Project title:	Carrots: Development of artificial inoculation techniques for cavity spot caused by *Pythium violae*
Project number:	FV 391a
Project leader:	John Clarkson
Report:	Final report
Previous report:	None
Key staff:	Nicole Pereira Kathryn Hales
Location of project:	Warwick Crop Centre, University of Warwick
Industry Representative:	Ian Holmes, Stawsons
Date project commenced:	01/04/2016
Date project completed (or expected completion date):	31/07/2017

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

AUTHENTICATION

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

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GROWER SUMMARY

Headline

Artificial inoculation of pot-grown carrot plants in the glasshouse with *Pythium violae* consistently resulted in the formation of small, stubby and stunted carrots. Typical cavity spot lesions were also observed on a large proportion of carrots in one experiment but the severity of the disease was generally low, with a maximum of six lesions per carrot. In a preliminary field experiment, artificial inoculation of carrots grown in macrocosms resulted in high incidence of cavity spot.

Background

Cavity spot of carrots is principally caused by the soilborne oomycete pathogen *Pythium violae* and continues to be the most economically important disease for UK growers with losses of at least £3-5 million per season. Control relies on the fungicide metalaxyl, but its efficacy in controlling the disease and the potential withdrawal of approval in the future are major concerns for the industry. However, previous AHDB-funded field trials have failed so far to reliably identify any new actives or approaches for control of *P. violae* due to no, or low levels of cavity spot development. One solution to the problem of low cavity spot levels is to artificially inoculate carrots in pots or the field with *P. violae* to ensure a high enough level of disease development such that activity of control treatments can be assessed reliably. However, a reproducible way of inducing cavity spot symptoms in carrots has yet to be developed, despite many attempts over decades of research. There is therefore still a clear need to try and develop artificial inoculation systems for *P. violae*. The overall aim of this project was therefore to develop methods for producing *P. violae* inoculum and determine the potential to cause cavity spot disease in glasshouse grown carrots. The specific objectives were:

1. Develop growth media and determine conditions to optimise inoculum production of *P. violae* mycelium / oospores by different isolates in controlled environment
2. Test the efficacy of different rates of *P. violae* inoculum in producing cavity spot symptoms in pot grown carrots in the glasshouse

Summary

Two methods were developed which allowed large numbers of *P. violae* oospores to be produced in both a liquid medium and a sand/oat-based solid substrate culture. The most spores were produced on the solid substrate after 10-15 weeks (47,639 oospores g^{-1}) and this medium was also the most amenable to inoculation as it could be easily mixed with compost or soil and carrots grown. However, oospore germination on agar was negligible as has been observed previously by other researchers.

In two long-term glasshouse experiments, artificial inoculation of the growing media using the *P. violae* solid substrate at five different rates (5, 10, 25, 50 and 75 oospores g^{-1}) initially resulted in some seedling death, reduced seedling size and a decrease in growth of foliage. However, at harvest, the principal effect of *P. violae* inoculation was the formation of small, stubby and stunted carrots with a much-reduced weight compared to the uninoculated control plants. These infected carrots were also characterised by a long hairy brown tap root with increased lateral root formation, many of which were collapsed. Typical cavity spot lesions were also observed in a large proportion of these stubby carrot roots in experiment 1 (up to 26%) but disease incidence was less in experiment 2. In both experiments, cavity spot severity was low with an average of approx. two lesions per carrot and a maximum of six lesions per carrot. *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.

A preliminary field experiment was also carried out as part of a PhD studentship and an approved follow-up project (FV 391b) whereby macrocosms (concrete pipes sunk in the ground) were filled with a soil / sand mix, artificially inoculated with *P. violae* solid substrate inoculum and carrots sown. In this situation, there was no effect of pathogen inoculation on either seedling survival or subsequent carrot growth but at harvest, a large proportion of the carrots (up to 40%) were affected by typical cavity spot symptoms.

Overall, inoculation with *P. violae* was very successful compared to previous attempts but further work is still required to reduce variation between experiments and increase the number of cavity spot lesions before the approach is used in extensive testing of control products in pot-based glasshouse tests or in the field.

Financial Benefits

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

Action Points

None at this time.

SCIENCE SECTION

Introduction

Cavity spot 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 491 (Gladders, 2014) tested a range of potential new fungicides, biological control treatments and pre-planting calcium applications while FV 405 (Clarkson, 2014) 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 therefore, there is a clear need to develop artificial inoculation systems for *P. violae* in order to test potential new products and approaches to cavity spot control. The overall aim of this project was to develop methods for producing *P. violae* inoculum and determine the potential to cause cavity spot disease in pot-grown carrots in the glasshouse. The specific objectives were:

1. Develop growth media and determine conditions to optimise inoculum production of *P. violae* mycelium / oospores by different isolates in controlled environment
2. Test the efficacy of different rates of *P. violae* inoculum in producing cavity spot symptoms in pot grown carrots in the glasshouse

Materials and methods

General procedures

Culturing and maintenance of *Pythium violae*

P. violae isolate HL derived from infected carrots (cv. Nairobi) collected in Holton, Lincolnshire was selected for all experiments as in preliminary experiments 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 unreplicated trial. Identity of isolate HL was confirmed as *P. violae* by sequencing of the ITS regions of the rDNA gene (Hales & Clarkson, 2016). *P. violae* HL was routinely cultured on cornmeal agar (CMA; Sigma-Aldrich, UK) or CMA with rifampicin (30 mg L^{-1}) 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 quantifying oospore production. V8B was prepared both with, and without 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 Bowers, UK) and oatmeal-based substrate was chosen as a solid medium for evaluating oospore production and as inoculum for glasshouse experiments. Oospore production by *P. violae* has been shown to be profuse on this type of medium and was used previously by Pettit (2002) and Hales & Clarkson (2016) for inoculum production. The solid substrate was prepared in 250 ml (125 g sand, 1.25 g oatmeal) or 1 L (500 g sand, 5 g oatmeal) conical flasks and sterile distilled water (SDW) added to achieve a final moisture content of approximately 13-15% w/w. Flasks were autoclaved twice for 15 min at 121°C with an interval of 24 h in-between each cycle.

Objective 1: Develop growth media and determine conditions to optimise inoculum production of P. violae mycelium / oospores

An experiment was set up to evaluate the production of *P. violae* oospores over time in liquid and solid media. Methods were developed to separate the oospores from the mycelium and kill mycelial fragments for accurate quantification and assessment of spore germination on CMA.

Oospore production, extraction and quantification in liquid medium

Agar plugs of mycelium (2 x 6 mm) from actively growing cultures of *P violae* HL on CMA were used to inoculate 20 ml aliquots of V8B and V8B+C in flat cell culture flasks (50 ml capacity; VWR, UK) with vented lids and incubated in the dark at 15°C. At intervals, mycelial mats were removed from each flask with sterile forceps and washed three times in 5 ml SDW by gentle pipetting. Mats were left in open Petri dishes overnight in a laminar flow hood to allow desiccation and to reduce viability of the mycelium. Each mat was then rehydrated with 5 ml SDW and transferred to a sterile bulbous flask (MSE, UK). The Petri dishes were washed with a further 20 ml SDW and washes added to the flasks. The blade of a motorised MSE homogeniser was sterilised by flaming in 100% ethanol and each mycelial mat homogenised on a low setting for 8 min. Homogenates were decanted into 40 ml sterile tubes and the blade and flask washed with 5 ml SDW. The homogenates were then sonicated (MSE Soniprep 150, UK) on ice for two 1 min bursts (amplitude setting 8) with a 1 min interval in between in order to dislodge spores and further reduce viability of mycelial fragments. The final total volume of the extract was then measured, a 5 ml aliquot taken and oospore concentration quantified using a modified Fuchs-Rosenthal haemocytometer. Three replicate V8B and V8B+C cultures were set up for oospore extraction and quantification at each of six time-points (5, 10, 15, 22 and 30 weeks) with four replicate oospore counts made for each extract.

Oospore production, extraction and quantification in solid substrate

Agar plugs of mycelium (5 x 6 mm) from actively growing cultures of *P violae* HL on CMA were used to inoculate sand/oatmeal substrate in 250 ml conical flasks and incubated in the dark at 15°C. At intervals, the contents of each flask were decanted into an open polythene grip seal bag and allowed to dry overnight in a laminar flow hood to help desiccate and reduce viability of the mycelium. Bags were sealed, the substrate shaken well after which two 20 g samples from each bag were weighed into 50 ml Falcon tubes, and 40 ml SDW added. The tubes were shaken vigorously for 3 min at full speed on a flask shaker (Stuart Scientific, UK) after which the sand was allowed to settle for a few seconds and the extract decanted into a

clean tube. A further three extracts were carried out for the remaining sediment using the same procedure using 30, 20 and 10 ml SDW respectively. All extracts were combined, the total volume recorded and sonication carried out (as described for liquid cultures) to dislodge spores and further reduce viability of mycelium. Oospore concentration was determined in a Sedgewick-Rafter chamber (1 ml extract) with extracts diluted in SDW if required. Three replicate flasks were set up for oospore extraction and quantification at each of four time-points (5, 10, 15 and 22 weeks) with two replicate counts made for each extract from the 20 g samples.

Assessment of oospore germination

Oospore extracts from V8B, V8+C liquid media and sand/oat solid substrate were successively filtered by vacuum through pre-sterilised 500, 250 & 80 µm nylon meshes to remove most mycelial fragments (Pettit, 2002). The filtrates were centrifuged at 1300 *x g* (2500 rpm, Sorvall, UK) for 5 min to concentrate the oospores and the supernatant poured off to leave a volume of approximately 5 ml. Oospores were then re-suspended by vortexing and 100 µl aliquots plated onto CMA + rifampicin (pH 8). Plates were incubated at 20°C in darkness and were observed under the microscope (x40) for any evidence of oospore germination over a period of seven days.

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

Two experiments were set up to determine if *P. violae* solid substrate inoculum could induce cavity spot symptoms in glasshouse grown carrots using different concentrations of oospores.

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 selected as the growing medium in all 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. Solid substrate inoculum of *P. violae* isolate HL (4-8 months old) was prepared as outlined above 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. 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 & 5000 oospore g^{-1} inoculum were then mixed with compost/sand growing medium in a cement mixer to obtain growing medium infested with final *P. violae* oospore concentrations of 5, 10, 25, 50 and 75 oospores g^{-1} (5.4, 10.8, 27.0, 54 & 81.0 oospores/cm^3). All growing medium/inoculum mixtures were prepared such that the compost/sand ratio was always 50:50 v/v. Plastic pots (5.5 l capacity, 20 cm diameter, 27.5 cm high) were filled with the infested growing medium (5.65 kg per pot) at each of the five 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 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 6-7 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, 16 replicate pots were prepared for each oospore concentration in a randomised block design consisting of four blocks each containing four replicate pots of each treatment. Two experiments were set up (Fig. 1); experiment 1 on 28/04/16 (21 weeks duration) and experiment 2 on 16/06/16 (23 weeks duration).

Figure 1. Pot-grown carrots inoculated with *P. violae* in experiments 1 and 2 (July 2016) 13 and 6 weeks after sowing.

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

In each experiment, carrot seedling emergence was recorded weekly for the first 6 weeks as well as any damping off symptoms. The fresh and dry weight of the seedlings that were removed as part of thinning out were recorded to determine any treatment-induced reduction in top growth at an early stage. Plants were harvested when carrots reached maturity (21 weeks for experiment 1 and 23 weeks for experiment 2). 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 two of the four pots from each oospore concentration treatment in each block (total eight roots per treatment), the long thin tap root excised and stored 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; total 24 root pieces per treatment). A 1-2 cm length of each section was then plated out onto CMA amended with rifampicin (30 mg L^{-1}; pH 6), incubated in the dark at 17°C and the number with *P. violae* 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 % seedling emergence, damping off, and cavity spot incidence and $\log_{10}$ transformation of number of cavity spot lesions.

Extra Objective: Preliminary trial to investigate the efficacy of different rates of P. violae inoculum in producing cavity spot symptoms in field grown carrots

As part of AHDB PhD FV432 and the follow up project to this work (FV 391b) an initial cavity spot field trial was carried out from May 2016 to February 2017 in 24 'macrocosm' plots located in the Wellesbourne quarantine field (Fig. 2). 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 was then added to provide a suitable sandy loam substrate for carrot growing and inoculation. *P. violae* HL sand/oatmeal 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. 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 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 carrot seeds (cv. Nairobi) and seeds covered with a 1 to 2 cm layer of sieved soil. Macrocosms were watered regularly throughout the growing season and sprayed periodically with Hallmark (lambda-cyhalothrin) to reduce damage from carrot root fly. Straw was applied in November 2016 to prevent frost damage. Carrots were harvested on 23/02/17 (eight months after inoculation) and both carrot yield and cavity spot symptoms recorded.

Figure 2. Macrocosms used for *P. violae* inoculation of carrots (July 2016).

Results

Objective 1: Develop growth media and determine conditions to optimise inoculum production of P. violae mycelium / oospores

Oospore production, extraction and quantification in a liquid medium

P. violae isolate HL grew well both in the liquid media (Fig. 3a) and the solid substrate with large numbers of oospores produced. However, different types of oospore were observed: i) 'immature' with no double wall present and a granular appearance, ii) 'small mature' which were double walled but were significantly smaller than iii) 'mature' with large, clear double wall and dark filling (Fig. 3b). Data is presented for mature (small and large) oospores only as these were assumed to have the capacity to germinate.

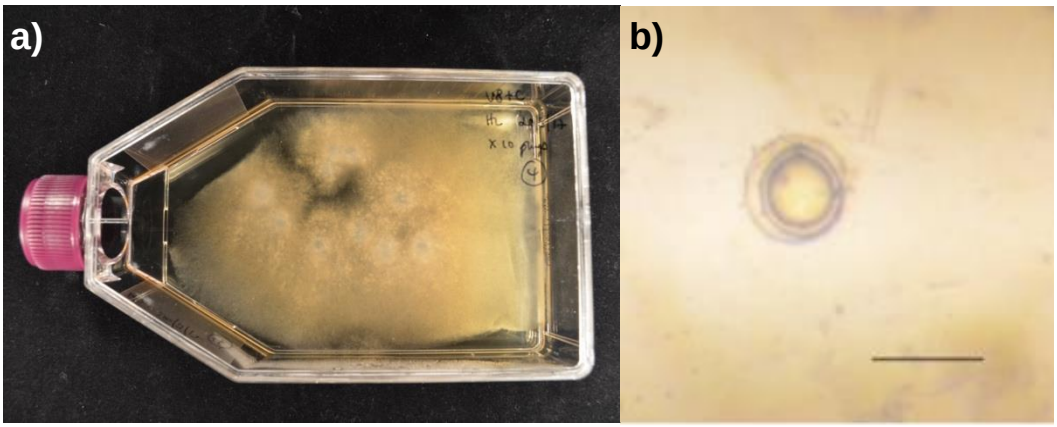


Figure 3. a) *P. violae* mycelial mat grown in V8B+C for 5 weeks and b) mature *P. violae* oospore. Bar = approx. 25 µm.

In liquid culture, oospore production by *P. violae* isolate HL ranged from 7498 spores ml^{-1} (V8B no cholesterol, 22 weeks) to 11,747 spores ml^{-1} (V8B with cholesterol, 10 weeks; Fig. 4a). On average, 2,500 more oospores were produced in V8B with cholesterol than without and this was consistent across the five time points where samples were quantified (Fig. 4a). There was also no apparent increase in oospore production after 10 weeks, and for the V8B only treatment, there was a small decline (Fig. 4a). In the solid substrate, the number of oospores increased from 10,246 to a maximum of 47,639 oospores g^{-1} after 15 weeks (Fig. 4b). Hence, a maximum total of 234,940 spores can be produced in a single liquid culture flask containing 20 ml medium compared to 5,954,875 spores in 125 g solid substrate.

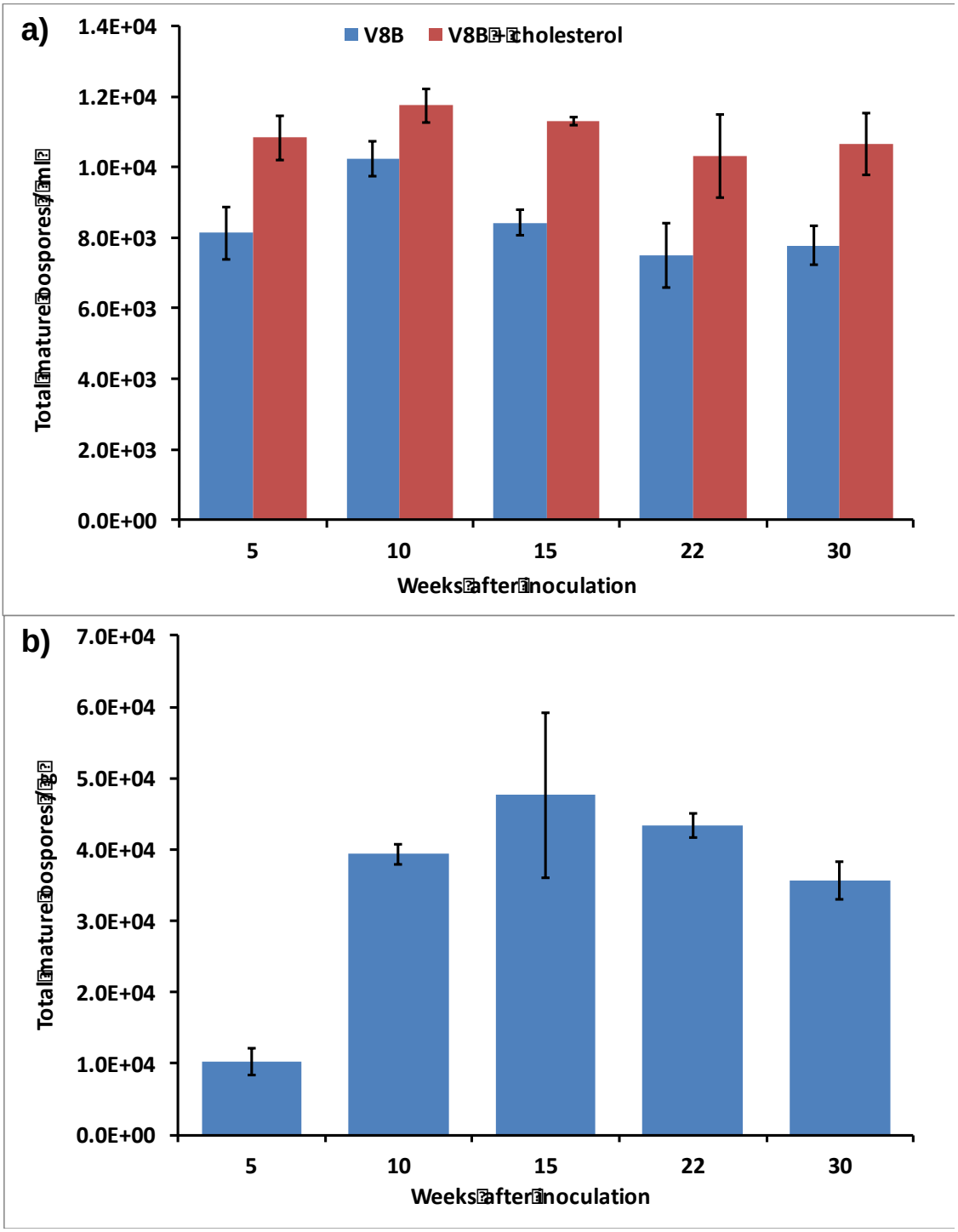


Figure 4. Oospore production by *P. violae* in a) liquid culture (V8B and V8B + cholesterol) and b) solid substrate (sand / oatmeal). Data are mean oospore counts from three replicate cultures flasks. Error bars represent the standard error of the mean (SEM).

Assessment of oospore germination

No germination was observed for oospores extracted from V8B or V8+cholesterol for samples taken at any of the timepoints while germination was only observed very occasionally for those produced on sand/oatmeal (Fig. 5) and was too low to accurately quantify.

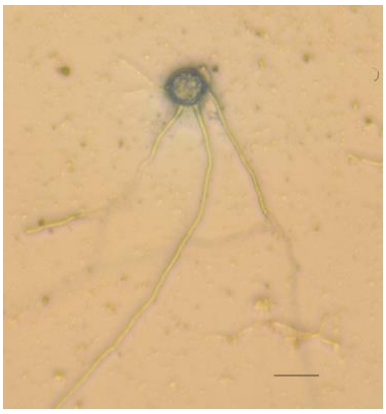


Figure 5: *P. violae* oospore from sand/oatmeal substrate germinating on CMA after 48 h at 20°C. Bar = approx. 25 µm).

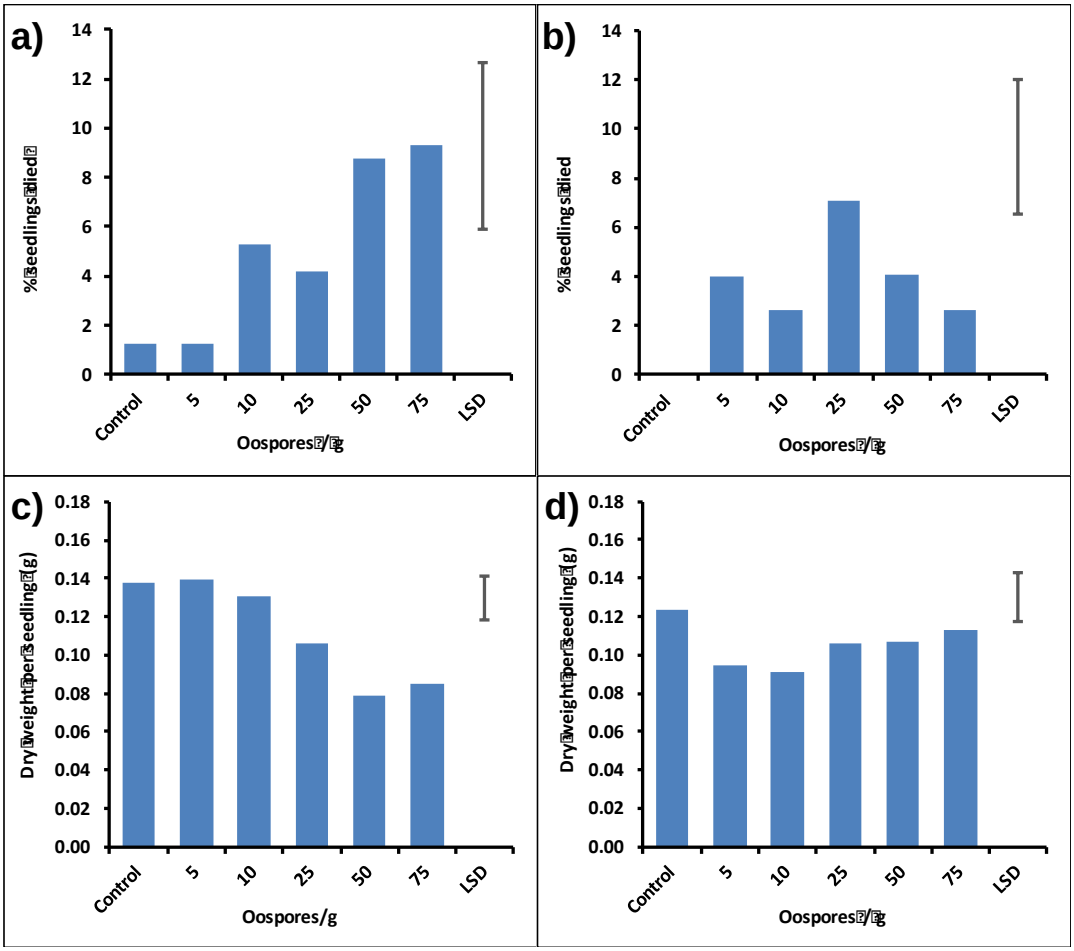
Objective 2: Test the efficacy of different rates of P. violae inoculum in producing cavity spot symptoms in pot grown carrots in the glasshouse

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

In the *P. violae* inoculated treatments in experiment 1, carrot seedling germination ranged from 82-86% for the five oospore concentrations and was similar to the 88% recorded in the control. In experiment 2, germination was slightly reduced to 73-77% for the *P. violae* treatments compared to 82% in the control (Table 1). However, this was not statistically significant. Some post-emergence damping off was observed in both experiments with a maximum of 7% and 5% in experiments 1 and 2 respectively (Fig. 6ab; Table 1). In experiment 1, more damping off was observed in the 50 and 75 oospore g^{-1} *P. violae* treatments (Fig. 6a; Table 1). The effect of *P. violae* treatment compared to the control was just outside the level of significance in experiment 1 ($P = 0.064$) and non-significant for experiment 2 ($P = 0.223$). However, in both experiments, some of the *P. violae* treatments, particularly the 50 and 75 oospore g^{-1} concentrations in experiment 1, significantly reduced seedling weight compared to the uninoculated control for those that were thinned out, ($P < 0.001$, $P < 0.05$ for experiments 1 and 2 respectively; Fig. 6cd; Table 1). It was also observed in experiment 1 that the growth of carrot plant foliage in *P. violae* inoculated treatments was reduced at 6-9 weeks compared to the untreated control with some visual evidence that this was greater with increasing oospore concentration (Fig. 7). However, this effect was not clearly observed in experiment 2 nor by the end of both experiments at harvest, where there

was no significant difference in foliage wet or dry weight between any of the treatments and the untreated control (Table 1).

Figure 6: Effect of *P. violae* on mean percentage damping off (a, experiment 1 and b, experiment 2;



angular transformed data) and mean seedling dry weight (c, experiment 1; d, experiment 2). Bars represent the least significant difference between treatments (LSD) at the 5% level.

Figure 7: Effect of *P. violae* on carrot foliage growth after 6 weeks in experiment 1. From L to R; untreated control, 5, 10, 25, 50 and 75 oospores g^{-1}.

When plants were harvested at the end of the two experiments, the most obvious effect of all the *P. violae* treatments was a marked stunting of the carrots which were small, stubby and also characterised by a long hairy brown tap root with increased lateral root formation, many of which were collapsed. *P. violae* treatments therefore had a significant effect on carrot weight compared to the untreated control in both experiments ($P < 0.001$; Fig. 9ab, Table 1), but there was no apparent effect of different oospore concentrations. Mean weight per carrot in the *P. violae* treatments ranged between 41-45 g and 34-42 g for experiments 1 and 2 respectively compared to 60 g and 48 g in the corresponding untreated controls (Fig. 9ab; Table 1).

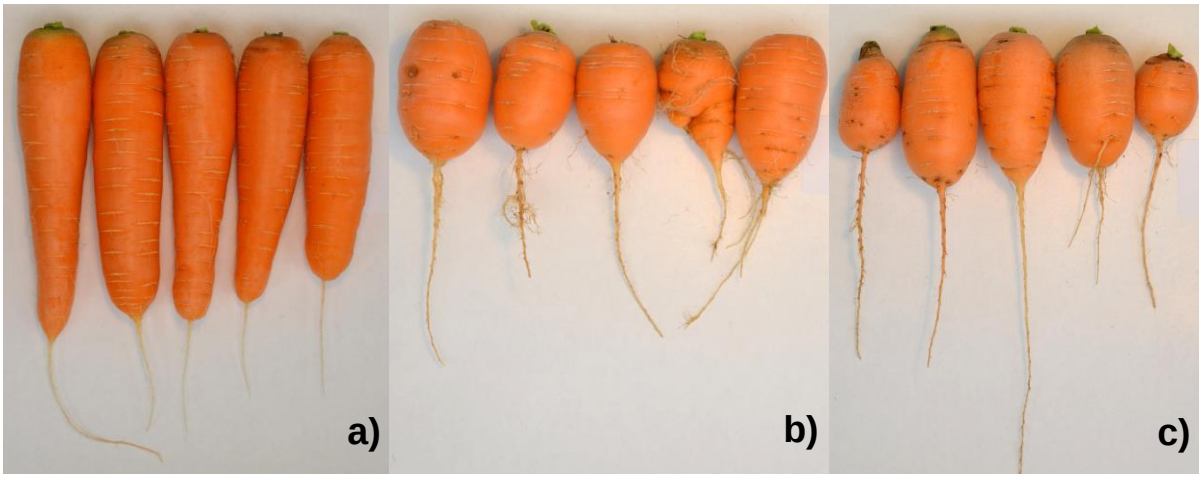


Figure 8: Effect of *P. violae* on carrot root growth (experiment 1); a) control, b) 25 oospores g^{-1}, c) 50 oospores g^{-1}.

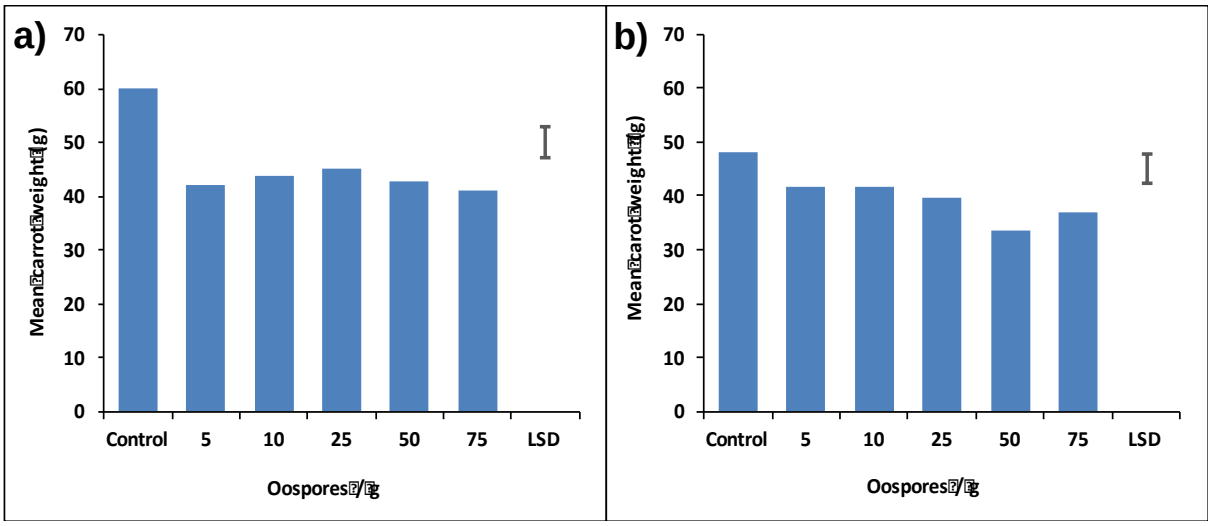


Figure 9: Effect of *P. violae* on carrot weight in a) experiment 1; b) experiment 2. Bars represent the least significant difference between treatments (LSD) at the 5% level.

Effect of P. violae inoculum on cavity spot incidence and severity

Typical cavity spot symptoms were observed in all *P. violae* treatments (Fig. 10) and incidence varied between 18-26% of carrots affected in experiment 1 and 4-13% in experiment 2 with no apparent effect of oospore concentration (Fig. 11ab; Table 1). No cavity spot symptoms were observed in uninoculated control treatments. However, the effect of *P. violae* inoculation on the incidence of cavity spot compared to the untreated control was only statistically significant for experiment 1 ($P < 0.001$), and just outside the 5% level of significance for experiment 2 ($P = 0.063$). Mean severity of cavity spot (across all carrots, including those unaffected) was low in both experiments ranging from 0.36-0.54 cavities per carrot in experiment 1 and 0.06-0.23 cavities per carrot in experiment 2 (Fig. 11cd; Table 1). Nonetheless the effect of *P. violae* inoculation on the number of cavities compared to the untreated control was statistically significant (log_{10} transformed data not shown) for experiment 1 ($P < 0.001$), and just outside the 5% level of significance for experiment 2 ($P = 0.088$). When only infected carrots were considered, then the number of lesions per carrot ranged between 1.84-2.35 in experiment 1 and 1.1-1.8 in experiment 2 (Fig 11ef, Table 1; statistical analysis not possible due to large number of missing values i.e. unaffected carrots). Maximum number of cavity spot lesions ranged from 4-6 and 2-5 in experiments 1 and 2 respectively (Table 1).

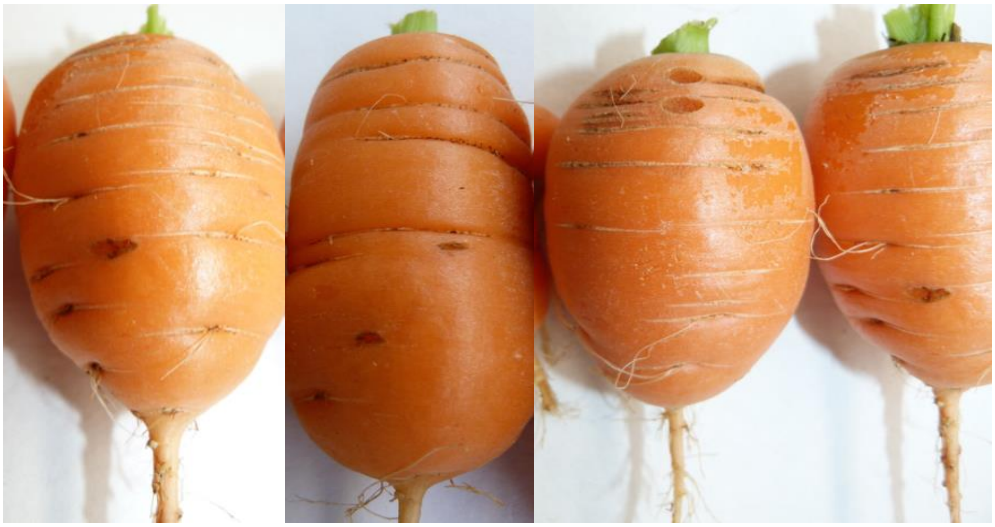


Figure 10. Cavity spot lesions on carrot roots from *P. violae* inoculated treatments.

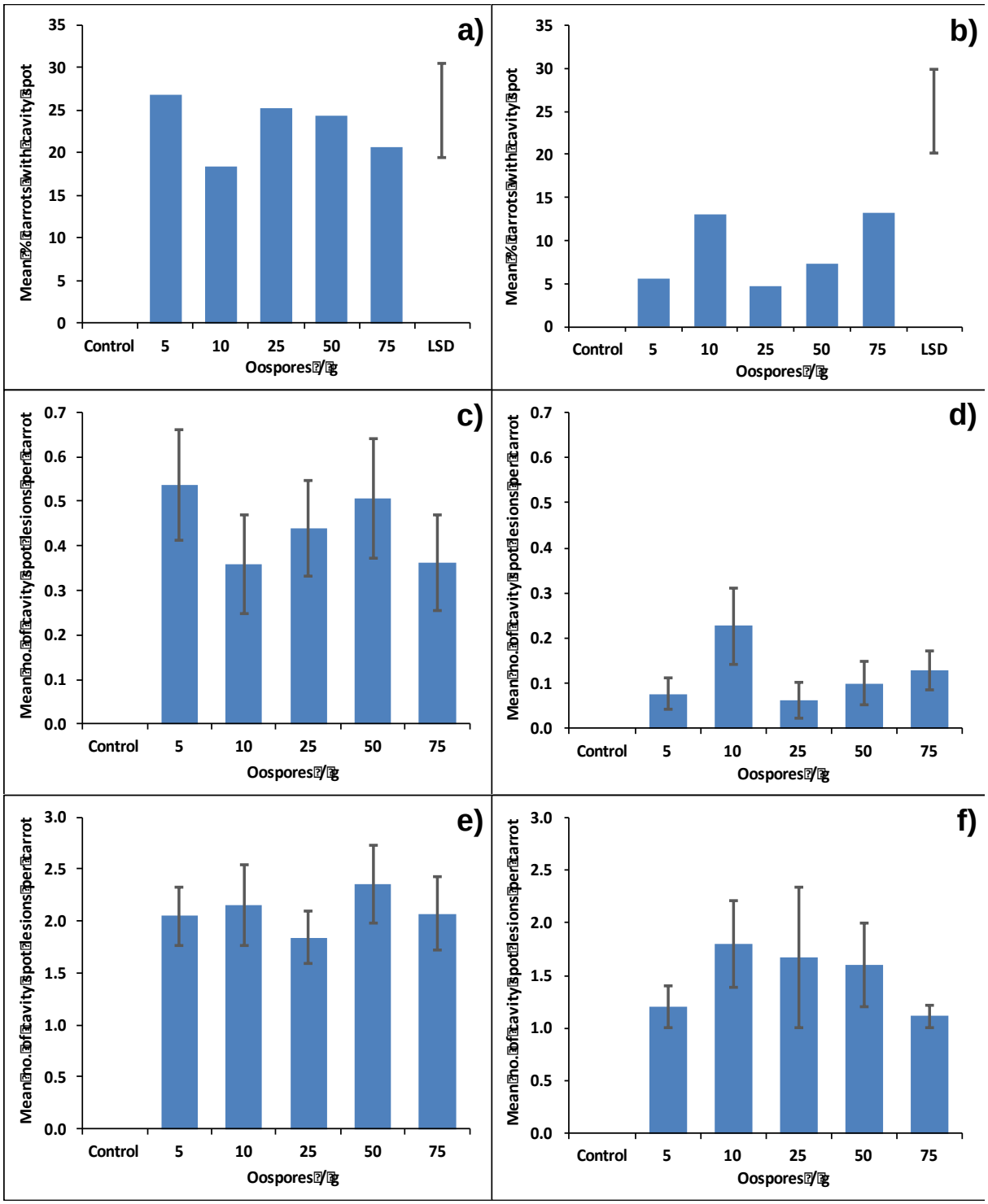


Figure 11. Effect of *P. violae* inoculation on cavity spot incidence (% carrots affected; a, experiment 1; b experiment 2; angular transformed data), severity (no. of lesions) across all carrots (c, experiment 1; d, experiment 2) and across affected carrots only (e, experiment 1; f, experiment 2). Bars in a) and b) represent the least significant difference between treatments (LSD) at the 5% level. Bars in c), d) e) and f) represent the standard error of the mean (SEM).

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

A high proportion of carrot tap root pieces from the inoculated carrots yielded *P. violae* colonies on agar (Fig. 12ab) demonstrating that the stunting of carrots was due to high levels of pathogen infection. The percentage of root pieces with *P. violae* ranged from 29-58% in experiment 1 and 12-25% in experiment 2 (Table 1). No *P. violae* was isolated from any of the root pieces from uninoculated control treatments. *P. violae* was also successfully isolated from selected cavity spot lesions (Fig. 12c).

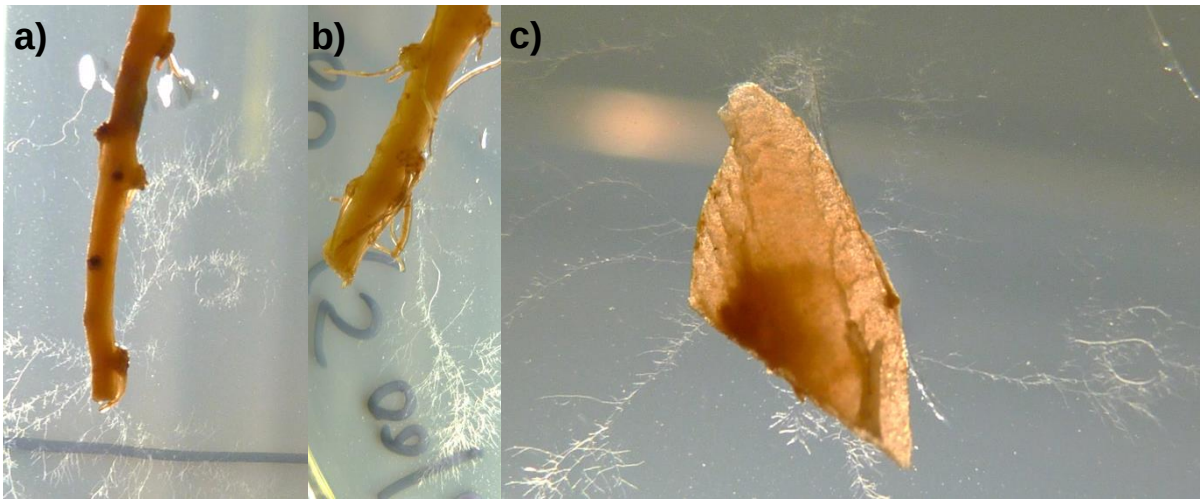


Figure 12. *P. violae* growing out from carrot tap root pieces (a, b) and cavity spot lesion (c) on agar.

Table 1. Summary of results from artificial inoculation of pot-grown carrots with *P. violae* in two glasshouse experiments

	Experiment 1							Experiment 2						
	Treatment (oospores g^{-1})						Level of	Treatment (oospores g^{-1})						Level of
	0	5	10	25	50	75	Significance	0	5	10	25	50	75	Significance
% seed germination	88.8	86.3	85.6	84.4	81.9	84.4	NS	81.9	72.5	73.1	77.5	76.3	76.3	NS
% damping off	0.7	0.7	3.3	2.8	6.1	6.9	NS	0.0	2.5	1.7	4.8	2.6	1.7	NS
Seedling dry wt (g)	0.14	0.14	0.13	0.11	0.08	0.09	<0.001	0.12	0.10	0.10	0.10	0.11	0.12	P < 0.05
Top growth dry wt (g)	10.8	11.3	10.9	10.3	11.1	11.5	NS	9.9	9.5	9.3	8.8	9.1	9.1	NS
Carrot wt (g)	59.7	41.5	42.9	43.9	42.3	41.1	<0.001	47.0	41.2	41.1	39.8	33.5	37.0	<0.001
% carrots with cavity spot	0.0	26.3	16.6	24.0	22.2	17.3	<0.001	0.0	5.6	12.5	3.5	6.3	11.3	NS
No. lesions per carrot (all roots)	0.00	0.54	0.36	0.44	0.51	0.36	<0.001	0.00	0.08	0.23	0.06	0.10	0.13	NS
No. lesions per carrot (infected roots)	0.00	2.05	2.15	1.84	2.35	2.07	N/A	0.00	1.20	1.80	1.67	1.60	1.11	N/A
Max. no. lesions per carrot	0	6	6	4	7	6	N/A	0	2	5	3	3	2	N/A
% root pieces with *P. violae* growth	0.0	58.3	37.5	29.2	33.3	41.7	N/A	0.0	25.0	12.5	12.5	12.5	20.8	N/A

Extra Objective: Preliminary trial to investigate the efficacy of different rates of P. violae inoculum in producing cavity spot symptoms in field grown carrots

Carrots grew well in the field macrocosms and there was no apparent effect of *P. violae* inoculation treatments on seed germination, seedling survival or carrot yield at harvest (Fig 14a). However, all the *P. violae* inoculated treatments resulted in typical cavity spot lesions forming on roots (Fig. 13). Cavity spot incidence ranged between 24% and 39% roots affected and there was no apparent effect of inoculum concentration although the 50 oospore g^{-1} treatment had a much greater incidence of cavity spot (39%) compared to the other treatments (Fig. 14b). There were very few or no cavity spot-type lesions in the control treatments. The full set of data for this experiment has yet to be summarised / statistically analysed and will be presented in the final report of the follow-up project FV 391b.

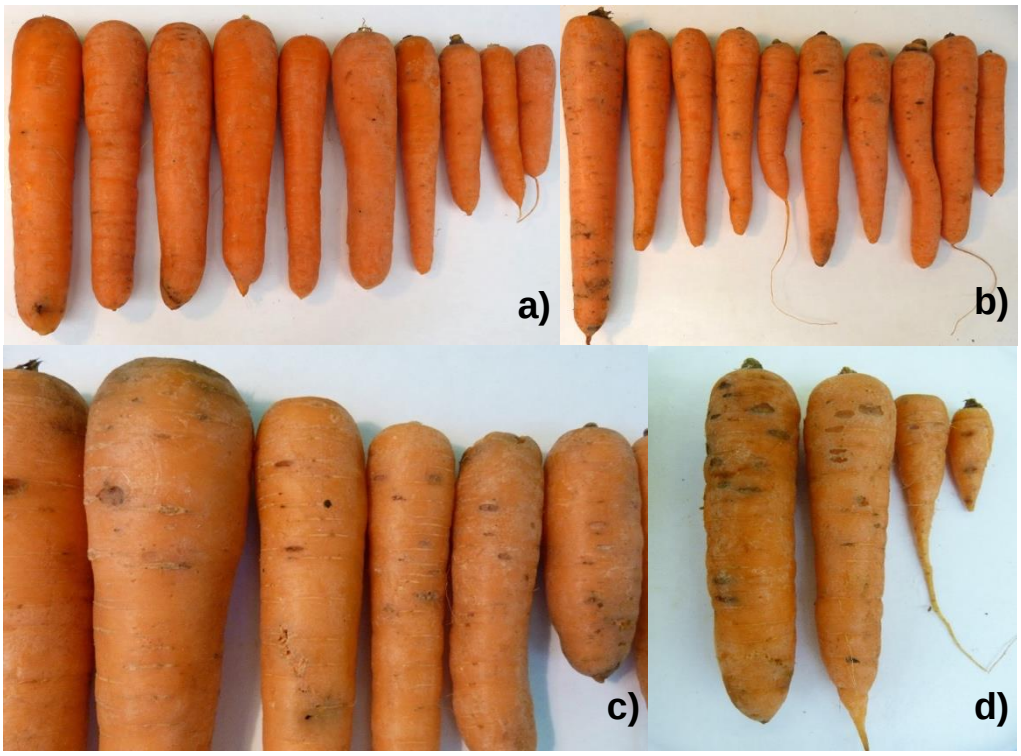


Figure 13. Carrots harvested from field macrocosms a) uninoculated control and b) inoculated with 50 *P. violae* oospores g^{-1} and carrots with typical cavity spot lesions from macrocosms inoculated with c) 30 *P. violae* oospores g^{-1} and d) 50 *P. violae* oospores g^{-1}.

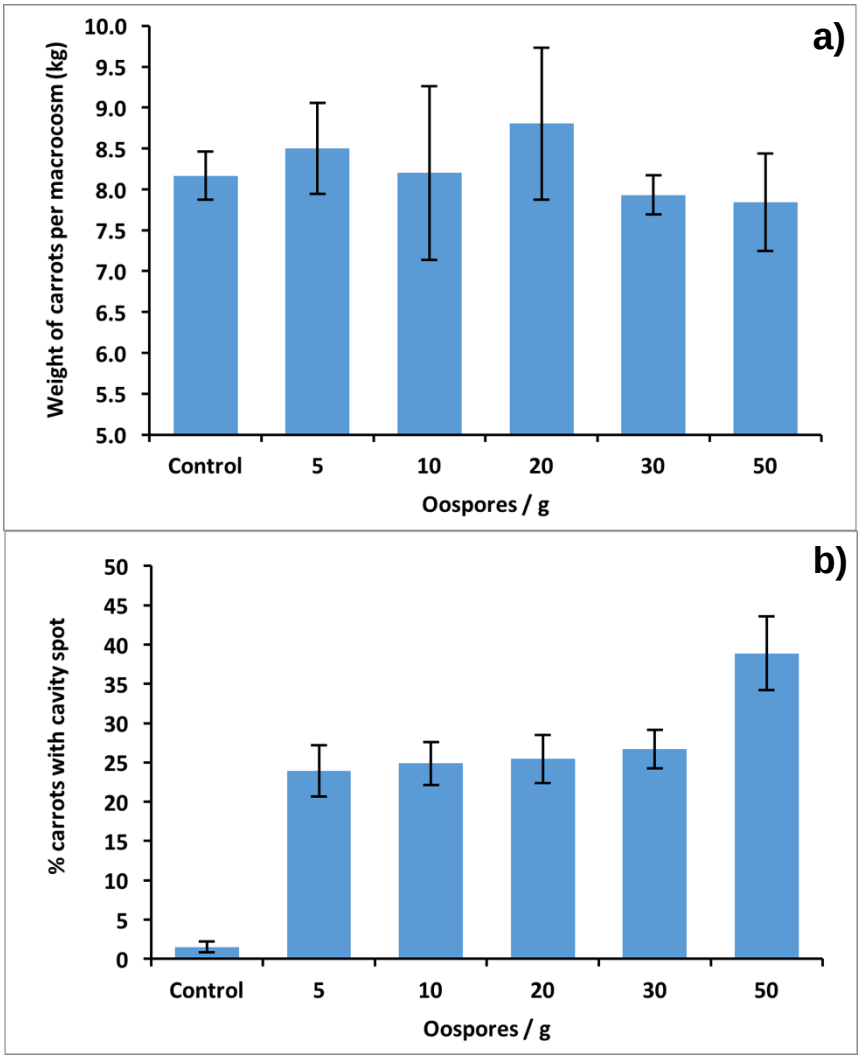


Figure 14. Effect of *P. violae* inoculation on a) total weight of carrots harvested per macrocosm and b) cavity spot) 50 *P. violae* oospores g^{-1}.

Discussion

Objective 1: Develop growth media and determine conditions to optimise inoculum production of P. violae mycelium / oospores

Results showed that both the V8 juice based liquid medium and the sand/oatmeal solid substrate were suitable for growth of *P. violae* and could support the production of large numbers of oospores. The addition of cholesterol to V8B also increased oospore production compared to V8 alone confirming the results of Ayers and Lumsden (1975) who made the same observation for three different *Pythium* species; *P. aphanidermatum*, *P. ultimum* and *P. myriotylum*. These researchers recorded oospore numbers between 52,000 - 77,000 spores ml^{-1} for *P. ultimum* and *P. myriotylum* respectively in V8B without cholesterol and between 307,000 and 309,000 spores ml^{-1} with cholesterol. This is considerably more than observed for *P. violae* in this project (up to 11,747 spores ml^{-1} in V8B with cholesterol). However, *Pythium* species and isolates can vary widely in their ability to produce oospores on different media and in the same study of Ayers and Lumsden (1975), *P. aphanidermatum* produced 1,146,000 oospores ml^{-1} in V8B with cholesterol, almost four times that of *P. ultimum* and *P. myriotylum*. Sterols have been reported to be required or to stimulate further oospore production in a range of different *Pythium* and *Phytophthora* species; however, it has also been suggested that some stress factors or other substances may also be implicated (Ko, 1998).

In this project, there was little if no germination of *P. violae* oospores observed for samples from either V8B or solid substrate at any of the timepoints assessed between 5 and 30 weeks. This is however a common phenomenon for *Pythium* and varies widely between species. Germination of oospores from wet or dry mycelial mats produced in V8B varied between 0 and 4% for *P. myriotylum*, 5-34% for *P. ultimum* and 89-90% for *P. aphanidermatum* (Ayers and Lumsden, 1975). In a previous AHDB project (FV 5f), Pettit (2002) observed low germination levels for *P. violae*, ranging from 5-14% depending on the age of culture, temperature at which culture was grown and the germination medium. However, Pettit (2002) suggested that this is a normal aspect of *P. violae* oospore survival behaviour and is not an accurate reflection of viability. This was confirmed in this project where the solid substrate oospore inoculum was demonstrated to result in carrot infection.

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

This is the first study to report the successful artificial inoculation of carrots with a *P. violae* solid substrate oatmeal/sand inoculum. Amending a compost / sand growing medium with different levels of this inoculum resulted in some seedling death, reduced seedling size, an initial decrease in growth of foliage and the formation of stubby, stunted carrot roots with typical cavity spot lesions as well as brown and hairy taproots.

Although *Pythium* spp. such as *P. ultimum* are often widely associated with damping off, this symptom has not been commonly associated with *P. violae*, although it has been observed in carrot seedlings previously by Pettit (2002) and Hales and Clarkson (2016). Pettit (2002) used a suspension of *P. violae* oospores in water (3 x 10^6 spores ml^1) to inoculate carrot seedlings sown in a sand contained in small modules. This resulted in up to 31% roots becoming colonised with *P. violae* as measured by plating onto agar media but no mortality was reported. However, Pettit (2002) also reported 100% pre-emergence damping off when carrot seed was sown in sand amended with two-week-old oatmeal/sand solid substrate inoculum as used in this study, but the number of oospores was not quantified. Howard et al., (1978) also reported damping off and root die back in carrot seedlings as being caused by at least 11 different *Pythium* species (but not *P. violae*) including *P. sulcatum* which is also associated with cavity spot in the UK (Hales and Clarkson, 2016). The same researchers also showed that carrot seedling death could be induced by artificial inoculation using a cornmeal/sand inoculum of both *P. sulcatum* and *P. irregulare*. However, in this study, the damping-off symptom caused by *P. violae* was not statistically significant, partly due perhaps to the relatively small number of seedlings in the test, but also because the levels of *P. violae* inoculum tested were deliberately chosen to avoid this, based on preliminary data that suggested that >75 oospores g^{-1} were required for significant damping off to occur (Hales and Clarkson, 2016).

One of the major of effects of *P. violae* inoculation using the solid substrate inoculum was on carrot plant growth, initially observed as a decrease in foliage (although this was only apparent in experiment 1) and subsequently more consistently with the formation of stubby, stunted carrots for all the different oospore concentration treatments. In addition, tap roots extending from the bottom of these carrots were brown and hairy compared to untreated control treatments and it was confirmed that these symptoms were due to *P. violae* as the pathogen was isolated from up to 58% of root pieces. This was a somewhat surprising result as *P. violae* has not generally been associated with stunted or malformed carrots. However,

Pratt and Mitchell (1972) previously reported that *P. sulcatum* was consistently isolated from carrots with 'forked and stunted' taproots and with 'brown root symptoms' in North America.

As well as a decrease in root weight, artificial inoculation with the *P. violae* solid substrate inoculum resulted in typical cavity spot lesions on carrots with 26% roots affected in experiment 1 with up to seven lesions per carrot, although the number of lesions overall was generally low. Similarly, White (1986) reported that artificial inoculation of sterile soil using a *P. violae* maizemeal inoculum at a rate of 33 g kg^{-1} soil resulted in 27% of carrots affected with cavities, also with a concomitant decrease in root weight. However, the number of cavities was not presented, nor was the experiment repeated. Two other comparable studies used millet seed colonised with different *Pythium* spp. associated with cavity spot (but not *P. violae*) as inoculum which was used to infest sterile soil before carrots were planted. In the first, a *P. sulcatum* treatment 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). This indicates that the ability to cause cavity spot symptoms can vary between different *Pythium* species.

Finally in this project, a high incidence of cavity spot was also observed in carrots grown in the inoculated field macrocosms which, along with the results from the pot tests, suggests that the solid substrate inoculum is a good approach for infecting carrots with *P. violae*. However, the data set has yet to be analysed and the same macrocosms were re-inoculated in May 2017 and carrots sown as part of the follow-on project FV 391b in order to determine if the results are reproducible.

Overall therefore, artificial inoculation with a solid substrate inoculum resulted in infection of carrots by *P. violae* resulting in root colonisation, stunting and typical cavity spot lesions. However, it was clear that results were variable between experiments and that disease severity was generally low. There are several potential reasons for this including variation in the viability / germination potential of different batches of inoculum and environmental conditions at different times of year despite attempts to control these. It may also be the case that *P. violae* isolates differ in their virulence on carrot and so far, only one isolate has been tested. Further work in the follow-on project FV 391b will aim to address some of these potential sources of variation in order to develop a more reproducible procedure to induce high levels of cavity spot in glasshouse-grown and field-grown carrots.

Conclusions

- High numbers of *P. violae* oospores can be produced in a liquid medium and solid substrate.
- Artificial inoculation of a compost / sand growing medium with a *P. violae* solid substrate 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.
- Artificial inoculation of soil contained in field macrocosms with the *P. violae* solid substrate inoculum resulted in a high incidence of cavity spot.

Knowledge and Technology Transfer

- Kat Hales presentation: SCI Young Researchers in Crop Sciences Conference, Syngenta, 14th July 2016.
- Kat Hales poster: AHDB Crops Studentship Conference, Stratford Upon Avon, 16th/17th November 2016.
- Kat Hales presentation: Carrot Advisor Experience Exchange Network (CAEEN), Thetford, 24th November 2016.
- Kat Hales presentation: 38th International Carrot conference, Bakersfield, California, 19th - 22nd March 2017.

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