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Development of inoculation methods for *Pythium violae* to evaluate resistance of carrot cultivars and efficacy of crop protection products for cavity spot control

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Abstract

Carrot is a major root vegetable crop grown in many parts of the world. In Europe, cavity spot disease continues to have a major impact on marketable yield. The disease is caused by several *Pythium* spp., with *P. violae* the main pathogen in the UK and results in small black lesions on mature carrot roots. The lack of reliable inoculation methods for these *Pythium* pathogens has, for many years, hampered the identification of new effective crop protection products or carrot varieties that are resistant to the disease. In this research, inoculation methods were developed for *P. violae* using mycelium or oospores, each of which successfully induced typical cavity spot symptoms in both pot- and field-grown carrots as well as consistent root stunting in the former. These methods were also used to successfully identify carrot cultivars with resistance to cavity spot and confirmed the efficacy of the fungicide metalaxyl-M against the disease. Results therefore demonstrated that the inoculation methods should be reliable for identifying the efficacy of crop protection products, assessing cavity spot resistance and for further studies investigating the biology and epidemiology of the pathogen.

KEYWORDS
carrot, cavity spot, inoculation, *Pythium violae*

1 | INTRODUCTION

Carrot (*Daucus carota*) is the UK's main root vegetable crop with a production area of 11,500 ha and a retail sales value of approximately £290 million (Hales, 2018). However, cavity spot disease continues to be a major constraint to production both in the UK and globally due to the potentially severe impact on carrot root quality, with losses in the UK estimated at £3–5 million per season (Hales, 2018). The disease is caused by several species of the oomycete pathogen *Pythium* and is typically characterized by dark sunken, elliptical lesions on the carrot root (Hiltunen & White, 2002). In the UK, *Pythium violae* is the main species associated with cavity spot followed by *Pythium sulcatum*, as is also the case in some other European countries (Hiltunen & White, 2002). However, *Pythium sylvaticum* and *Pythium intermedium* have also been isolated from cavity spot lesions in the UK (Hales, 2018) and Norway (Hermansen et al., 2007) and were the most common species associated with the disease in Canada (Allain-Boulé et al., 2004). Other reported *Pythium*

species associated with cavity spot include *Pythium coloratum* (El-Tarabily et al., 1997; Suffert & Guibert, 2007), *Pythium irregulare* (Suffert & Guibert, 2007), *Pythium ultimum* (El-Tarabily et al., 2004) and *Pythium recalcitrans* (Lu et al., 2013).

Management of cavity spot has, for years, relied on applications of metalaxyl and metalaxyl-M fungicides, but efficacy can decline significantly with repeated applications in the same fields due to enhanced microbial degradation (Davison & McKay, 1999; Kenny et al., 2001) and there have also been reports of pathogen populations developing resistance to these fungicides (Allain-Boulé et al., 2004). The historic reliance on this single fungicide for control of cavity spot, the decreasing efficacy in controlling the disease, and the potential withdrawal of registration of these products in some countries are major concerns for the sustainability of carrot production. Hence there is an urgent need to identify alternative effective fungicides or other approaches for control of cavity spot.

Carrot resistance to cavity spot would be of great benefit to the industry but although there are some commercially available cultivars with good partial resistance, other characteristics are also important for achieving high yield and marketable roots. For instance, the cv. Nairobi, which does not have high tolerance to cavity spot, is still very widely grown in the UK. There has been ongoing research to develop resistant carrot cultivars since the 1980s (reviewed by du Toit et al., 2019), and although cultivars such as Nandor have consistently been shown to have higher levels of resistance than others such as Nanco (Hiltunen & White, 2002), no complete resistance has ever been identified. In addition, as only a few cavity spot lesions make roots unmarketable, the level of resistance available in commercial cultivars is often insufficient to prevent economic loss.

A major obstacle to identify effective crop protection products or carrot germplasm with resistance to cavity spot is the lack of a reliable standard inoculation method for consistently inducing high levels of cavity spot symptoms. The life cycle of *P. violae* is not well defined and, unlike other *Pythium* species, isolates have never been observed to produce zoospores (Hiltunen & White, 2002). Although oospores are formed readily and assumed to be the primary source of inoculum in the field (Suffert & Montfort, 2007), efforts to induce and observe high levels of oospore germination have not been successful. Various inoculation approaches for *Pythium* species associated with cavity spot have also met with variable results. One technique often employed to test pathogenicity or assess resistance of carrot germplasm involves inoculating freshly harvested carrot roots with agar plugs colonized by mycelium (El-Tarabily et al., 1996, 2004; Guerin et al., 1994; Hermansen et al., 2007; Suffert & Guibert, 2007; Zamski & Peretz, 1995). Although this can give an indication of the pathogenicity of isolates of different *Pythium* spp., it is not appropriate or practical for testing control treatments such as fungicides or biological control agents, as this method does not always result in typical cavity spot lesions and also may not always accurately reflect the response of cultivars in field conditions (Vivoda et al., 1991). More realistic approaches to inoculation have therefore been investigated such as the use of naturally infested field soil or incorporation of infected carrots into soils (Suffert & Montfort, 2007; White, 1986). However, without being able to quantify inoculum accurately, results can be variable and unpredictable. An alternative method is to produce *Pythium* inoculum on an organic substrate and grow carrots in a medium amended with this inoculum. Although this approach has not been tested widely, it was used successfully to induce cavity spot lesions on carrot for *P. violae* grown on barley grain and *P. sulcatum*/*P. coloratum* grown on millet grain (El-Tarabily et al., 1996, 2004).

The main aim of this research was to develop reliable inoculation methods for *P. violae* that are suitable for carrots grown either in pots in the glasshouse or in small field plots ('macrocosms'). Through a series of experiments, we demonstrated that *P. violae* mycelial and oospore inocula could be used to induce high incidences of cavity spot in glasshouse and field experiments, respectively. Moreover, we showed that these methods successfully identified carrot cultivars with partial resistance to cavity spot and confirmed the activity of metalaxyl-M. This study therefore provides a way forward for evaluating the efficacy of crop protection products and assessing cavity spot resistance in carrot germplasm and for further studies investigating the biology and epidemiology of the pathogen.

2 | MATERIALS AND METHODS

2.1 | Culturing and maintenance of *Pythium* spp.

P. violae isolate HL (P10), derived from a cavity spot lesion on the Nantes-type carrot cv. Nairobi collected from a field in Holton, Lincolnshire, in 2013, was used in all experiments. In addition, *P. sulcatum* isolate P67, isolated from a cavity spot lesion on an unknown Chantenay-type carrot cultivar collected from Carlton in Lindrick, Nottinghamshire, in 2014, was used in one glasshouse experiment. *Pythium* species identity of the two isolates was confirmed by sequencing the internal transcribed spacer regions of the rDNA gene (Levesque & de Cock, 2004) and part of the cytochrome oxidase subunit II gene (*CoxII*; Martin, 2000). Sequences were deposited in GenBank (ITS: PP813757, PP813758; *CoxII*: PP849121, PP849122). *Pythium* isolates were cultured routinely on cornmeal agar (CMA; Sigma-Aldrich) or CMA with rifampicin (30 mg/L; CMA-Rif) at 17°C for 7 days in the dark. Plugs of mycelium excised from the colony margins were submerged in sterile water and maintained at 4°C for long-term storage.

2.2 | Preparation of *P. violae* oospore inoculum

A solid medium comprising horticultural sand and oatmeal was used for production of an oospore-based *P. violae* inoculum, similar to that used by Howard et al. (1978). The substrate was prepared by mixing dry horticultural sharp sand (425 g; J. Arthur Bowers) with finely milled organic oatmeal (5 g) in 1 L conical flasks and adding 75 mL sterile distilled water (SDW) to achieve a final moisture content of

15% wt/wt. Flasks were autoclaved twice for 15 min at 121°C with an interval of 24 h between each cycle, after which the medium was inoculated with agar plugs taken from the edge of an actively growing culture of *P. violae* isolate HL (15 plugs per 1 L flask) and incubated at 20°C in the dark for up to 7 months.

2.3 | Preparation of *P. violae and P. sulcatum* mycelial inoculum

Millet seed was used for production of *P. violae* and *P. sulcatum* mycelial inoculum (El-Tarabily et al., 2004). White millet seed (Cotswolds Seeds Ltd; 70 g) was moistened with 112 mL SDW in 500 mL conical flasks to achieve a final moisture content of 62% wt/wt. Flasks were autoclaved three times at 121°C for 30 min with 24 h between each cycle. After each cycle, the flasks were shaken well to avoid clumping of the moist grain. Each flask was inoculated with 14 agar plugs taken from an actively growing culture of *P. violae* HL or *P. sulcatum* P67 and incubated at 20°C for 3 weeks in the dark.

2.4 | Testing *P. violae* oospore and mycelial inocula and evaluating suitability for assessing cultivar resistance and efficacy of metalaxyl-M

A series of experiments (Table 1) was set up to test the effect of *P. violae* oospore inoculum on producing cavity spot symptoms for carrots grown in pots in the glasshouse (Experiments 1a,b) and in the field (small-plot macrocosms; Experiments 5a,b). *P. violae* mycelial inoculum was tested only in the glasshouse (Experiment 2) with further experiments then evaluating its suitability for assessing carrot cultivars for cavity spot resistance (Experiment 3) and the efficacy of metalaxyl-M in combination with susceptible and partially resistant cultivars (Experiment 4). Two additional macrocosm experiments also evaluated carrot cultivars for resistance in the field (Experiments 6a,b). The emphasis of the approach was to demonstrate development of robust *P. violae* inoculation methods through different experiments where carrot cultivars and metalaxyl-M were later introduced as a means of testing applicability for identifying control approaches in glasshouse and field environments.

2.4.1 | Experiments 1a/1b: Effect of *P. violae* oospore inoculum on producing cavity spot symptoms in glasshouse-grown carrots

Oospore inoculum of *P. violae* HL was prepared as described above and the contents of four 1-L flasks thoroughly mixed in a grip-seal bag to dislodge oospores and ensure homogeneity of the inoculum. Oospore concentration was then estimated by vortexing 1 g of this inoculum in 10 mL SDW for 1 min and counting oospores in a Sedgewick-Rafter counting chamber (two replicate counts for each of four 1 g samples). This inoculum was then diluted in horticultural grade sharp sand (Westland Horticulture Ltd) and mixed in a cement mixer (Belle Minimix 150; drum volume 130 L) for 3 min to obtain starter batches of inoculum at concentrations of approximately 1000

TABLE 1 Summary of experiments to test the effect of *Pythium* oospore and mycelial inocula on producing cavity spot symptoms in carrots grown in pots in the glasshouse and in field macrocosms and to evaluate their suitability for evaluating carrot cultivars for resistance and efficacy of metalaxyl.

Expt. no.	Expt. type[a]	Inoculum type[b]	*Pythium* sp. and inoculum concentration	Treatment and cultivar
1a	Glasshouse	Oospores	*P. violae* oospores (5–75 oospores/g)	Nairobi
1b	Glasshouse	Oospores	*P. violae* oospores (5–75 oospores/g)	Nairobi
2	Glasshouse	Mycelium	*P. violae*/*P. sulcatum* (5 & 50 mg/g)	Nairobi
3	Glasshouse	Mycelium	*P. violae* (5 mg/g)	10 carrot cultivars incl. Nairobi
4	Glasshouse	Mycelium	*P. violae* (10 & 30 mg/g)	4 carrot cvs incl. Nairobi ± metalaxyl-M (1.3 L/ha)
5a	Field (2017)	Oospores	*P. violae* oospores (5–50 oospores/g)	Nairobi
5b	Field (2018)	Oospores	*P. violae* oospores (5–50 oospores/g)	Nairobi
6a	Field (2020)	Oospores	*P. violae* (no inoculation)	4 carrot cvs incl. Nairobi
6b	Field (2021)	Oospores	*P. violae* (no inoculation)	4 carrot cvs incl. Nairobi

[a]Field dates indicate year of harvest.
[b]Oospores produced on sand/oat substrate; mycelium produced on millet seed.

and 5000 oospores/g. Appropriate amounts of these two concentrations of *P. violae* oospores were then mixed with a growing medium consisting of a 50:50 vol/vol mix of compost (John Innes No. 3) and horticultural grade sharp sand (Westland Horticulture Ltd) in a cement mixer to obtain final *P. violae* oospore concentrations of 5, 10, 25, 50 and 75 oospores/g in the growing medium. Each growing medium/inoculum mixture was prepared such that the compost:sand ratio was always 50:50 vol/vol. Round, plastic pots (5.5 L capacity, 20 cm diameter, 27.5 cm high) were filled with inoculated growing medium (5.65 kg per pot) at each of the five *P. violae* oospore concentrations. Non-inoculated control pots received a mixture of 50:50 vol/vol compost/sand only. Each pot was placed in a saucer (21 cm diameter, 3 cm deep) in a glasshouse compartment maintained at 18/16°C (day/night) with a 16 h photoperiod and watered to ensure a high moisture content before sowing with carrot seed (10 seeds per pot, cv. Nairobi; Elsoms Seeds Ltd). Initially, the growing medium was kept damp by gentle overhead watering while the carrot seedlings emerged, with additional weekly watering by filling the saucers. After 6 weeks, seedlings were thinned to five plants per pot. Once plants had established, watering was increased from above and below (via the saucers), depending on conditions, to keep the growing medium damp. Established carrot plants were watered weekly with a nutrient solution via the saucers from 9 weeks after sowing, alternating 2 N:1P:4 K (Vitafeed; Vitax Ltd) with 0 N:1 P:3 K (Solufeed Ltd). Aphiline (*Aphidius colemani*), Amblyline (*Amblyseius cucumeris*), Encarline (*Encarsia formosa*), Exhibitline Sf (*Steinernema feltiae*) and Hypoline (*Hypoaspis miles*) biocontrol agents (Bioline Agrosciences Ltd) were applied routinely for controlling aphids, thrips, whiteflies and sciarid flies. In total, 16 replicate pots of five carrots were prepared for each oospore concentration (80 carrots per treatment) in a randomized block design consisting of four blocks each containing four replicate pots of each treatment. The experiment was carried out twice.

Mature carrot roots were harvested 21 weeks after planting in the first experiment and 23 weeks after planting in the second. Carrots were removed gently from the soil, washed, and total root weight recorded. Each carrot was assessed for cavity spot incidence, recorded as the presence of one or more cavity spot lesions, and, as lesions were generally of uniform size, severity was recorded as the total number of lesions (El-Tarabily et al., 2004). Cavity spot lesions were only recorded if they were >2 mm in diameter and if the periderm was sunken or fully ruptured.

To confirm whether lesions were caused by *P. violae*, reisolation of the pathogen was attempted both from cavity spot lesions on the main body of the carrot root as well as from the long thin tap root. This was done for one carrot selected from two of the four pots per oospore concentration in each block (total eight roots per treatment). Small pieces of carrot tissue excised from selected cavity spot lesions in each treatment were plated directly onto CMA-Rif, incubated in the dark at 17°C, and examined for *P. violae* growth after 5–10 days. The long thin tap roots were surface sterilized in 70% ethanol for 1 min, washed twice in SDW and then cut into three sections of roughly equal length (top: nearest the base of the carrot, middle and bottom) for a total 24 root pieces per treatment. A 1–2 cm length of each section was then plated onto CMA-Rif and incubated as described previously.

2.4.2 | Experiment 2: Effect of *P. violae* and *P. sulcatum* mycelial inoculum on producing cavity spot symptoms in glasshouse-grown carrots

Mycelial inoculum of both *P. violae* HL and *P. sulcatum* P67 was prepared using millet seed as described above and mixed with a 50:50 vol/vol mix of the compost/sand growing medium in the cement mixer for 3 min to obtain final concentrations of 5 and 50 mg colonized millet seed/g. In addition, a 50:50 wt/wt mixture of *P. violae* HL and *P. sulcatum* P67 millet inoculum was autoclaved for 1 h to create two sets of 'dead inoculum' as control treatments, also at concentrations of 5 and 50 mg/g. This was to allow for the potential effect of the millet seed as a source of nutrients for carrot growth. Before setting up the experiment, a few grains of live and dead millet inocula were plated onto CMA and incubated for 5 days at 20°C to confirm that *P. violae* and *P. sulcatum* had successfully colonized the grain and that no live *Pythium* remained on the autoclaved inoculum. Inoculated compost was dispensed into eight replicate 20 cm diameter pots for both *P. violae* HL and *P. sulcatum* P67 for each millet concentration (5 and 50 mg/g). Similarly, eight pots were set up for dead inoculum of each pathogen at both millet concentrations. In addition, 16 untreated control pots were set up comprising non-amended compost/sand growing medium. Pots were arranged in the glasshouse in a randomized block design consisting of eight blocks, each with eight pots comprising two non-inoculated control treatments (no millet), *P. violae* and *P. sulcatum* millet inoculum at each of the two concentrations (four pots) and two dead inoculum treatments at each of the two concentrations. Carrot seeds (cv. Nairobi) were sown in each pot (10 seeds per pot to accommodate for any damping off caused by the *Pythium* isolates) and seedlings thinned to five plants per pot after 6 weeks (40 plants per treatment). Plants were maintained, harvested after 21 weeks, and assessed for cavity spot incidence and severity as described previously.

2.4.3 | Experiment 3: Assessment of glasshouse-grown carrot cultivars for cavity spot resistance using *P. violae* mycelial inoculum

The use of *P. violae* isolate HL mycelial inoculum was assessed as a means of identifying carrot cultivars with resistance to cavity spot. Seed of eight cultivars (Table 2) was obtained from both the UK Vegetable Genebank (Warwick, UK) and commercial sources, with selections including the widely grown commercial cv. Nairobi as well as the cv. Eskimo, which is recognized by the industry as highly resistant to cavity spot. Two breeding cultivars (T1308086 and T1308403), previously confirmed to be susceptible to cavity spot, were also included. The remaining six carrot cultivars were selected

TABLE 2 Carrot cultivars and landraces evaluated for cavity spot resistance.

Diversity set no.	Cultivar	Description
CDS002	Autumn King 2 Vita Longa	Flakee-type elite cultivar
CDS007	Nairobi	Nantes-type elite cultivar
CDS012	Red Elephant	St Valery-type heirloom advanced cultivar
CDS025	Azerbaijan landrace	Traditional landrace red/white carrot
CDS026	Daghestan landrace	Traditional landrace white carrot
CDS034	Criolla	Chantenay-type advanced cultivar
CDS040	Royal Star	Advanced cultivar
CDSX01	Eskimo	Nantes-type elite cultivar

based on their relative susceptibility to cavity spot as identified in a field trial in which a diverse set of 90 cultivars was evaluated in a field with a previous history of moderate cavity spot pressure (authors' unpublished data). Millet seed inoculum of *P. violae* HL was prepared as described previously and incorporated into the compost/sand growing medium to achieve a final concentration of 5 mg colonized millet seed/g. Eight replicate 20 cm diameter pots, each with six carrot plants, were set up for each cultivar (48 carrots per treatment), except for the standard cv. Nairobi for which 16 replicate pots were prepared. Four replicate pots of dead (autoclaved) inoculum control treatments were included for each cultivar. Pots were arranged in the glasshouse in a randomized block design consisting of four blocks, each with 32 pots comprising two of each of the nine carrot cultivars with *P. violae* millet inoculum, four of the cv. Nairobi with *P. violae* millet inoculum, and one each of all 10 cultivars with dead inoculum. Twelve carrot seeds were sown in each pot and seedlings thinned to six plants per pot after 6 weeks. Plants were maintained, harvested (21 weeks after planting) and assessed for cavity spot incidence and severity as described previously.

2.4.4 | Experiment 4: Assessment of glasshouse-grown carrot cultivars for cavity spot resistance in combination with metalaxyl-M treatment using *P. violae* mycelial inoculum

The use of *P. violae* isolate HL mycelial inoculum was also assessed as a means of identifying carrot cultivars with resistance to cavity spot alone and in combination with the crop protection product metalaxyl-M. The carrot cultivars used were Criolla (highly susceptible), T1308403 (a susceptible breeding cultivar), the industry standard Nairobi (moderately resistant) and Eskimo (highly resistant). Millet seed inoculum of *P. violae* HL was prepared as described previously and incorporated into the compost/sand growing medium to achieve final concentrations of 10 and 30 mg colonized millet seed/g for each cultivar and metalaxyl-M treatment. The experiment consisted of 16 treatments (four carrot cultivars × two *P. violae* inoculum concentrations with and without metalaxyl-M application) with eight replicate pots of six carrots per treatment (48 carrots per treatment). Non-inoculated control treatments comprising four replicate pots each of 10 and 30 mg/g dead (autoclaved) *P. violae* inoculum for each carrot cultivar were also set up. The 160 pots in the experiment were grouped into eight blocks each containing 20 pots, with four blocks placed on two benches on either side of the glasshouse compartment. The carrot cultivars were grouped into pairs of susceptible (cvs Criolla and T1308403) and partially resistant (cvs Nairobi and Eskimo) to cavity spot. The susceptible pair of cultivars was allocated to four blocks and the partially resistant pair of cultivars to the other four blocks, with two blocks of each type on each side of the compartment, and one block of each type at a different position along the length of the compartment. This arrangement allowed potential variation between the two sides of the glasshouse compartment and along the length of the benches to be separated from any treatment effects. Within each block, each of the pair of cultivars was allocated to 10 pots, comprising two pots for each of the four combinations of the two inoculum rates with and without metalaxyl-M, and one pot at each inoculum rate for the dead inoculum treatment. Carrots were sown in each pot (25 seeds per pot to accommodate potential damping off caused by *P. violae*), and seedlings thinned to six plants per pot 5 weeks after planting. Spray applications of SL 567A (metalaxyl-M) were made at 1.3 L/ha in 1000 L water (product label rate; equivalent to approx. 7 mL spray volume per pot) to the soil surface 1 day after sowing and again 3 weeks after sowing. The pots were briefly watered from above 1 day before and after metalaxyl-M application to maintain soil moisture. Plants were maintained as described previously with the exception that fertilizer applications were reduced to a single application of 2 N:1 P:4 K (Vitax Vitafeed) once every 2 weeks from 10 weeks after sowing. Carrots were harvested 18 weeks after planting and assessed for cavity spot incidence and severity as described previously.

2.4.5 | Experiments 5a/5b: Effect of *P. violae* oospore inoculum in producing cavity spot symptoms in field-grown carrots

Two field experiments were carried out to determine the effect of *P. violae* oospore inoculum in producing cavity spot symptoms, one from May 2016 to February 2017 (Year 1), and the second from May 2017 to March 2018 (Year 2), in 24 macrocosm field plots located at the University of Warwick, Wellesbourne, UK. Each macrocosm comprised a sunken concrete tube 100 cm in diameter and 60 cm deep that was filled with a 10 cm deep gravel layer, followed by a 20 cm layer of sandy silt loam ('Wick' series, Wellesbourne, UK; 225 kg) mixed with 40 kg horticultural sand (Westland). A 30 cm deep top layer consisting of 328 kg sieved soil and 60 kg horticultural sand was then added to provide an ideal sandy loam substrate for successive crops of carrot and inoculation with *P. violae*. In Years 1 and 2,

flasks of *P. violae* HL sand/oat oospore inoculum were produced and incubated for 4 and 3 months, respectively, as described previously. After determining the oospore concentration, dilutions were made with sand using a cement mixer to obtain bulk batches of inoculum at concentrations of approximately 1000 and 5000 oospores/g. These were further diluted in sand to provide 5 kg batches of inoculum, which were raked into the soil to achieve final *P. violae* oospore concentrations of 5, 10, 20, 30 and 50 oospores/g in the top 10 cm of soil in the appropriate macrocosms. Non-inoculated control plots received 5 kg sand alone. Each treatment was replicated across four macrocosms in a randomized complete block design. Each macrocosm was sown with approximately 280 carrot seeds of the cv. Nairobi, which were then covered with a 1–2 cm deep layer of sieved soil. In Year 2, macrocosms were reinoculated with 2-month-old *P. violae* HL oospore inoculum to achieve the same oospore concentrations as in Year 1 in the same macrocosms. Each was then sown with approximately 280 carrot seeds of the cv. Nairobi. In both years, macrocosms were watered regularly via an oscillating line and sprayed periodically with Hallmark (lambda-cyhalothrin; Syngenta) to prevent damage from carrot root fly (*Psila rosae*). In Year 2, macrocosms received a maintenance fertilizer dressing of 59 g 0:20:30 N:P:K and 14 g N (Nitram; CF Industries). A layer of straw was applied to the surface of each macrocosm in November 2016 (Year 1) and in early December 2017 (Year 2) to prevent winter frost damage. Carrots were harvested in February 2017 (Year 1) and March 2018 (Year 2) and roots washed and assessed for cavity spot incidence and severity as described previously. Isolations from typical cavity spot lesions on 20 carrots harvested in Year 2 were made by plating out carrot tissue on CMA-Rif as described above.

2.4.6 | Experiments 6a/6b: Assessment of field-grown carrot cultivars for cavity spot resistance using *P. violae* oospore inoculum

The *P. violae*-inoculated field macrocosms described above were also used to evaluate five carrot cultivars tested previously in the glasshouse experiment for cavity spot resistance over two growing seasons (May 2019–April 2020 and June 2020–Feb 2021): T1308086 (highly susceptible), the industry standard cvs Nairobi and Autumn King (moderately resistant) and cvs Eskimo and CDS025 (highly resistant). As described above, the field macrocosms were inoculated with *P. violae* HL oospore inoculum in 2016 and 2017 but no further inoculum was applied for this experiment. However, another carrot crop of the cv. Nairobi was grown in these macrocosms from May 2018 to March 2019 with high incidence and severity levels of cavity spot observed and little variation among the inoculated macrocosms (45%–52% disease incidence, data not shown). Prior to sowing seed of the five carrot cultivars, the soil in each macrocosm was dug over to remove any compaction, and a maintenance fertilizer dressing was applied (60 and 30 g 0:20:30 N:P:K in 2018 and 2019, respectively, with 14 g Nitram) in both years. Each macrocosm was sown with approximately 300 carrot seeds, with four macrocosms planted per cultivar in a randomized block design. To minimize any possible residual effect of the original inoculum concentration, each cultivar was allocated across macrocosms previously inoculated with four different oospore concentrations. In addition, the cv. Nairobi was allocated to the non-inoculated set of macrocosms. Crop maintenance, harvest and cavity spot disease assessment were carried out as described previously.

2.5 | Statistical analysis

Data were analysed by analysis of variance (ANOVA) using GenStat (22nd edition, VSN International Ltd), taking account of the blocking structures described for each experiment. Treatment factors (fixed terms) were inoculation rate, cultivar and metalaxyl-M rate (presence/absence), with some experiments involving combinations of these and hence interaction terms, while random terms were block (replicate) and plot/pot as can be inferred from the experimental design descriptions. Treatment comparisons were made at the 5% significance level ($\alpha = 0.05$). Cavity spot incidence data were expressed as the percentage of roots with one or more cavity spot lesions and prior to analysis were subjected to angular transformation, with the exception of the glasshouse and macrocosm experiments testing different carrot cultivars for cavity spot resistance (Experiments 3; 6a,b) where a logit transformation was used, to satisfy the ANOVA assumptions of homogeneity of variance and normality of data. Disease severity data were expressed as the mean number of cavity spot lesions per root averaged across all carrots, and subjected to a logarithmic transformation in all experiments prior to analysis, to satisfy the ANOVA assumptions. Carrot weight (g) was analysed similarly. Data for cavity spot incidence and severity presented in the text and figures are back-transformed values. Data presented in the tables in the supporting information are the transformed values used for statistical analyses. Where appropriate, statistical analyses examined differences between resistant and susceptible carrot cultivars, among cultivars within resistance categories, and between the non-treated control plots and the different combinations of *P. violae* inoculum rates and metalaxyl-M treatments.

3 | RESULTS

3.1 | Experiments 1a and 1b: Effect of *P. violae* oospore inoculum on producing cavity spot symptoms in glasshouse-grown carrots

In both experiments, a low incidence of post-emergence damping-off of carrot seedlings was observed, with a maximum of 2.6% and 1.5% recorded in the first and second experiments, respectively (data not shown). In Experiment 1a, there was weak evidence of differences in damping-off incidence among different oospore concentrations ($p = 0.064$), and at the two highest *P. violae* oospore concentrations of 50 and 75 oospores/g, the seedling mortality was 2.3% and 2.6%, respectively, which was significantly higher than

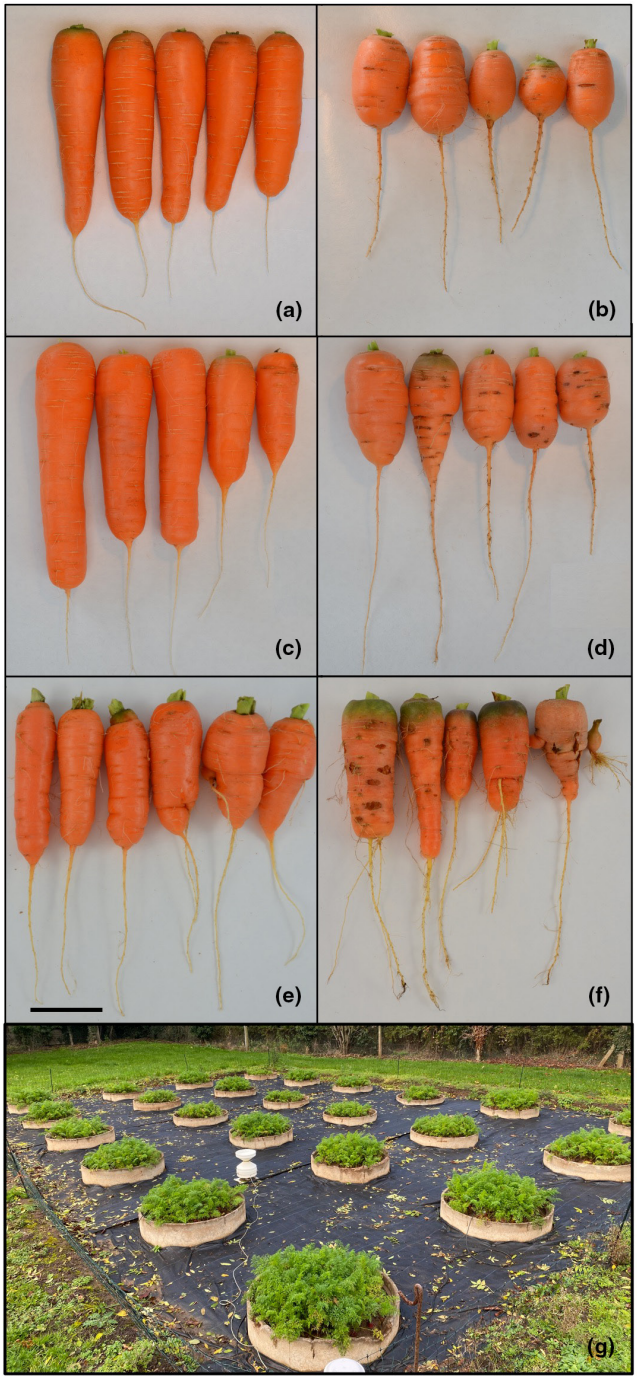

FIGURE 1 Pot-grown carrots from experiments inoculated with *Pythium violae* isolate HL illustrating root stunting and typical cavity spot lesions. (a) Normal growth of carrots for non-inoculated control treatment compared with (b) stunted carrots with cavity spot lesions grown in medium inoculated with *P. violae* sand/oat oospore inoculum at 50 oospores/g; (c) normal growth of carrots in medium amended with autoclaved (dead) inoculum compared with (d) stunted carrots with cavity spot lesions in medium inoculated with *P. violae* millet mycelial inoculum at 5 mg/g; (e) few cavity spot lesions observed on roots of partially resistant carrot cv. Eskimo compared with (f) severe, expanded cavity spot lesions on roots of the susceptible carrot cv. Criolla following inoculation with *P. violae* millet inoculum at 30 mg/g, (g) macrocosm field plots used to evaluate the effectiveness of *P. violae* oospore inoculum at inducing cavity spot symptoms in the field. Black bar for (a) to (f) = 5 cm.

for the non-inoculated control treatment and the lowest oospore concentration (5 oospores/g) (both had 1.2% seedling mortality). However, in the second Experiment 1b, there was no evidence of differences in carrot seedling survival among the different oospore concentrations and the non-inoculated control treatment ($p = 0.223$). At harvest, carrot roots from all the *P. violae*-inoculated treatments were distinctly stunted and characterized by a long, brown tap root with increased lateral root formation, many of which were collapsed (Figure 1b). In contrast, carrot roots from the non-inoculated treatment were longer and had healthy white tap roots (Figure 1a). Mean weight per carrot in the *P. violae* treatments was therefore significantly decreased ($p < 0.001$; Figure 2c, Table S1) compared to the non-inoculated control treatments in both experiments, ranging from 41 to 45 g and 34 to 42 g for Experiments 1a and 1b respectively, compared to 60 g and 48 g in the corresponding non-inoculated control treatments (Figure 2c). However, there was no apparent effect of different oospore concentrations on carrot weight.

Typical cavity spot symptoms of dark brown-black lesions that were sunken and round or elliptical in shape were observed on carrot roots in all the treatments inoculated with *P. violae* oospores for both experiments. In more expanded lesions, the periderm had completely ruptured to form cavities that were more irregular in shape. Characteristic *P. violae* colonies grew out from tap root pieces and from cavity spot lesions for selected carrots from different oospore concentration treatments when plated onto CMA-Rif, demonstrating successful colonization by the pathogen. Cavity spot incidence ranged from 10% to 20% of carrots in Experiment 1a and from 1% to 5% in Experiment 1b (Figure 2a), while cavity spot symptoms were not observed in non-inoculated control treatments. The effect of *P. violae* inoculum across all oospore concentrations on the incidence of cavity spot compared to the non-inoculated control was highly significant for Experiment 1a ($p < 0.001$), but just outside the 5% level of significance for Experiment 1b ($p = 0.063$; Table S1). Mean severity of cavity spot lesions in both experiments in all *P. violae*-inoculated treatments (across all carrots, including those without symptoms) was low and ranged from 0.18 to 0.31 cavities per carrot in Experiment 1a and from 0.03 to 0.09 cavities per carrot in Experiment 1b (Figure 2b). Nonetheless, the effect of *P. violae* inoculum on the number of cavities per carrot compared to the non-inoculated control treatments was highly significant for the first experiment ($p < 0.001$), and just outside the 5% level of significance for the second experiment ($p = 0.088$; Table S1).

3.2 | Experiment 2: Effect of *P. violae* and *P. sulcatum* mycelial inoculum on producing cavity spot symptoms in glasshouse-grown carrots

Overall, the dead *P. violae* millet seed inoculum used as the control treatment promoted carrot growth when used at the higher concentration in this experiment. Incorporation of the dead *Pythium* spp.

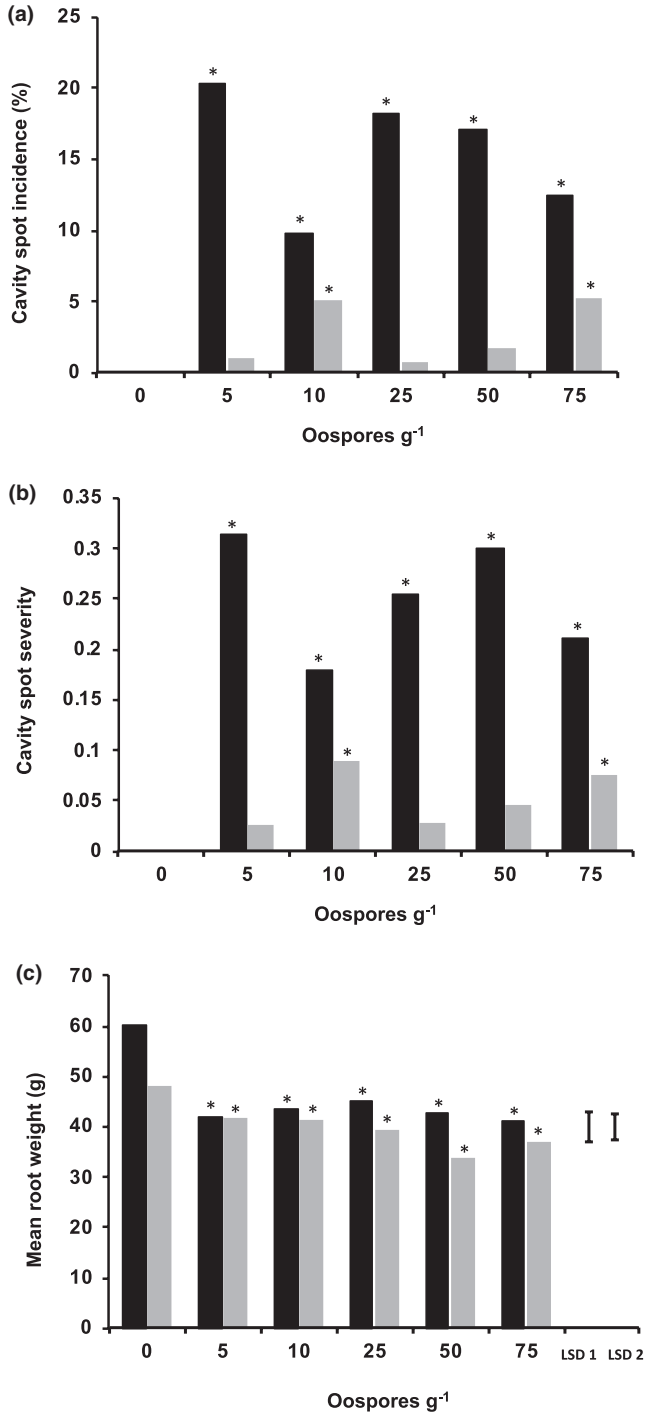

FIGURE 2 Experiments 1a, b: Effect of *Pythium violae* isolate HL inoculation at different concentrations of sand/oat oospore inoculum on (a) cavity spot incidence (% of carrot roots with symptoms, back-transformed data), (b) severity (no. of lesions per carrot averaged across all carrots, back-transformed data), and (c) mean carrot root weight (g) in two experiments. Black bars represent results for experiment 1; grey bars for experiment 2. Line bars represent the least significant difference (LSD) at the 5% level for the first and second experiments. Asterisks indicate treatments with significant differences compared with the non-inoculated control treatments. Statistical analyses are presented in Table S1.

inoculum (50% *P. violae*, 50% *P. sulcatum*) at 50 mg/g resulted in a significant increase in mean carrot root weight (129.1 g per carrot) compared to the non-inoculated control roots (no millet; 88.5 g per carrot; Figure 3c, $p < 0.05$, Table S2). In contrast, the mean root weight of carrots from the dead millet inoculum treatment applied to the growing medium at 5 mg/g (84.2 g) was not significantly different from that of the non-inoculated control roots (88.5 g, Figure 3c; $p > 0.05$, Table S2). Therefore, results below compare the *Pythium* millet mycelial inoculum treatments with the corresponding concentration of dead (autoclaved) inoculum control treatment to take account of the nutritional effect of the millet seed. Carrots inoculated with live *P. violae* inoculum at 50 mg/g had a significantly lower mean carrot root weight (88.2 g) compared with those in the corresponding dead inoculum control treatment (129.1 g, Figure 3c; $p < 0.05$, Table S2), while carrots inoculated with *P. sulcatum* P67 at the same inoculum concentration had complete pre-emergence damping-off and hence both carrot root weight and cavity spot data could not be recorded for this treatment. Inoculation of carrots with *P. violae* HL and *P. sulcatum* P67 at 5 mg/g also significantly reduced mean carrot root weight, to 63.4 and 62.0 g, respectively, compared with the corresponding dead inoculum control treatment of 84.4 g ($p < 0.05$; Figure 3c). Although there was an overall significant effect of *Pythium* isolate and inoculum concentration on mean carrot root weight ($p < 0.001$), there was no significant isolate × inoculum concentration interaction ($p = 0.101$; Table S2).

Typical cavity spot lesions were observed on carrots inoculated with mycelial inoculum of either *P. violae* HL or *P. sulcatum* P67 (Figure 1d). These lesions were generally larger and deeper than those seen for roots grown with the *P. violae* oospore inoculum in the previous experiments. Cavity spot incidence was also much greater for the *P. violae* mycelial inoculum treatments than for the oospore inoculum treatments with 49.5% and 82.0% of carrots affected at concentrations of 5 and 50 mg/g, respectively, compared with 17.4% for *P. sulcatum* P67 at 5 mg/g (Figure 3a). Cavity spot lesions were not observed on carrots from the dead inoculum control treatments or in the non-inoculated (no millet) control treatment. Overall, there were significant differences in cavity spot incidence between *P. violae* HL and *P. sulcatum* P67 ($p < 0.001$) and among the different inoculum concentrations ($p = 0.006$; Table S2). A significant isolate × inoculum concentration interaction was also evident ($p = 0.021$; Table S2) indicating that disease incidence varied depending on *Pythium* species and inoculum concentration. However, some caution must be exercised in interpreting the interaction effect, as values for the *P. sulcatum* P67 treatment at 50 mg/g were missing. As observed for cavity spot incidence, cavity spot severity was greater for the *P. violae* mycelial inoculum than for oospore inoculum described previously, with mean values of 0.80 and 1.85 cavities per carrot (across all carrots) at inoculum concentrations of 5 and 50 mg/g, respectively, compared with 0.22 cavities per carrot for the 5 mg/g *P. sulcatum* P67 treatment (Figure 3b). Overall, there were significant differences in cavity spot severity between the two *Pythium* species ($p < 0.001$) and among the inoculum concentrations ($p = 0.017$) and

there was also a significant isolate × inoculum concentration interaction ($p = 0.042$; Table S2). Again, some caution must be exercised in interpreting the interaction results, as there were no roots present for assessment for 50 mg/g of the *P. sulcatum* P67 treatment because of 100% pre-emergence damping-off.

3.3 | Experiment 3: Assessment of glasshouse-grown carrot cultivars for cavity spot resistance using *P. violae* mycelial inoculum

Overall, addition of *P. violae* millet inoculum significantly reduced carrot root weight ($p < 0.001$; Table S3) in this experiment. Mean carrot root weight of the inoculated carrots ranged from 39.1 g (Red Elephant) to 105.7 g (White Dagestan landrace), compared to 47.3 and 110.2 g, respectively, for these cultivars in non-inoculated control treatments (Figure 4c). Although there was a consistent negative effect of the inoculation on carrot root weight, some cultivars showed a greater response than others; for example, Royal Star and Eskimo carrots inoculated with *P. violae* had mean root weights of 51 and 55 g, respectively, which were significantly less than their respective non-inoculated control treatments of 71 and 78 g (Figure 4c; Table S3).

Typical cavity spot lesions were observed on all the carrot cultivars in inoculated treatments, while disease symptoms were not observed on carrot roots from any of the dead millet inoculum control treatments. The overall effect of inoculation with *P. violae* on cavity spot disease incidence was highly significant ($p < 0.001$, Table S3). Carrot cultivar also had a significant effect on cavity spot incidence ($p = 0.013$, Table S3), but the inoculum × cultivar interaction was not significant ($p = 0.194$, Table S3). Among the 10 carrot cultivars, mean cavity spot incidence ranged from 5.8% for cv. Azerbaijan landrace to 35% for Criolla (Figure 4a). Three cultivars, Azerbaijan landrace, Eskimo and Royal Star, had significantly less cavity spot incidence ($p < 0.05$; 5.8%, 6.6% and 8.6%, respectively) than the standard cv. Nairobi (21.5%; Figure 4a; Table S3).

Mean cavity spot severity across all carrot cultivars was low and ranged from 0.02 (cv. White Dagestan landrace) to 0.49 lesions per carrot (T1308403; Figure 4b), but *P. violae* inoculation still had a statistically significant effect on disease severity ($p < 0.001$; Table S3). A significant effect of cultivar on cavity spot severity was also evident ($p = 0.004$, Table S3). Disease severity for White Dagestan landrace, Azerbaijan landrace and Royal Star was 0.02, 0.04 and 0.06 lesions per carrot, respectively, and significantly lower than for Nairobi ($p < 0.05$; 0.22 lesions per carrot; Figure 4b; Table S3).

3.4 | Experiment 4: Assessment of glasshouse-grown carrot cultivars for cavity spot resistance in combination with metalaxyl-M treatment using *P. violae* mycelial inoculum

In this experiment, mean cavity spot incidence ranged from 2% to 99% of carrots affected across the four carrot cultivars when not treated with metalaxyl-M (Figure 5a), and there was a significant overall effect of inoculation with *P. violae* compared with the non-inoculated control carrots ($p < 0.001$; Table S4). There was also a significant overall effect of inoculum concentration ($p < 0.001$), with the higher concentration of 30 mg/g resulting in a greater incidence of cavity spot than the 10 mg/g concentration across all carrot cultivars. The same pattern was also observed for disease severity, where the mean number of cavity spot lesions ranged from 0.03 to 3.00 lesions per carrot across the carrot cultivars when not treated with metalaxyl-M (Figure 5b, Table S4). Cavity spot symptoms did not develop in any of the non-inoculated (dead inoculum) control treatments. Inoculation with *P. violae* also significantly reduced overall root weight compared to the non-inoculated (dead inoculum) control treatments for all the carrot cultivars ($p = 0.003$; Table S4). However, when comparing root weight for individual carrot cultivars inoculated with *P. violae* with the corresponding non-inoculated control treatment, a general trend of reduction in root weight was observed, but this was only significant for the susceptible cv. T1308403 inoculated at 10 mg/g (Figure 5c; Table S4).

Overall, there was a significant effect of carrot cultivar (resistant or susceptible pairs) on cavity spot incidence and severity ($p < 0.001$), and the susceptible carrot cvs Criolla and T1308403 consistently developed more disease than the more resistant cvs Nairobi and Eskimo (Figure 5a,b; Table S4). It was also noted that cvs Criolla and T1308403 had larger and more expanded lesions than Nairobi and Eskimo (Figure 1e,f). At the higher *P. violae* inoculum concentration of 30 mg/g, mean cavity spot incidence was 87% and 99%, respectively, for the susceptible cvs Criolla and T1308403 compared with 42% and 18% for the more resistant cvs Nairobi and Eskimo, respectively (Figure 5a). At the lower concentration of inoculum of 10 mg/g, cavity spot incidence was 21% and 14% for cvs Criolla and T1308403 compared with 3% and 2% for cvs Nairobi and Eskimo, respectively. There was a significant interaction between carrot variety and *P. violae* inoculum concentration ($p < 0.001$) such that the more resistant cvs Eskimo and Nairobi exhibited fewer lesions compared with the corresponding non-inoculated (dead inoculum) control treatments than the more susceptible cvs Criolla and T1308403, especially at the lower concentration of inoculum (Table S4). The same pattern of results was also observed for disease severity.

Treatment with metalaxyl-M significantly reduced the incidence of cavity spot across the carrot cultivars ($p < 0.001$) compared with untreated (inoculated) carrots of these cultivars (Table S4). For instance, for the susceptible cv. Criolla, mean cavity spot incidence was reduced by metalaxyl-M from 21% to 5% carrots affected at 10 mg/g *P. violae* inoculum concentration and from 87% to 28% carrots affected for 30 mg/g inoculum (Figure 5a). Disease incidence for cv. T1308403 was also reduced from 13% to 1% and from 99% to 13% for the same two inoculum concentrations, respectively. There were significant interactions between metalaxyl-M treatments and carrot cultivars ($p < 0.001$), and metalaxyl-M treatments and *P. violae* inoculum rate ($p < 0.001$) such that the effect of the fungicide tended to be less evident or nonsignificant on the more resistant cvs Nairobi and Eskimo at the lower inoculum level.

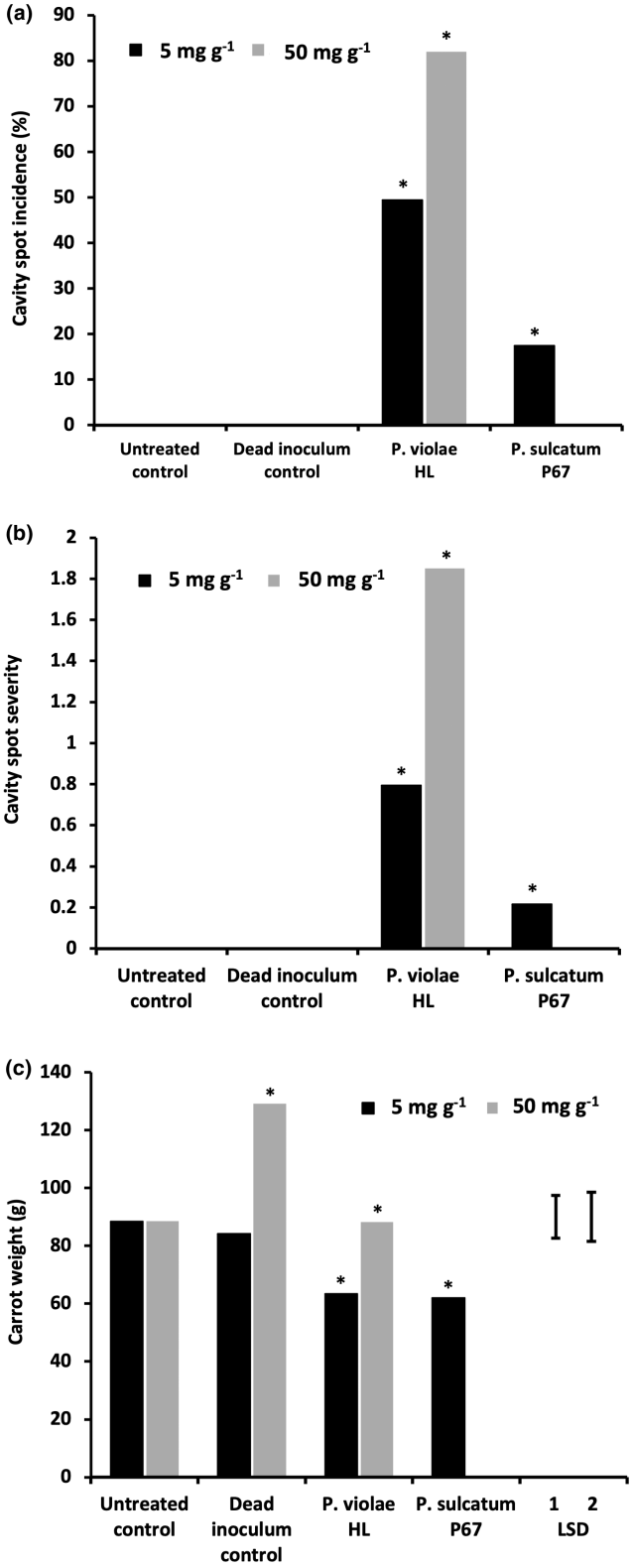

FIGURE 3 Experiment 2: Effect of different concentrations (5 mg/g and 10 mg/g) of millet seed mycelial inoculum of *Pythium violae* isolate HL and *P. sulcatum* isolate P67 on (a) cavity spot incidence (% of carrot roots with symptoms, back-transformed data), (b) severity (no. of lesions per carrot averaged across all carrots, back-transformed data) and (c) mean carrot root weight (g). Line bars represent LSDs (5% level) for comparison between the untreated control treatment (no millet; 16 replicates) and inoculated treatments (eight replicates; LSD 1) and for comparison among inoculated treatments only (eight replicates; LSD 2). Asterisks indicate treatments with significant differences from the corresponding untreated (no millet) control treatments or autoclaved (dead) inoculum control treatments. Statistical analyses are presented in Table S2.

However, at the higher *P. violae* inoculum level of 30 mg/g, metalaxyl-M significantly reduced cavity spot incidence from 42% to 3% for the moderately resistant cv. Nairobi and from 18% to 1% for the resistant cv. Eskimo ($p < 0.001$; Figure 5a; Table S4). The same pattern was also observed for cavity spot severity, for example, where applying metalaxyl-M significantly reduced the mean number of cavity spot lesions from 3.1 to 0.2 lesions per carrot for the susceptible cv. T1308403 at 30 mg/g millet inoculum concentration ($p < 0.001$; Figure 5b; Table S4). However, overall there was no significant difference in mean carrot root weight between *P. violae*-inoculated plants treated with metalaxyl-M and those that were untreated ($p = 0.783$).

3.5 | Experiments 5a and 5b: Effect of *P. violae* oospore inoculum on producing cavity spot symptoms in field-grown carrots

In both experiments harvested in 2017 and 2018, there was no evidence of the stunting of carrot roots observed in the glasshouse experiments where *P. violae* oospore inoculum was also tested and, therefore, there were no significant differences in carrot weight among inoculated and non-inoculated treatments (data not shown). However, in both field seasons, typical cavity spot lesions were observed on carrot roots, and across all the inoculated macrocosms, disease incidence ranged from 24% to 39% roots for the carrots harvested in 2017 (Experiment 5a) and from 30% to 36% for those harvested in 2018 (Experiment 5b; Figure 6a). In both experiments, all the inoculated treatments resulted in significantly greater cavity spot incidence than the non-inoculated control plots ($p < 0.001$, Table S5), although there was some background cavity spot in the non-inoculated plots, with a mean incidence of 1% in 2017 (Experiment 5a), which increased to 6% in 2018 (Experiment 5b; Figure 6a). Despite the high cavity spot incidence, disease severity was low and ranged from 0.46 to 0.72 lesions per root in 2017, and from 0.69 to 0.86 lesions per root in 2018 (Figure 6b), which was significantly greater than for the non-inoculated control plots ($p < 0.001$; Table S5). However, as observed for the initial glasshouse experiments, there were no significant differences in cavity spot incidence or severity among macrocosms inoculated with different oospore concentrations. Following isolation of *Pythium* spp. from typical cavity spot lesions on carrots harvested from inoculated macrocosms in 2018, all were identified as *P. violae*, while *P. violae*, *P. sulcatum* and *P. intermedium* were all identified in the few carrots with lesions from non-inoculated control plots (data not shown).

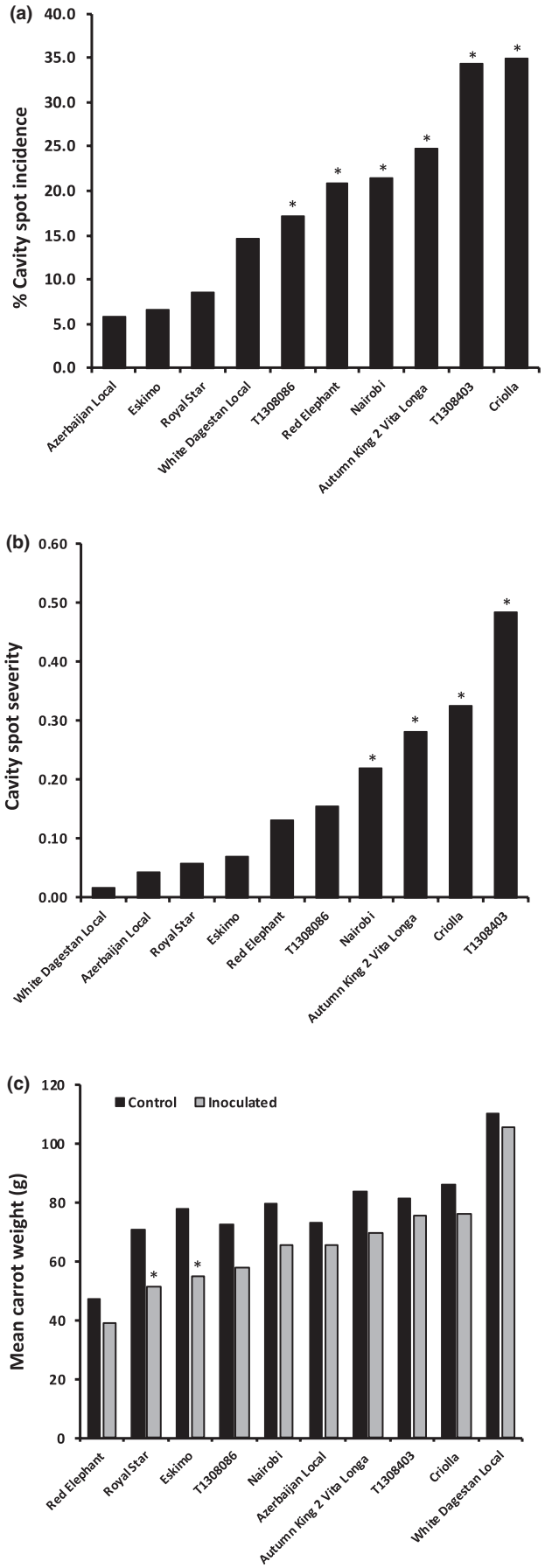

FIGURE 4 Experiment 3: Effect of millet seed mycelial inoculum of *Pythium violae* isolate HL (5 mg/g) on (a) cavity spot incidence (% of carrot roots with symptoms; back-transformed data), (b) severity (no. lesions per carrot averaged across all carrots; back-transformed data) and (c) mean carrot root weight for 10 cultivars. Asterisks indicate treatments significantly different from the autoclaved (dead) inoculum control treatment. Statistical analyses are presented in Table S3.

3.6 | Experiments 6a and 6b: Assessment of field-grown carrot cultivars for cavity spot resistance using *P. violae* oospore inoculum

In both experiments harvested in 2020 and 2021, characteristic cavity spot lesions were observed on all carrot cultivars grown. Interestingly, the background level of cavity spot incidence in non-inoculated control plots of the cv. Nairobi increased from 6% in 2018 (Experiment 5b; Figure 6a) to 18% and 20% in 2020 (Experiment 6a) and 2021 (Experiment 6b), respectively (Figure 7a). Overall, there was a significant effect of carrot cultivar on cavity spot incidence and severity in both 2020 and 2021 ($p < 0.001$; Table S6). At harvest in April 2020 (Experiment 6a), cavity spot incidence for cvs Azerbaijan landrace and Eskimo was 20% and 24%, respectively, which was significantly less than for cvs T1308086, Nairobi and Autumn King 2 Vita Longa (46%, 46% and 53%, respectively; $p < 0.05$ Figure 7a; Table S6) but not significantly different from that of cv. Nairobi grown in the non-inoculated control plots (18%). Although cavity spot incidence was lower in 2021 overall (Experiment 6b), the same pattern of results was observed among the five carrot cultivars. Cavity spot incidence for cvs Azerbaijan landrace and Eskimo was 13% and 16%, respectively, which was significantly less than for cvs T1308086, Nairobi and Autumn King 2 Vita Longa (33%, 32% and 40%, respectively; $p < 0.05$, Figure 7a; Table S6). The same pattern of results was observed for cavity spot severity in each experiment as cvs Azerbaijan landrace and Eskimo had significantly fewer lesions (both had 0.3 lesions per carrot in 2020 and 0.2 lesions per carrot in 2021) compared to cvs T1308086, Nairobi and Autumn King 2 Vita Longa in both 2020 (0.8, 0.9 and 1.2 lesions per carrot, respectively) and 2021 (0.6, 0.6 and 0.9 lesions per carrot, respectively; $p < 0.05$, Figure 7b; Table S6).

4 | DISCUSSION

This research successfully developed inoculation methods for inducing cavity spot symptoms in carrots grown in the glasshouse and field using either *P. violae* oospores (oatmeal/sand inoculum) or mycelium (millet seed inoculum). Given the time taken to grow carrots to maturity to observe cavity spot symptoms (including in the glasshouse), these experiments could not all be repeated, which was a limitation in our approach. Nevertheless, results identifying carrot cultivars with partial resistance to cavity spot and the efficacy of metalaxyl-M in follow-up glasshouse experiments with the mycelial millet inoculum

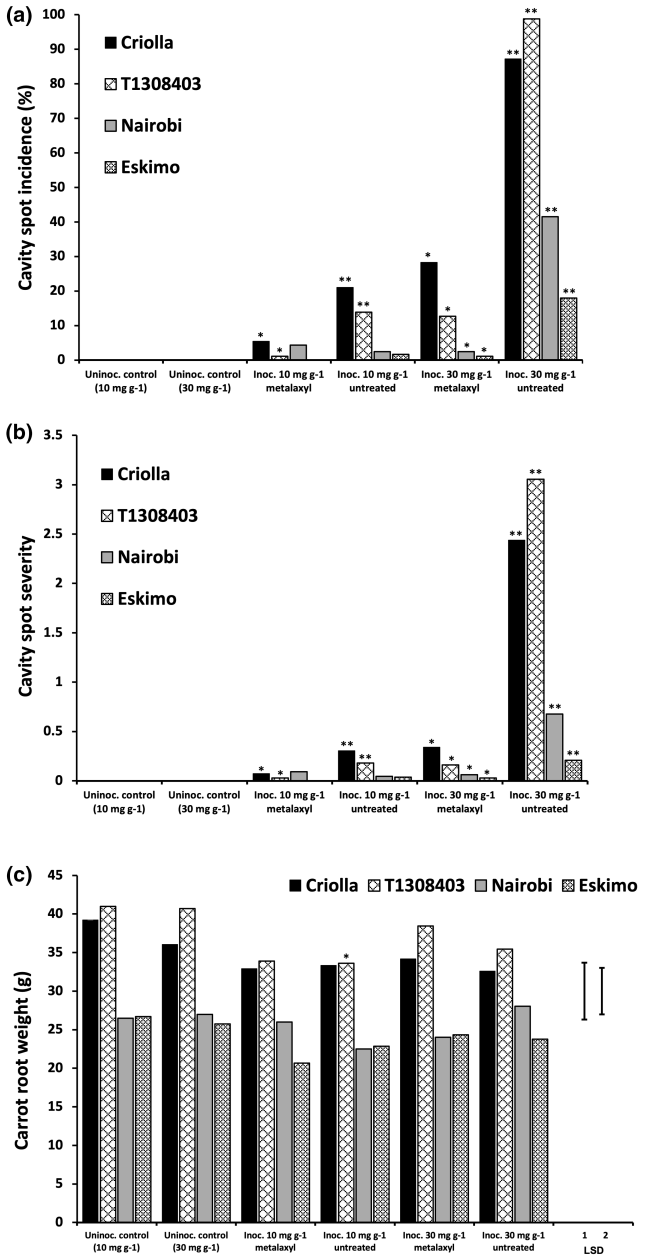

FIGURE 5 Experiment 4: Effect of carrot cultivar and metalaxyl-M treatment for two concentrations at 10 mg/g and 30 mg/g *Pythium violae* millet seed mycelial inoculum (isolate HL) on (a) cavity spot incidence (% of carrot roots with symptoms, back-transformed data), (b) severity (no. of lesions per carrot averaged across all carrots, back-transformed data) and (c) mean carrot root weight (g). Line bars represent least significant differences (LSD) at the 5% level for comparison between the *P. violae*-inoculated treatments (eight replicates; LSD 1) and the non-inoculated (dead inoculum) control treatments (four replicates), and for comparison between metalaxyl-M treated and untreated inoculated carrots (eight replicates; LSD2). *indicates metalaxyl-M treatments for each carrot cultivar that are significantly different from *P. violae*-inoculated untreated plants. **indicates *P. violae*-inoculated treatments for each carrot cultivar that are significantly different from the non-inoculated control treatment. Statistical analyses are presented in Table S4.

demonstrated that this method was robust at producing consistent disease symptoms in cv. Nairobi across a total of three experiments. Similarly, it was demonstrated that *P. violae* oospore inoculum resulted in consistent cavity spot symptoms in carrots grown in field macrocosms (cv. Nairobi and other cultivars) in 4 years between 2017 and 2021 following two rounds of inoculation in 2016/2017. Overall, this demonstrated that our inoculation methods were robust and therefore should provide a reliable and realistic means of identifying carrot cultivars with resistance to cavity spot and testing crop protection chemicals, which has previously been challenging. However, as the focus of this research was on method development, more research should be carried out to confirm the utility of these inoculation methods in identifying further sources of carrot cultivar resistance and new products or approaches to cavity spot control such as microbial biological control agents and biostimulants.

In the glasshouse, both inoculum types resulted in typical cavity spot lesions, but use of *P. violae* millet seed mycelial inoculum resulted in higher and more consistent levels of cavity spot compared with the *P. violae* oat/sand oospore inoculum. There was also a clear effect of mycelial inoculum concentration on disease but this was not the case with the oospore inoculum. The use of inoculated millet grain has previously been shown to induce cavity spot lesions in carrots using *P. sulcatum* and *P. coloratum* (El-Tarabily et al., 1996). However, in contrast to that study, artificial inoculation with *P. violae* in this research also caused some seedling death as well as the formation of stubby, stunted carrot roots with brown, malformed, hairy taproots in the glasshouse experiments. Such large effects on carrot growth have not been reported commonly but similar symptoms were observed in two studies (Pratt & Mitchell, 1973; White, 1986). The *P. violae* millet seed mycelial inoculum had several advantages compared with the oospore inoculum in that it was much quicker to produce (3–4 weeks as opposed to 3–4 months), can readily be quantified and overcomes problems associated with oospore viability or providing correct environmental or plant factors required for oospore germination. These are also potential reasons for the lack of a significant association between oospore concentration and cavity spot incidence or severity observed in both the glasshouse and field experiments. Suffert and Montfort (2007) reported the potential for secondary infections from carrots already affected that might influence response to inoculum concentration. As *P. violae* is not known to produce zoospores (Hiltunen & White, 2002) and oospores have not been observed to germinate readily on agar media, it is difficult to readily assess viability, which also suggests that the factors noted above may be important for germination. Poor germination of oospores is common among different *Pythium* spp., and this has been attributed to various reasons relating to constitutive dormancy (Higginbotham et al., 2004; Van der Plaats-Niterink, 1981), oospore age or exposure to soil (Ayers & Lumsden, 1975; Mondal et al., 1996) or germination only in the presence of root exudates (Hendrix & Campbell, 1973; Huisman, 1982). None of these factors has been investigated for *P. violae* although Suffert and Lucas (2008) demonstrated that carrot root exudates did not enhance plant infection or mycelial growth.

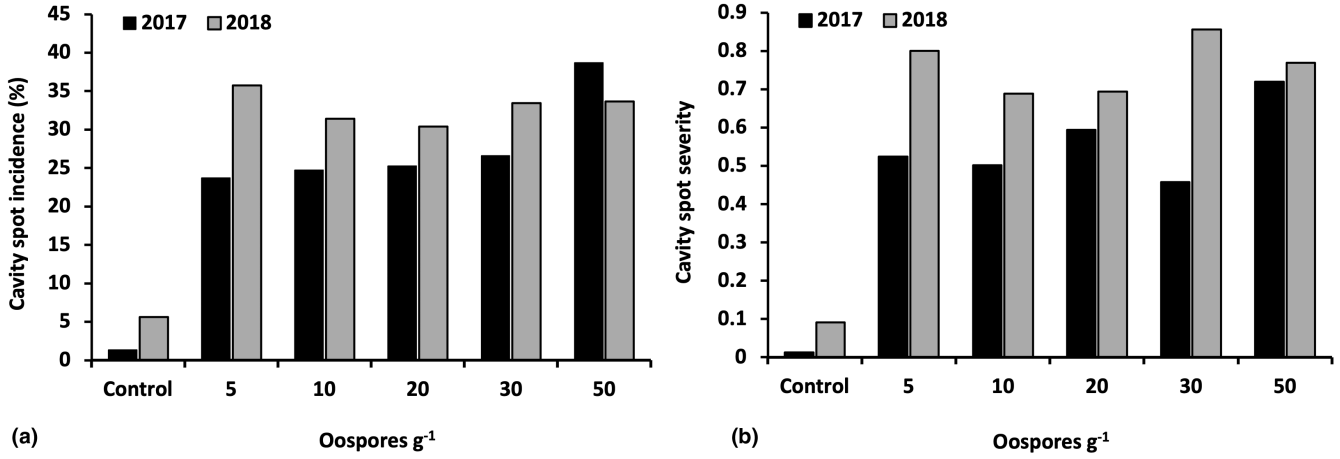

FIGURE 6 Experiments 5a, b. Effect of *Pythium violae* isolate HL inoculation with different concentrations of sand/oat oospore inoculum in field macrocosms (2017, 2018) on (a) cavity spot incidence (% of carrot roots with symptoms, back-transformed data) and (b) severity (no. of lesions per carrot averaged across all carrots, back-transformed data). Statistical analyses are presented in Table S5.

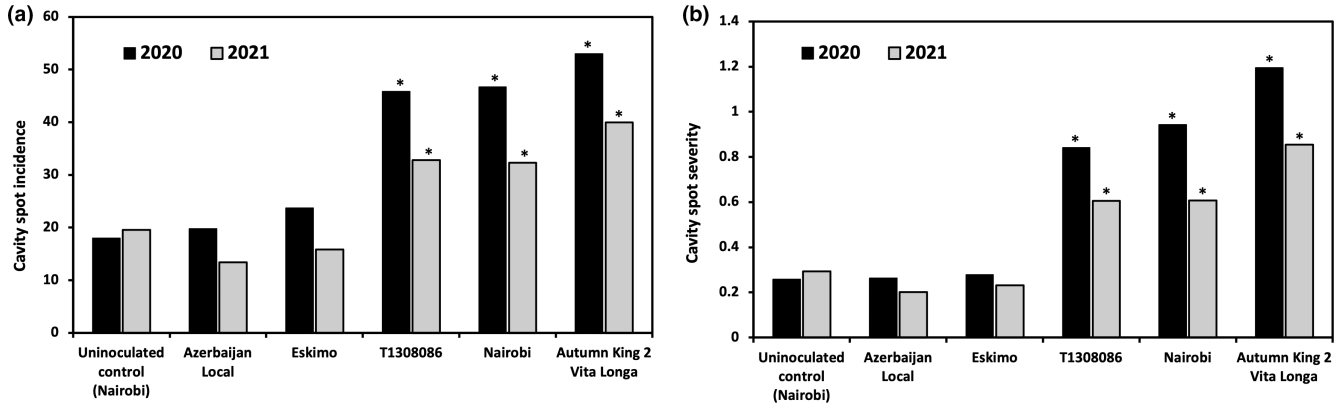

FIGURE 7 Experiments 6a, b: Assessment of (a) cavity spot incidence (% of carrot roots with symptoms, back-transformed data) and (b) cavity spot severity (no. of lesions per carrot averaged across all carrots, back-transformed data) for five carrot cultivars grown in 2020 and 2021 in field macrocosms inoculated with *Pythium violae* isolate HL oospores. Asterisks indicate treatments significantly different from the corresponding non-inoculated control plots. Statistical analyses are presented in Table S6.

The utility of the *P. violae* millet seed mycelial inoculum inoculation method for identifying cavity spot control approaches in glasshouse-grown carrots was demonstrated in this study in Experiments 3 and 4, in which the resistance status of different carrot cultivars was assessed and the efficacy of the fungicide metalaxyl-M against *P. violae* confirmed. The cvs Eskimo and Royal Star, as well as Azerbaijan and White Daghestan landraces, all exhibited a high level of resistance compared with cv. Autumn King, which was moderately resistant, while cv. Criolla and the breeding cv. T1308403 were highly susceptible. While cv. Eskimo is known by the industry to have a high level of cavity spot resistance, the identification of resistance in the two landraces represents potentially new material for future breeding programmes and highlights the importance of maintaining diversity in carrot genetic resources for development of such beneficial traits (Allender, 2019). The widely used commercial cv. Nairobi was moderately resistant to cavity spot as has been reported in numerous field trials at the Muck Crops Research Station of the University of Guelph (Ontario, Canada) at a site with a high level of disease pressure (McDonald et al., 2022).

In the glasshouse experiment testing cultivar effects in combination with metalaxyl-M (Experiment 4), the resistance of cv. Eskimo and the susceptibility of both cvs Criolla and T1308403 were confirmed, while the fungicide significantly reduced disease incidence and severity across all cultivars, with the greatest effect observed for the more susceptible cultivars. An interaction between metalaxyl-M and carrot cultivar was observed as the efficacy of the fungicide was less evident on the more resistant cultivars Eskimo and Nairobi at the lower *P. violae* inoculum concentration, as expected. Nevertheless, the results demonstrated that combining metalaxyl-M application with use of a partially resistant carrot cultivar is a very effective strategy for managing cavity spot compared with each control approach used alone. For instance, at the higher level of inoculum, cv. Eskimo treated with metalaxyl-M had 1% cavity spot incidence compared with 18% without the fungicide treatment,

while for the more susceptible cv. Criolla, incidence was 28% in the metalaxyl-M treatment and 87% in the untreated carrots. Although such combined control approaches are recommended, there are apparently no reports in which this has been demonstrated for cavity spot. The efficacy of metalaxyl-M in this study validated our mycelial-based inoculation method as being suitable for evaluating crop protection products for control of cavity spot. The efficacy of this fungicide as well as the original metalaxyl compound has been well documented. For instance, Lyshol et al. (1984) reported a decrease in cavity spot incidence from 46% to 4% in field-grown carrots when applied as a spray at sowing, and early applications within 4 weeks of sowing have been shown to be essential for optimum control (Gladders & McPherson, 1986; Lyshol et al., 1984). By comparison, for the susceptible cv. Criolla, metalaxyl-M reduced cavity spot incidence from 27% to 13% for the 10 mg/g *P. violae* inoculum concentration and from 69% to 32% carrots for the 30 mg/g inoculum concentration in this study. However, the long-term use of metalaxyl-M for cavity spot control globally has resulted in decreased efficacy in some areas either due to enhanced microbial degradation or development of fungicide tolerance in some pathogen populations (Allain-Boulé et al., 2004). These problems, alongside the risk of withdrawal of registration of these fungicides for use on carrot crops, emphasizes the need to identify effective crop protection products for cavity spot control.

The field soil macrocosm experiments using *P. violae* sand/oat oospore inoculum successfully induced cavity spot symptoms, with incidence ranging from 30% to 36% of the roots of cv. Nairobi in the second year (2018). As observed for the glasshouse experiments using oospore inoculum, there was little or no effect of inoculum concentration on cavity spot incidence or severity and there was also no stunting of carrot roots, with larger lesions perhaps more typical of those observed in commercial carrot crops. These results suggest that the field soil environment is more conducive to cavity spot development from oospore inoculum compared to using growing medium in the glasshouse, perhaps due to exposure to certain soil abiotic/biotic factors or environmental conditions that promote activation of oospores and infection of carrot roots. The macrocosms were also employed successfully in Experiments 6a and 6b to assess the relative resistance of several carrot cultivars used in the glasshouse test which confirmed that cvs Eskimo and Azerbaijan landrace had good levels of resistance compared with the more susceptible cvs Autumn King, Nairobi and the breeding cultivar T1308403. This also indicated that field soil inoculation with *P. violae* oospores results in comparable results to the glasshouse experiment with mycelial inoculum.

Overall, the millet seed *P. violae* mycelial inoculum represented the most efficient approach for inoculation of carrots in the glasshouse studies while the sand/oat oospore inoculum may be more appropriate for field inoculation where disease pressure can be maintained through successive crops of carrots. Both approaches successfully distinguished differences in carrot cultivar resistance and also have the potential to be used to identify effective fungicides or other crop protection products such as biological control agents. However, the concentration of mycelial inoculum in the glasshouse may need to be considered carefully such that either very susceptible carrot cultivars are used to test crop protection products at the lower level of *P. violae* inoculum or higher levels of inoculum are used for more resistant varieties such as the widely grown commercial cv. Nairobi.

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DATA AVAILABILITY STATEMENT

The datasets generated during this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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