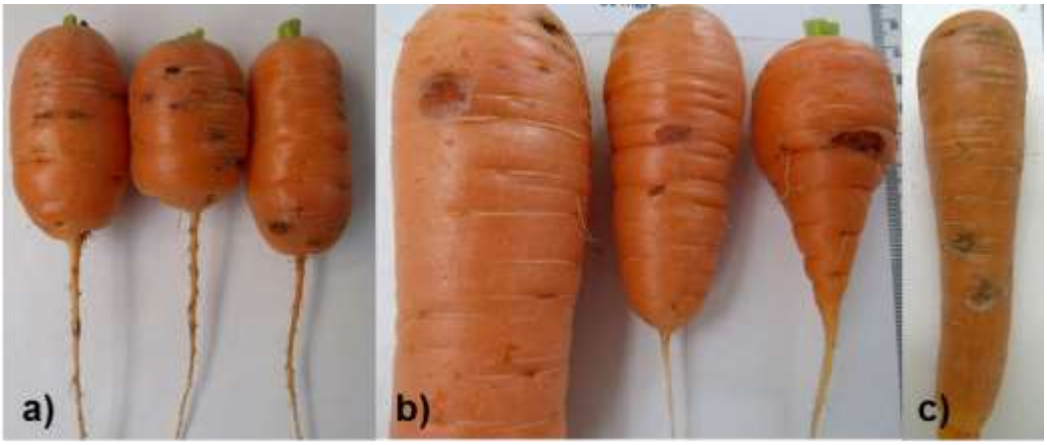

Figure 27: Typical cavity spot lesions and root browning in pot-grown carrots inoculated with a, b) *P. violae* HL and c) *P. sulcatum* P67 (c) millet seed inocula.

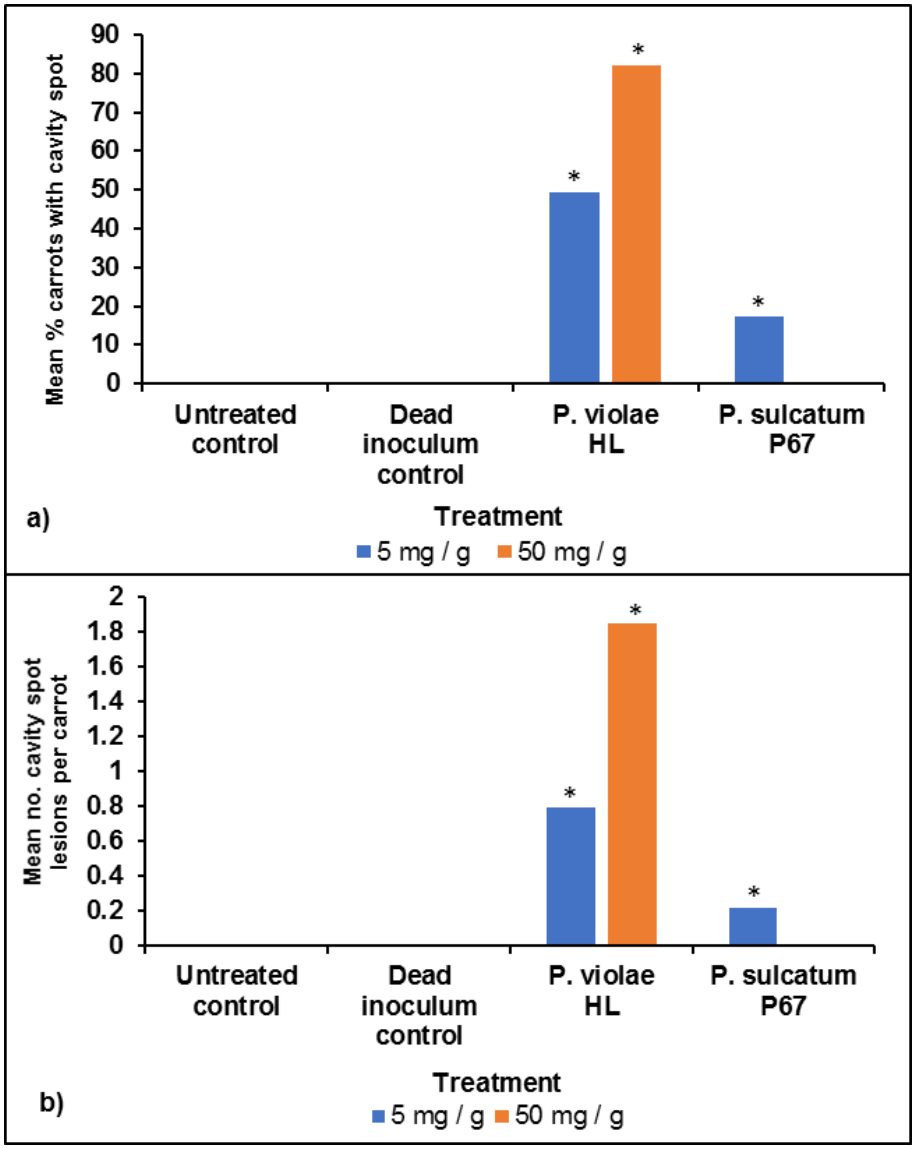

Figure 28: Effect of *P. violae* isolate HL and *P. sulcatum* isolate P67 inoculation with millet seed inoculum at two concentrations (5 mg g^{-1} and 50 mg g^{-1}) on a) cavity spot incidence (% carrots affected) and b) severity (no. lesions per carrot across all carrots). Data presented are back transformed means and asterisks indicate significant increase compared to appropriate control.

Isolation of *P. violae* from roots

When plated onto CMA/Rif, *Pythium* colonies grew from tap root pieces from carrots inoculated with both *P. violae* HL and *P. sulcatum* P67 (Appendix 3), but the results did not follow the same pattern as for cavity spot incidence and severity. The percentage of root pieces infected with *P. sulcatum* P67 was 37.5%, which was higher than for *P. violae* HL treatments which were 20.8 and 4.2% at 5 and 50 mg g^{-1}, respectively. A χ^2 analysis revealed

that infection between the uninoculated controls and *P. violae*-inoculated treatments across all root pieces tested was statistically different (χ^2, $P < 0.001$). *P. violae* and *P. sulcatum* were also successfully isolated from the root tissue of several cavity spot lesions.

Objective 3: Test the efficacy of different rates of solid *P. violae* inoculum in producing cavity spot symptoms in field grown carrots

In this experiment, field macrocosms were inoculated with different concentrations of a sand/oat inoculum of *P. violae* isolate HL.

Effects on seed germination and carrot growth

Good carrot growth was observed in both 2017 (Year 1) and 2018 (Year 2) with no obvious effects of *P. violae* inoculation on seed germination or seedling survival. The mean total carrot yield was similar across the two years ranging from 7.8-8.5 kg and 7.3-8.3 kg in 2017 and 2018, respectively across all treatments (Fig 29 a; Appendix 4). *P. violae* inoculation had no effect on mean total carrot weight in either year, as no significant differences were found between any of the treatments (2017 and 2018; $P = 0.735$ and $P = 0.381$). Mean carrot weight ranged from 62.0-84.6 g and 30.5-39.8 g in 2017 and 2018, respectively (Fig 29 b; Appendix 4). The comparatively low mean carrot weight across all treatments in 2018 can be attributed to the higher mean number of carrots in each treatment in this year compared to 2017 (Fig 29 c; Appendix 4). *P. violae* inoculation however had no significant effect on mean carrot weight (2017 and 2018; $P = 0.770$ and $P = 0.316$) or total carrot number (2017 and 2018; $P = 0.542$ and $P = 0.230$).

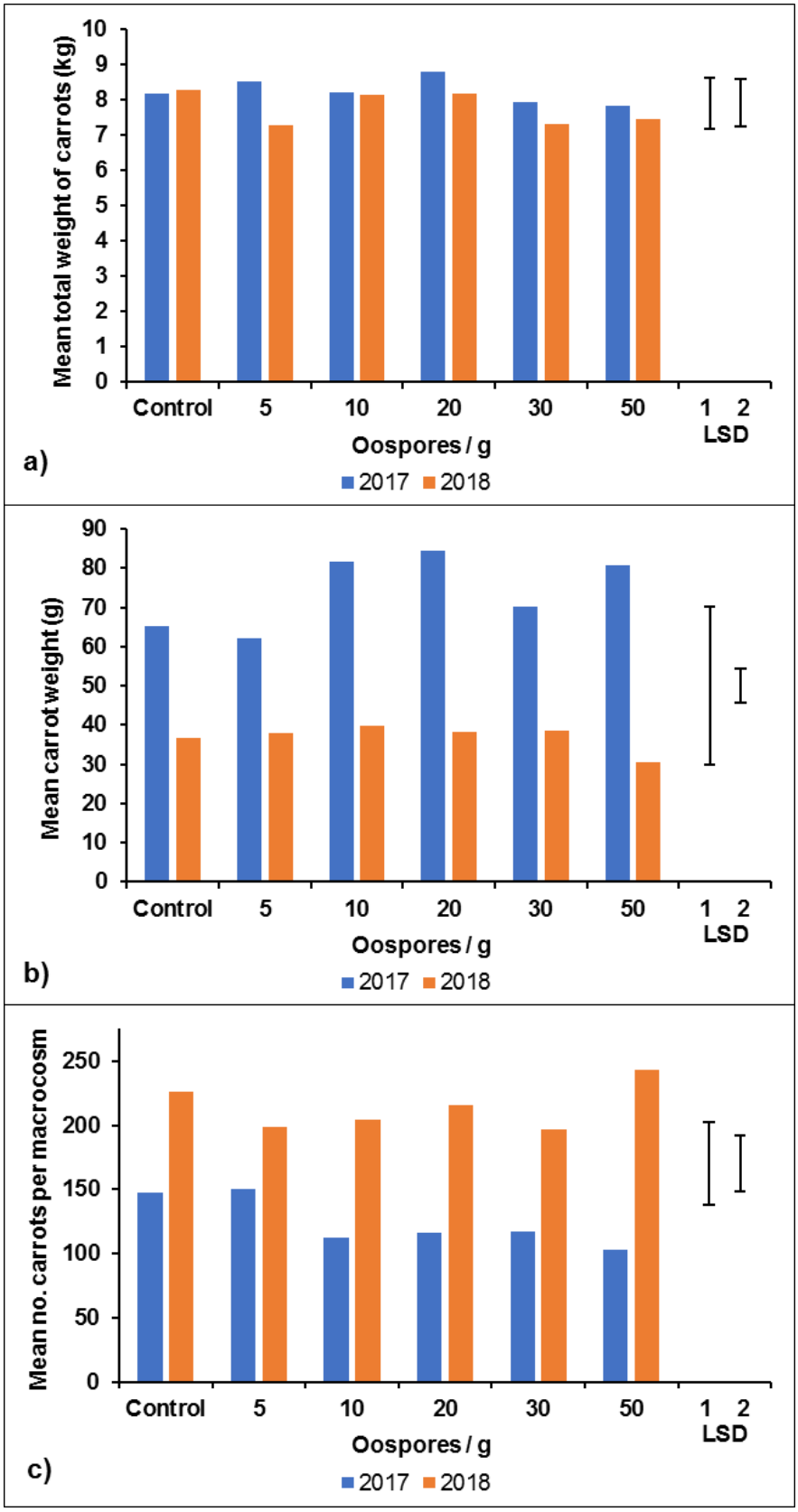

Figure 29: Effect of *P. violae* HL inoculation of field macrocosms with sand/oat inoculum at different concentrations on a) mean total carrot root weight, b) mean root weight and c) mean number of carrots harvested per field macrocosm in 2017 and 2018. Error bars represent the LSD at the 5% level.

Effects on cavity spot incidence and severity

In both years, typical cavity spot lesions were observed on carrot roots (Fig. 30 and 31). Across all the macrocosms inoculated with *P. violae* HL, cavity spot incidence ranged between 23.7-38.7% roots affected in 2017 and 30.4-35.7% roots affected in 2018 (Fig. 32 a). The background level of cavity spot incidence in control macrocosms was 1.4% in 2017, which increased slightly to 5.6% in 2018. A significant difference in incidence was found across all treatments in both years ($P < 0.001$ for both 2017 and 2018; Appendix 4) with carrots from all inoculated treatments showing significantly greater incidence than the uninoculated control plots in both 2017 and 2018 (Fig. 32 a), with strong evidence of a linear dose response effect particularly in 2017 ($P < 0.001$, statistical data not shown).

Cavity spot severity across all roots from *P. violae* inoculated macrocosms ranged between 0.46-0.72 lesions per root in 2017, and 0.69-0.86 lesions per root in 2018 (Fig. 32 b). A significant difference in severity was found across all treatments in both years ($P < 0.001$ for both 2017 and 2018; Appendix 4) with carrots from all inoculated plots having significantly higher cavity spot severity in both years compared with the uninoculated controls (Fig. 32 b). However, no significant differences in terms of severity were evident between macrocosms inoculated with different oospore concentrations.

Cavity spot severity across infected roots only was 1.9-2.4 lesions per carrot in 2017, and 2.2-2.6 lesions per carrot in 2018 for the *P. violae* inoculated plots (Fig. 32 c). In 2017, cavity spot severity across treatments was found to be significantly different ($P = 0.008$; Appendix 4), due to the fact that all inoculated treatments showed significantly greater cavity spot severity compared to the uninoculated control. However, in 2018, disease severity across infected roots did not vary significantly across the treatments ($P = 0.109$; Appendix 4), which can be attributed to the fact that roots from uninoculated control macrocosms were affected by low levels of cavity spot in the second year.

Figure 30: Carrots harvested in 2017 from field macrocosms. (a) uninoculated control; b, c, d) carrots with typical cavity spot lesions from macrocosms inoculated with *P. violae* HL at concentrations of 20, 30 and 50 oospores g^{-1} respectively.

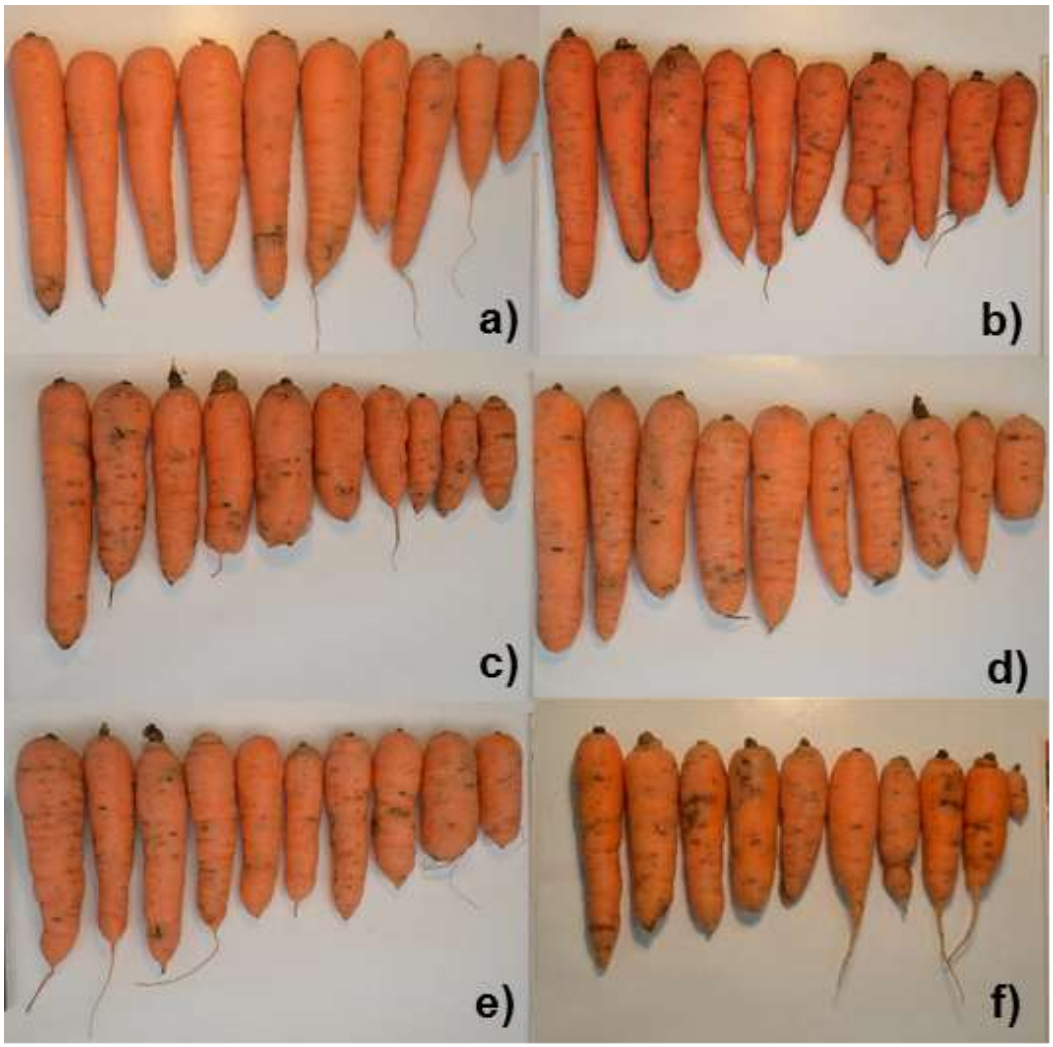

Figure 31: Representative selection of carrots harvested in 2018 from field macrocosms; a) uninoculated control and b - f) carrots with typical cavity spot lesions from macrocosms inoculated with *P. violae* at concentrations of 5, 10, 20, 30 and 50 oospores g^{-1} respectively.

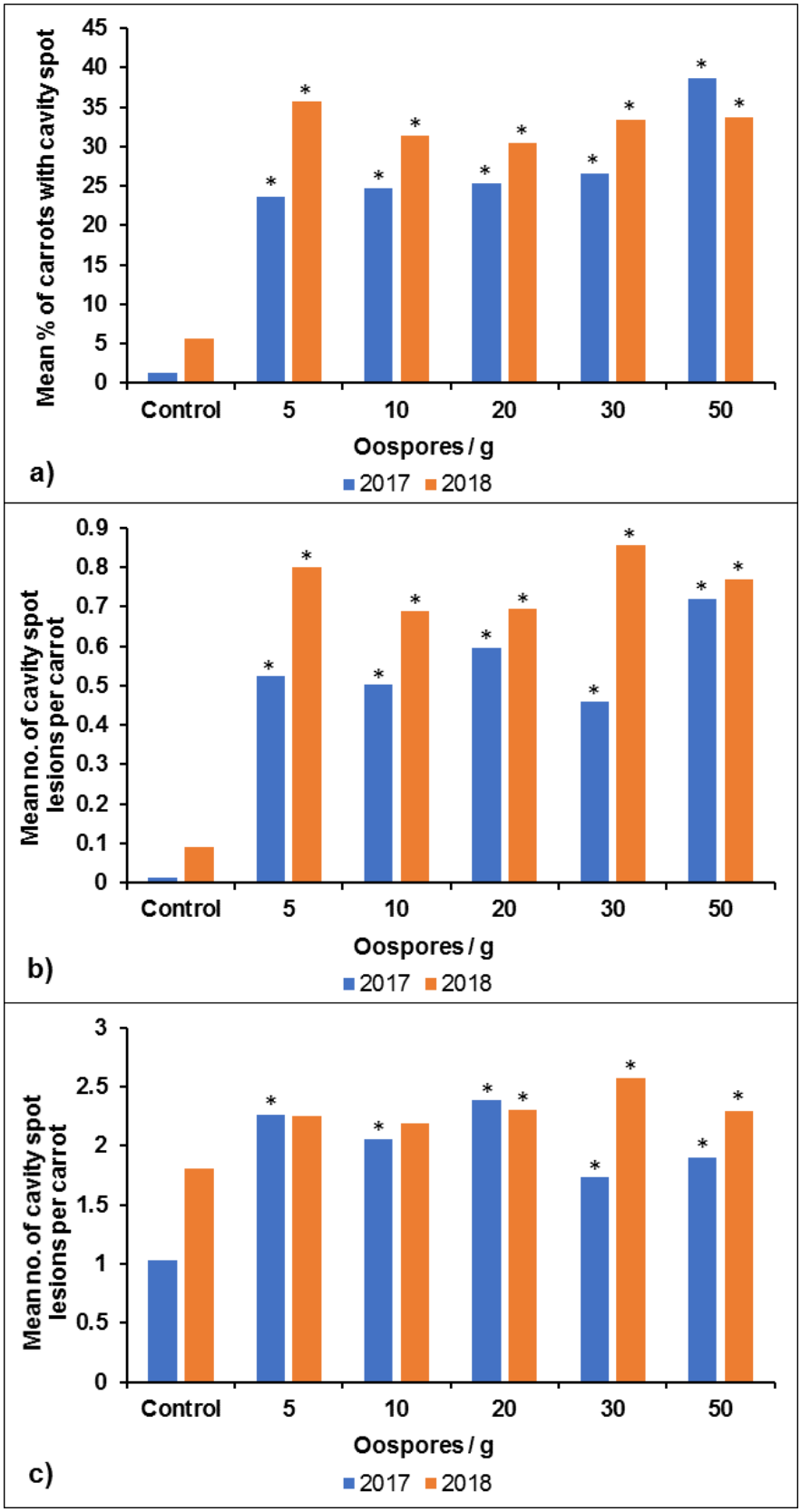

Figure 32: Effect of *P. violae* HL inoculation of field macrocosms with sand/oat inoculum at different concentrations **a) on** cavity spot incidence, b) severity (mean no. of lesions per carrot across all carrots) and c) severity (mean no. of lesions per carrot for infected carrots only). Asterisks indicate significant increase relative to appropriate untreated control.

Sequencing of isolates obtained from atypical and typical lesions

In 2018, a few carrot tissue samples from typical cavity spot lesions (Fig. 33 a) and from 'atypical' superficial lesions (Fig. 33 b) from the uninoculated control macrocosms were plated out onto CMA/Rif to check for presence of *P. violae* and identify other fungal / oomycete species. DNA was extracted from cultures of each isolate and the ITS regions of the rDNA sequenced after amplification by PCR (Hales & Clarkson, 2016). Of the six isolates from atypical lesions, four were identified as *Ilyoncectria* spp., one was identified as *P. sulcatum* and one as a Zygomycete. Of the four typical cavity spot lesions, *P. intermedium, P. violae, P. sulcatum* and *Ilyonectria* sp. were identified.

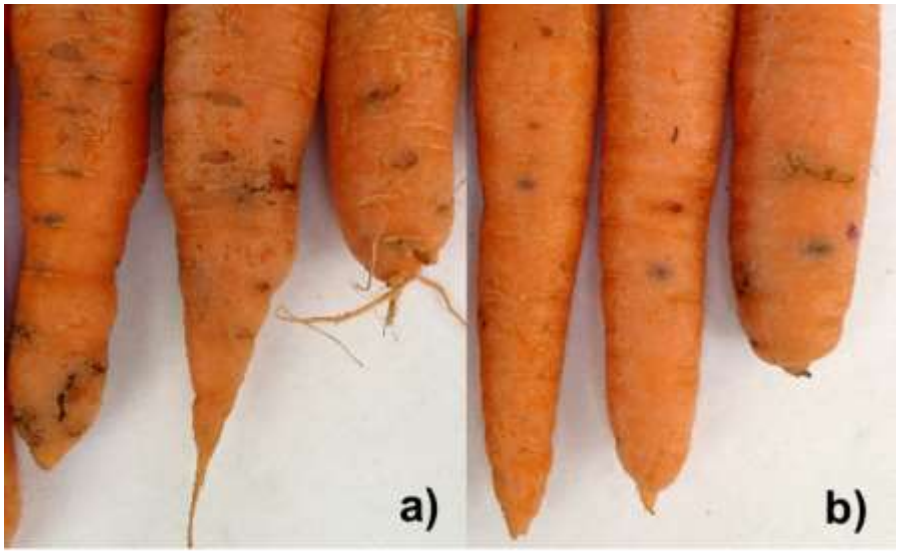

Figure 33: Carrots harvested from an uninoculated control field macrocosm in 2017 showing a) typical elliptical cavity spot lesions and b) 'atypical' very dark superficial lesions.

Objective 4. Examine the feasibility of large-scale *P. violae* inoculum production in solid state fermentation

The bioreactor for large scale production of *P. violae* sand/oat inoculum has been modified and set up ready for inoculation which will now be carried out mid-October 2018. Oospore counts will be carried out 4-6 weeks later.

Discussion

Testing of methods to assess *Pythium* oospore viability

Methods were investigated to assess *P. violae* oospore viability as a way of checking the quality of inoculum as this was hypothesised to be a potential source of the variation in cavity spot disease levels and impact on carrot growth observed in experiments in FV 391. Two methods were examined for oospores from several *P. violae* and *P. sulcatum* isolates; a MTT vital stain and a plasmolysis method.

MTT staining has been widely used for assessing viability of oomycete oospores (Sutherland & Cohen, 1983, El-Hamalawi and Erwin, 1986 and Widmer, 2010) with spores staining purple or rose-pink considered to be viable. High proportions of live oospores from both *P. violae* and *P. sulcatum* stained purple in this study in contrast to dead oospores which were stained black or colourless. This therefore suggests that MTT staining is appropriate for testing viability of *Pythium* oospores. There is however some inconsistency in the literature with regards to interpretation of staining results. Blue-stained oospores have previously been designated as activated or ready to germinate (Sutherland & Cohen, 1983), but Boutet *et al*. (2010) found that blue-stained oospores of *Phytophthora ramorum* did not have normal morphological features and hence were considered non-viable. In this study, *P. sulcatum* oospores staining blue appeared abnormal and therefore were also designated as non-viable. Although live spores of *P. violae* and *P. sulcatum* stained purple in this study, there was some difficulty distinguishing between very dark purple (viable) and black (non-viable) oospores. This could account for the large discrepancy in viability between the MTT and plasmolysis tests for the *P. sulcatum* isolates, particularly P127 where 57.7 ± 0.8% were deemed viable using MTT compared to 3.9 ± 0.5% using plasmolysis. A tendency for overestimating oospore viability using MTT has been reported for *Phytophthora* spp. (Etxeberria *et al.*, 2011) and Flier *et al.* (2001), which is therefore in agreement with the findings in this study. Therefore viability assessment results obtained with MTT staining must be viewed with some caution as the interpretation of the different colours is subjective and the staining reaction can vary depending on the permeability of the oospore. As some authors have suggested (Etxeberria *et al.* 2011; Dyer and Windels, 2003) MTT can be used in combination with other methods such as plasmolysis and microscopic observation of the cytoplasm to gain a more accurate measure of oospore viability.

The plasmolysis method to measure viability relies on observation that when live (viable) oospores are incubated in a saline solution, the cytoplasm contracts into a ball-like structure due to the resulting loss of water. In contrast, dead (non-viable) spores do not display plasmolysis due to the lack of functional cell membranes (Jiang & Erwin, 1990). The

plasmolysis method has been used to determine oospore viability in other oomycetes such a *Pseudoperonospora humuli* (Gent *et al*., 2017) and *Peronospora effusa* (Kunjeti *et al*., 2016). The method has advantages over MTT staining as it does not involve a harmful staining reagent and the reaction time is much quicker (45 min as opposed to a 24-h staining reaction). In this study, plasmolysis was very clearly visible in live, viable *P. violae* oospores and absent in the dead autoclaved non-viable oospores. This was also the case for *P. sulcatum*, but care was required to due to the smaller size of the oospores (van der Plaats-Niterink AJ, 1981).

Separation of *Pythium* oospores from mycelium to assess germination

An effective method for producing *Pythium* oospore preparations free of mycelial fragments was developed as any live, residual mycelium hampers microscopic assessment of germination as it quickly overgrows oospores. Initial oospore extraction methods were developed in FV 391 for sand /oat inoculum and liquid V8 cultures which both employed a sonication step to dislodge the spores from the mycelium and help kill mycelial fragments, with extraction from V8B also involving a long homogenisation step. However, *P. violae* HL oospores extracted using these methods showed very poor or no germination on CMA or V8A, which was attributed to innate dormancy, or damage from the extraction methods. Furthermore, some mycelial fragments survived these processes and therefore hindered the germination assessments.

In the present study, it was found that Glucanex, a mixture of lysing enzymes from *Trichoderma harzianum*, effectively digested and deactivated mycelial fragments in *P. violae* and *P. sulcatum* oospore preparations whilst the walls of oospores were left intact. The survival of oospores during this process was confirmed as there was little or no loss of viability as assessed by the plasmolysis assay. Glucanex has been used previously to produce ‘clean’ oospore preparations from *Phytophthora ramorum* (Boutet *et al.*, 2010) and *Phytophthora capsici* (Hurtado-Gonzales, 2008).

Factors affecting oospore germination

On addition to lysing enzymes being effective for producing mycelium-free oomycete oospore preparations, Flier *et al.* (2001) suggested that they may also break oospore dormancy and enable germination. Following treatment with NovoZym 234 (mixture of lysing enzymes; Novo Biolabs), an increase in activated (blue stained) oospores of *Phytophthora infestans* compared to dormant (purple stained) oospores was observed, although germination was still low in the isolates tested and ranged between 0 and 11%. The chemical and/or physical factors which specifically induce *P. violae* oospore germination are not known and significant germination has never been observed.

Treatment of oospores with potassium permanganate ($KMnO_4$) has been shown to enhance oospore germination in some *Phytophthora* and *Pythium* spp. (El-Hamalawi & Erwin, 1986, Guo & Ko, 1994, Ruben *et al*., 1980). Guo & Ko (1994) reported increased germination of *Pythium splendens* oospores when $KMnO_4$ treatment was combined with incubation on V8 agar or media consisting of basal salts, lecithin and glucose. The phosphatidyl choline, esterified fatty acids and sterols found in natural sources of lecithin are thought to be stimulatory as oospores may require an external source of phospholipids for germ tube synthesis. In addition, phosphatidyl choline may act as an emulsifier and aid absorption of other compounds through the oospore wall (Ruben *et al*., 1980). Desiccation and heat shock have also been identified as factors which promote oospore germination (El-Hamalawi and Erwin, 1986 & Ruben *et al*., 1980). Such treatments, including pre-incubation with $KMnO_4$, are thought to induce conformational changes in cell wall structure, lipids and/or proteins, although the exact mechanisms responsible for the germination-enhancing effects are unknown.

To provisionally examine the potential of different treatments to break dormancy of *Pythium* oospores in this project, the effect of the Glucanex treatment on germination of both *P. violae* and *P. sulcatum* oospores was evaluated while a combination treatment of Glucanex followed by $KMnO_4$ was tested for its effect on oospore germination of *P. violae*. Despite retaining viability, Glucanex-treated oospores of *P. violae* HL and with or without $KMnO_4$ (0.1% w/v, 15 min) failed to germinate on either CMA/Rif or V8A/Rif. In contrast, Glucanex-treated oospores of *P. sulcatum* P67 germinated within 2 to 3 days on CMA/Rif at 20°C without any additional treatment. The percentage spore germination recorded for *P. sulcatum* P67 oospores was 43.5% which was consistent with a percentage viability of 44.8 ± 4.8% as measured using the plasmolysis assay. This suggests that all viable *P. sulcatum* P67 oospores germinated and a lack of any dormancy mechanism or need for any environmental conditioning.

It is well documented that the oospores of many oomycetes do not exhibit high levels of germination (Ayers and Lumsden, 1975) and that some are unresponsive to $KMnO_4$ (Gent *et al*. 2017; *Pseudoperonospora humuli*). It must be noted, however that only one concentration of $KMnO_4$ and one incubation period was tested in this study, and other factors such as desiccation, heat shock, exposure to soil and plant exudates may be important for oospore germination. The outcomes of this series of experiments also highlighted that the requirements for oospore germination potentially vary considerably between *P. violae* and *P. sulcatum* and this may also be the case for different isolates within a species. Hence, much more extensive research is required to investigate potential triggers of oospore germination in multiple isolates of *P. violae* and *P. sulcatum*.

Development of inoculation methods to produce cavity spot symptoms in pot grown carrots in the glasshouse

Experiments were carried out to continue the initial work in FV 391a to develop an artificial inoculation system for *P. violae* for pot-grown carrots in the glasshouse. An initial experiment using a liquid oospore inoculum resulted in no cavity spot symptoms and was also difficult to apply consistently. We therefore focussed on further developing the use of a solid sand/oat *P. violae* inoculum which had shown some promise in FV 391a. Initially, an experiment was set up to test the effect of metalaxyl seed treatment, firstly to investigate whether it would control cavity spot and secondly to determine if it could delay early infection of *P. violae* yet allow symptoms to develop later on in mature carrot roots. A follow up experiment using the same type of inoculum then evaluated the ability of different isolates of both *P. violae* and *P. sulcatum* to induce cavity spot symptoms to examine if there were more virulent isolates that could induce higher levels of cavity spot than observed in previous tests.

Overall, the major finding from these first two glasshouse experiments was that artificial inoculation of carrots with the sand/oat *P. violae* oospore inoculum resulted in a reduction in seed germination and foliage development as well as the formation of short stubby carrots with a reduced root weight. Typical cavity spot lesions were also formed while tap roots were misshapen and brown. The pathogen was consistently isolated both from lesions and taproots, confirming that infection was successful. These observations are consistent with the results from FV 391a where the same range of symptoms was also evident and similar effects of *P. violae* on seed germination and carrot growth have been reported previously (e.g. Pettit, 2002; Pratt and Mitchell, 1972; White, 1986; see FV 391 a report for details).

In addition, results from this project and FV 391a also indicated that increasing the concentration of *P. violae* oospores did not result in a corresponding increase in subsequent disease metrics across the range of 10-100 oospores g^{-1} soil. Significantly, the extent of disease indicators between experiments both in this project and FV 391a was variable despite careful quantification of *P. violae* inoculum. For instance, reduction in carrot weight for *P. violae* inoculated treatments was significant in both the experiments in FV 391a but not in the first two experiments in this project. Cavity spot incidence for *P. violae* was 0.75-23% and 5-20% in this project and FV 391a respectively, with corresponding disease severity of 1-3 and 1-2 lesions per carrot for those roots affected. Overall therefore, while inoculation of carrots with the *P. violae* sand/oat inoculum always results in some cavity spot disease, the level at which this occurs is unpredictable. In this project, similar growth effects on carrot and cavity spot symptoms were observed following inoculation with one isolate of *P. sulcatum* while another two failed to cause any infection.

The reasons for this variability in cavity spot disease are unclear and may be related to oospore viability, the ability of oospores to germinate or environmental factors (e.g. water availability) in the glasshouse. Viability and germinability of oospores used in these experiments was not directly assessed as the methods described above to achieve this were only developed in the latter stages of the project. However, viability of *P. violae* and *P. sulcatum* oospores is unlikely to have been a significant factor given subsequent results of the plasmolysis assay. Cavity spot disease development was particularly low in the experiment testing different isolates of *P. violae* and *P. sulcatum* although there were apparent differences in virulence as one of the two *P. violae* isolates and two of the three *P. sulcatum* isolates resulted in no disease symptoms at all. The low disease level associated with *P. sulcatum* P67 was somewhat surprising given that oospores of this isolate germinate readily *in vitro* in contrast to *P. violae* isolate HL. Previous studies have also reported variation both between species and within-species variation in virulence for *P. violae* and *P. sulcatum* (e.g. Suffert and Guibert, 2007).

Finally, results from the experiment testing metalaxyl treated seed suggested this had little overall benefit in reducing cavity spot which is in contrast to the findings of Petch *et al*., (1991) where film-coating with the fungicide reduced cavity spot incidence by up to approx. 50%. However, there was some protective effect of metalaxyl at 1 oospore g^{-1} only, which may suggest some benefit at low disease pressure. There was also some evidence that in the absence of the pathogen, metalaxyl reduced carrot seed germination which has also been noted previously (Petch *et al*.,1991).

Given the variability in cavity spot disease development and plant growth effects in experiments using the sand/oat oospore inoculum, a new approach was tested in a third glasshouse experiment where a millet seed mycelium-based inoculum of *P. violae* and *P. sulcatum* was tested at two different concentrations. Although there was a significant carrot growth promoting effect of the millet itself at the high concentration of 50 mg g^{-1} soil, which was not evident at the low concentration of 5 mg g^{-1} soil, both levels of inoculum resulted in very high incidence of cavity spot lesions for *P. violae* isolate HL with 49.5 and 82% carrots affected for low and high inoculum levels respectively. Cavity spot incidence was lower for *P. sulcatum* isolate P67 at the lower inoculum concentration (17 %) while no carrots survived to maturity at the higher inoculum level due to extensive damping off. Overall therefore, the millet inoculum resulted in much higher cavity spot disease incidence and severity than observed in any of the experiments using the sand/oat inoculum either in this study or the previous project FV 391a. This suggests that the millet-based *Pythium* inoculum may be a better approach for inoculation of carrots in the glasshouse although further testing is required to confirm this. The likely reason for this is that the millet inoculum largely comprises active *Pythium* mycelium

(with a potential additional food source for the pathogen) and in contrast to the sand/oat inoculum does not rely on the ability of oospores to germinate. The millet inoculum also has the advantage that it is much quicker to produce (3-4 weeks as opposed to 3-4 months) and can be easily incorporated into growing medium. Although successful, the severity of cavity spot obtained with the millet inoculum with *P. violae* and *P. sulcatum* was still lower than in two similar studies which used the same inoculum. In the first, millet inoculum of an isolate of *P. sulcatum* resulted in a mean of five cavities per carrot compared to 24 cavities per carrot for *P. coloratum* (another species causing cavity spot; El-Tarabily et al., 1996) while in the second, *P. sulcatum* inoculation resulted in 35 cavities per root compared to seven cavities per root for *P. ultimum* (El-Tarabily et al., 2004). However these studies were carried out using steam sterilised soil which would have enhanced infection levels but they also further confirm that the ability to cause cavity spot symptoms can vary between different *Pythium* species.

Development of an inoculation method to produce cavity spot symptoms in field grown carrots

Experiments were set up over two years to test the effect of different concentrations of *P. violae* isolate HL sand/oat oospore inoculum on development of cavity spot disease in field macrocosms. Overall, this approach was extremely successful and in contrast to the pot-based experiments resulted in high cavity spot incidence ranging from 30.4-35.7% roots affected in the second year (2018) with similar levels in year 1. Again however, mean disease severity was low with a mean of 2-3 lesions per carrot. Nonetheless this is the first report of artificial inoculation with *P. violae* resulting in significant cavity spot disease under field conditions. As observed in the pot tests, overall there was little effect of oospore inoculum concentration except in year 1, but significantly, unlike in the pot experiments, there was no effect of *P. violae* inoculation on carrot growth and lesions were perhaps more typical of those observed in commercial field crops. These results may suggest that *P. violae* oospores germinate more readily in field soil than in the growing medium employed in the pot tests, perhaps due to exposure to certain soil factors or environmental conditions that are more conducive to activation of oospores and infection of carrot roots.

Conclusions

- High numbers of *P. violae* and *P. sulcatum* oospores can be produced in a liquid medium and solid substrate.
- A plasmolysis assay was developed to determine viability of *P. violae* and *P. sulcatum* oospores.

- Treatment with a Glucanex enzyme mix or potassium permanganate did not promote germination of *P. violae* oospores.
- Oospores of a *P. sulcatum* isolates germinated readily on agar in contrast to an isolate of *P. violae*
- Artificial inoculation of a compost / sand growing medium with a *P. violae* solid substrate sand/oat oospore inoculum resulted in successful infection of pot grown carrots. Symptoms included some seedling death, reduced seedling size, a decrease in growth of foliage and the formation of small, stubby and stunted carrots with brown tap roots and typical cavity spot lesions. *P. violae* was consistently isolated from tap roots and cavities.
- Metalaxyl seed treatment had little effect or no effect on cavity spot levels or on reducing effects on carrot plant growth in pot tests following artificial inoculation with a *P. violae* sand/oat oospore inoculum.
- Different isolates of *P. violae* and *P. sulcatum* varied in their ability to cause cavity spot symptoms or reduce carrot plant growth in pot tests following inoculation with sand/oat oospore inoculum.
- A solid substrate millet seed mycelial inoculum of *P. violae* resulted in high incidence of cavity spot level in pot tests.
- Artificial inoculation of soil contained in field macrocosms with the *P. violae* solid substrate sand/oat oospore inoculum resulted in a very high incidence of cavity spot.

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Knowledge and Technology Transfer

Appendix 1: Soil analysis results (NRM Laboratories, Cawood Scientific Ltd, UK)

Table a: Measurements of soil pH and P, K and Mg in sieved John Innes 3/sand (JI3/sand) growing medium used for glasshouse (GH) experiments 2.2 and 2.3, and in field macrocosm soil taken from surface and cored (20 cm depth) samples

		Index			mg L^{-1} (available)		
Sample	**SOIL pH**	**P**	**K**	**Mg**	**P**	**K**	**Mg**
GH Experiment 2 (JI3/Sand)	7.7	1	1	2	11.4	94	95
GH Experiment 3 (JI3/Sand)	7.2	2	2-	3	18	128	121
Field surface macrocosms	7.2	4	2+	3	64.6	213	118
Field core macrocosms	7.1	4	2+	3	54.2	202	144

Table b: Measurements of micronutrients, electrical conductivity and cation exchange capacity in sieved JI3/sand growing medium used for GH experiments 2.2 and 2.3, and in field macrocosm soil taken from surface and cored (20 cm depth) samples

Measurement	**GH Experiment 2 (JI3/Sand)**	**GH Experiment 3 (JI3/Sand)**	**Field surface macrocosms**	**Field core macrocosms**
Copper (EDTA Extractable) mg L^{-1}	1.2	1.3	3.2	3.5
Boron (Hot Water Soluble) mg L^{-1}	0.3	0.3	0.5	0.4
Sodium (Ammonium Nitrate Extractable) mg L^{-1}	56.5	24	103.5	83.5
Zinc (EDTA Extractable) mg L^{-1}	0.7	0.6	2.4	2.2
Calcium (Ammonium Nitrate Extractable) mg L^{-1}	1180	1315	1100	990
Iron (DPTA Extractable) mg L^{-1}	12.5	7.1	46	58.6
Organic matter (LOI) %	2.1	2.3	1.8	2
Sulphate (Phosphate Buffer Extractable) mg L^{-1}	143	254.9	20.5	15.6
Manganese (DPTA Extractable) mg L^{-1}	2.6	2.5	4.1	16.1
Electrical Conductivity (Sat. $CaSO_4$) µS/cm	2213	2650	2216	2172
Estimated Cation Exchange Capacity meq/100 g	11.4	10.5	11.8	11.3

Appendix 2: Summary of results for glasshouse experiments

Table a: Summary of results for Experiment 2.2 to determine the effect of artificial inoculation with *P. violae* isolate HL sand/oat inoculum on carrot growth and cavity spot disease development in pot-grown carrots grown using metalaxyl and untreated seed.

EXPERIMENT 2.2	Metalaxyl treated seed					Untreated seed						
	Oospores g^{-1}					Oospores g^{-1}						
Variable measured	**Control**	**1**	**10**	**50**	**100**	**Control**	**1**	**10**	**50**	**100**	**LSD (5%)**	**Level of significance**
% Seed germination[1]	59.6	76.0	61.0	70.4	72.6	74.43	62.25	59	72.36	68.91	10.7	0.009
Foliage dry weight per plant (g)	3.40	2.95	2.92	3.18	3.20	3.34	3.18	3.14	3.22	3.04	0.247	0.12
Carrot weight (g)	115.5	105.7	106.1	108.7	100.2	110.8	102.8	112.1	110.8	99.7	11.22	0.697
% Carrots with cavity spot (incidence)[1]	9.23	21.84	20.59	9.71	18.66	0	21.44	4.43	8.75	28.38	14.94	0.158
No. lesions per carrot (severity; all roots)[2]	-0.793	-0.586	-0.526	-0.810	-0.623	-1	-0.633	-0.895	-0.794	-0.320	0.307	0.03

Table b: Summary of results for Experiment 2.3 to determine the effect of artificial inoculation with *P. violae* isolates HL and P34 and *P. sulcatum* isolates P91, P67 and P127 using sand/oat inocula on carrot growth and cavity spot disease development in pot-grown carrots.

EXPERIMENT 2.3		*Pythium* isolate and inoculum concentration (oospores g^{-1})												
		P. violae HL		*P. violae* P34		*P. sulcatum* P91		*P. sulcatum* P67		*P. sulcatum* P127		LSD 1 (5%)	LSD 2 (5%)	
Variable measured	**Untreated control**	**10**	**100**	**10**	**100**	**10**	**100**	**10**	**100**	**10**	**100**	**max-min**	**min-rep**	**Level of significance**
% Seed germination[1]	69.79	54.22	59.2	57.78	63.24	52.45	59.65	83.09	68.97	81.05	70.45	8.59	9.9	0.004
Foliage dry weight per plant (g)	1.73	1.92	1.85	1.73	1.95	2.03	1.83	1.4	1.49	1.77	1.72	0.135	0.155	0.359
Carrot weight (g)	48.57	53.74	51.33	49.89	52.88	59.25	53.32	45.44	40.75	48.67	50.89	4.689	5.415	0.698
% Carrots with cavity spot (incidence)[1]	0	0	5.112	0	11.25	0	0	3.321	7.36	0	0	9.68	11.2	0.608
No. lesions per carrot (severity; all roots)[2]	-1	-1	-0.863	-1	-0.731	-1	-1	-0.940	-0.867	-1	-1	0.226	0.3	0.572

[1]Angular transformed values; [2]$\log_{10}$ transformed values. LSD 1 allows comparison between the untreated control (16 replicates) and inoculated treatments (8 replicates) and LSD 2 allows comparison between inoculated treatments only (8 replicates).

Appendix 2 continued

Table c: Summary of results for Experiment 2.4 to determine the effect of artificial inoculation with *P. violae* isolate HL and *P. sulcatum* isolate P67 using millet seed inocula on carrot growth and cavity spot disease development in pot-grown carrots.

EXPERIMENT 2.4		**Treatment and inoculum concentration (mg g^{-1})**								
		Dead inoculum control		***P. violae* HL**		***P. sulcatum* P67**		**LSD 1 (5%)**	**LSD 2 (5%)**	
Variable measured	**Untreated control**	**5**	**50**	**5**	**50**	**5**	**50**	**max-min**	**min-rep**	**Level of significance**
% Seed germination[1]	73.22	65.65	42.81	64.53	49.5	61.39	2.3	8.45	9.8	< 0.001
Foliage dry weight per plant (g)	2.39	2.35	3.96	1.87	2.88	1.85	*	0.423	0.489	0.085
Carrot weight (g)	88.51	84.22	129.05	63.42	88.2	61.99	*	14.71	17.06	0.101
% Carrots with cavity spot (incidence)[1]	0	0	0	44.7	64.9	24.67	*	10.39	12.0	0.021
No. lesions per carrot (severity; all roots)[2]	-1	-1	-1	-0.048	0.29	-0.499	*	0.199	0.2	0.042

[1]Angular transformed values; [2]$\log_{10}$ transformed values. LSD 1 allows comparison between the untreated control (16 replicates) and inoculated treatments (8 replicates) and LSD 2 allows comparison between inoculated treatments only (8 replicates).

Appendix 3: Summary of cavity spot severity and root plating results from glasshouse experiments

Summary of results from three artificial inoculation experiments (2.2-2.4) to determine the effect of *Pythium* sand/oat inoculum (2.2, 2.3) and millet seed inoculum (2.4) on cavity spot disease severity (actual mean no. of lesions per root across infected roots only; statistical analysis not possible) in carrots grown from metalaxyl (M) or untreated (U) seed. Results are also presented for the percentage of tap root pieces plated onto CMA/Rif colonised by *Pythium*.

EXPERIMENT 2.2				
Treatment	**Seed**	**Inoculum concentration (oospores g $^{-1}$)**	**No. lesions per carrot (infected roots only)**	**% Root pieces with *Pythium* growth**
Untreated control	M	0	2.5	4.2
***P. violae* HL**	M	1	1.3	54.2
***P. violae* HL**	M	10	2.5	37.5
***P. violae* HL**	M	50	1.3	4.2
***P. violae* HL**	M	100	1.3	62.5
Control	U	0	0	0.0
***P. violae* HL**	U	1	2.2	62.5
***P. violae* HL**	U	10	1.5	16.7
***P. violae* HL**	U	50	2	8.3
***P. violae* HL**	U	100	2.3	50
EXPERIMENT 2.3				
Control	U	0	0	0
***P. violae* HL**	U	10	0	4.8
***P. violae* HL**	U	100	2.7	9.5
***P. violae* P34**	U	10	0	4.8
***P. violae* P34**	U	100	10.4	19.0
***P. sulcatum* P91**	U	10	0	0
***P. sulcatum* P91**	U	100	0	0
***P. sulcatum* P67**	U	10	1.0	52.4
***P. sulcatum* P67**	U	100	1.0	28.6
***P. sulcatum* P127**	U	10	0	0
***P. sulcatum* P127**	U	100	0	0
EXPERIMENT 2.4				
Control	U	0	0	0
Dead inoculum control	U	5	0	0
Dead inoculum control	U	50	0	0
***P. violae* HL**	U	5	2.10	20.8
***P. violae* HL**	U	50	2.75	4.2
***P. sulcatum* P67**	U	5	1.33	37.5
***P. sulcatum* P67**	U	50	missing value	missing value

Appendix 4: Field macrocosm results

Summary of results from artificial inoculation of field-grown macrocosm carrots with *P. violae* isolate HL sand/oat inoculum in 2017 and 2018. Means calculated from four replicate field macrocosms for each treatment.

2017 (YEAR 1)	**Inoculum concentration (oospores g^{-1})**							
Variable measured	**Control**	**5**	**10**	**20**	**30**	**50**	**LSD (5%)**	**Level of significance**
Total weight of carrots (kg)	8.17	8.5	8.2	8.8	7.93	7.84	1.457	0.735
Carrot weight (g)	65.3	62	81.6	84.6	70.3	80.8	40.28	0.77
Number of carrots	147.8	150.5	112.2	116.5	117.5	102.8	64.99	0.542
% Carrots with cavity spot [1]	6.67	29.12	29.81	30.17	31.03	38.47	6.421	<0.001
No. of lesions per carrot (all roots) [2]	-1.912	-0.281	-0.300	-0.226	-0.339	-0.143	0.312	<0.001
No. of lesions per carrot (infected roots) [2]	0.015	0.354	0.313	0.378	0.240	0.278	0.182	0.008
No. of lesions per carrot (infected roots) [3]	1.04	2.33	2.12	2.61	1.78	1.92	N/A	N/A
2018 (YEAR 2)	**Inoculum concentration (oospores g^{-1})**							
Variable measured	**Control**	**5**	**10**	**20**	**30**	**50**	**LSD (5%)**	**Level of significance**
Total weight of carrots (kg)	8.28	7.27	8.15	8.17	7.31	7.45	1.342	0.381
Carrot weight (g)	36.6	37.8	39.8	38.2	38.5	30.5	8.72	0.316
Number of carrots	226.2	198.5	204.2	216	197	243.5	43.78	0.23
% Carrots with cavity spot[1]	13.74	36.71	34.1	33.44	35.33	35.44	4.646	<0.001
No. of lesions per carrot (all roots)[2]	-1.039	-0.096	-0.166	-0.159	-0.067	-0.114	0.284	<0.001
No. of lesions per carrot (infected roots)[2]	0.256	0.352	0.341	0.362	0.410	0.360	0.102	0.109
No. of lesions per carrot (infected roots)[3]	1.85	2.25	2.20	2.30	2.59	2.32	N/A	N/A

[1]Angular transformed values; [2]$\log_{10}$ transformed values are presented for severity; [3]actual mean values for severity across infected roots only.