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Demonstration of secondary infection by *Pythium violae* in epidemics of carrot cavity spot using root transplantation as a method of soil infestation

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Cavity spot of carrot (CCS), one of the most important soilborne diseases of this crop worldwide, is characterized by small sunken elliptical lesions on the taproot caused by a complex of pathogens belonging to the genus *Pythium*, notably *P. violae*. In most soilborne diseases the soil is the source of inoculum for primary infections, with diseased plants then providing inoculum for secondary infections (both auto- and alloinfection). Using fragments of CCS lesions to infest soil, it was demonstrated that CCS lesions on carrot residues can cause primary infection of healthy roots. Using a novel soil infestation method, in which an artificially infected carrot root (the donor plant) was placed close to healthy roots (receptor plants) the formation of typical CCS lesions were induced more efficiently than the use of classical soil inoculum and showed that CCS can spread from root to root by alloinfection from transplanted diseased roots. The method also demonstrated the polycyclic nature of a CCS epidemic caused by *P. violae* in controlled conditions. Secondary infections caused symptoms and reduced root weight as early as two weeks after transplantation of the diseased carrot. This reproducible method may be used for delayed inoculation and for studying the effect of cropping factors and the efficacy of treatments against primary and secondary cavity spot infections.

Keywords: alloinfection, carrot cavity spot, *Daucus carota*, epidemiology, *Pythium* spp, soilborne pathogen

Introduction

Cavity spot (CCS) is one of the most damaging fungal diseases of carrot worldwide, causing severe yield losses and quality deterioration (Guba *et al*., 1961; Hiltunen & White, 2002). This soilborne disease is characterized by small sunken elliptical lesions on the taproot caused by a complex of pathogens belonging to the genus *Pythium*. In France, cavity spot is mostly caused by *P. violae* (Montfort & Rouxel, 1988).

Soilborne plant pathogens are transmitted by various biological and physical mechanisms, including mycelial growth from organic residues in soil or from an infected host plant, spore dispersion in soil water, and the growth of root systems bringing infected and healthy roots into contact. Several soilborne diseases have two epidemiological phases – a monocyclic 'simple interest' phase (single infection cycle) and a polycyclic 'compound interest' phase (sequence of at least two infection cycles within a growing season) (Zadoks & Schein, 1979). For these diseases, soil inoculum (spores, sclerotia, mycelium) is responsible for the primary infections (the monocyclic phase). Diseased plants then provide inoculum for secondary infections mediated by direct root-to-root contact or mycelial growth in soil (the polycyclic phase).

Secondary infection cycles have been demonstrated and quantified for some soilborne fungal diseases: white rot due to *Sclerotium cepivorum* in onion and garlic (Scott, 1956a,b; Crowe & Hall, 1980), sunflower wilt due to *Sclerotinia sclerotiorum* (Huang & Hoes, 1980), fusarium crown and root rot of tomato caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Rekah *et al*., 1999, 2001), pea root rot due to *Aphanomyces euteiches* (Pfender & Hagedorn, 1983), wheat take-all due to *Gaeumannomyces graminis* var. *tritici* (Colbach *et al*., 1997; Bailey & Gilligan, 1999), and diseases caused by *Phytophthora parasitica* var. *nicotianae* (Shew, 1987) or by *Phymatotrichum omnivorum* attacking roots of cotton (Koch *et al*., 1987).

Differentiation between primary and secondary infection cycles has also been modelled. The model produced for take-all of wheat was based on epidemiological data and depended on two parameters reflecting the importance of the primary and secondary infection cycles (Colbach *et al*., 1997). Bailey & Gilligan (1999) suggested

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that the initial phase of primary *G. graminis* var. *tritici* infection in winter wheat occurs when the seminal roots grow through the soil and encounter inoculum. Mycelial growth causes secondary autoinfection (infection from lesions on the same root) or alloinfection (root-to-root contamination). Other non-linear models of epidemics involving differentiation between primary and secondary infection cycles have been proposed, for the damping-off of cress caused by *Pythium ultimum* (Brassett & Gilligan, 1988), *Polymyxa betae* infection in sugar beet (Webb *et al*., 1999) and *Rhizoctonia solani* infection causing damping-off in radish seedlings (Otten *et al*., 2003).

A theoretical distinction should be made between auto- and alloinfection in the development of epidemiological models (Zadoks & Schein, 1979), but few experiments have provided formal proof of the existence of these two phases and empirical evidence for the plant-to-plant spread of the diseases. Pfender (1982) argued that an understanding of the disease cycle is important and that useful information could be obtained by investigating the existence of secondary infection cycles, rather than observing disease progression over time, as the effects of the disease cycle and of other factors may be confounded in progression analyses.

Suffert (2006) recently suggested that CCS might resemble other soilborne plant pathogens in displaying primary infection and secondary infection (autoinfection and alloinfection). Some carrot roots have many lesions whereas others have few or none (Guba *et al*., 1961). Increases in CCS incidence *i*, density of lesions *d*, and total disease area *tda* over time, and the nature of the clustering of lesions, suggest that CCS pathogens are involved in a 'reproductive process', possibly based on mycelial growth over root surfaces (Phelps *et al*., 1991). Correlations have been found between disease measurements: the nature and form of these disease relationships may provide clues to epidemiological processes and dispersal mechanisms (Large, 1966; McRoberts *et al*., 2003). For CCS, using the model $i = b \cdot (1 - \exp(a(t) \cdot tda))$, it was shown that a(t) varied over time t, reflecting a balance between epidemiological phases: soil inoculum mobilization leading to primary infection, disease intensification on the root giving autoinfection, and disease extensification on neighbouring roots due to the plant-to-plant spread of the pathogen, resulting in alloinfection (Suffert, 2006).

The objective of the present study was to demonstrate the occurrence of secondary infection in CCS caused by *P. violae*. The first step was to show that CCS lesions on carrot residues were potentially infectious for healthy roots. The second step was to show that CCS can spread from root to root, by the transplantation of a diseased root within a healthy carrot population.

Materials and methods

Infection experiments

A series of experiments conducted in controlled conditions was carried out to demonstrate and compare three infectious processes in soil: i) method A – primary infection via soil inoculum to healthy root, using a classical mixed inoculum of mycelium and oospores of *P. violae* cultured *in vitro* in dishes, ii) method B – primary infection via infected carrot residues to healthy root, using fragments of carrot roots with CCS lesions induced by *P. violae* in a natural field epidemic, and iii) method C – secondary infection via diseased root to healthy root, achieved by the transplantation of a diseased root artificially inoculated with *P. violae* into the soil. Adapting the terminology of Huang & Hoes (1980), the infectious carrot root transplanted into the soil was considered to be the 'donor plant' (infected host) and carrot roots in the vicinity of this infectious root to be 'receptor plants' (healthy hosts). The donor root exhibited CCS lesions following artificial inoculation with *P. violae* and was still living and physiologically active.

Inoculum, plants and disease assessment

An isolate of *P. violae* Pv490 (CBS 102·609), obtained in 1994 from CCS-affected carrots symptoms in a carrot field in Normandy, France, was used as inoculum for each infestation method. The specific protocols of infestation are explained below. Statistical analyses were carried out using the SAS statistical package, version 8·1 (SAS, 2000). Treatment effects (inoculum doses) were compared using the ANOVA procedure.

Each microcosm for the three experiments consisted of a circular 4 L pot (17·5 cm diameter, 21·8 cm high) containing a steam sterilized reconstituted soil (50% sand, 25% compost, 25% organic soil). Seven carrot seeds (cv. Nanco) were sown in each pot. Pots were placed in controlled environment conditions favouring carrot growth and compatible with the temperature requirements of *P. violae* (14 h daylight at 20°C and 8 h darkness at 12°C). They were watered as needed to adjust soil moisture to the water holding capacity of the pot.

CCS symptoms were assessed by measuring disease incidence *i* (proportion of diseased roots per pot), lesion density *d* (mean number of lesions per root), symptom intensity *si* (mean diameter of lesions per root) and total disease area *tda* (total necrotic area on the root surface), as described by Suffert (2006). Mean root weight was also noted to estimate the impact of the disease on root growth.

Method A – primary infection via soil inoculum to healthy root

The soil inoculum consisted of *P. violae* mycelium and oospores produced on carrot juice agar (CJA) (20 g agar, 200 mL centrifuged carrot juice (Nature Bio, CORA©), made up to 1 L with distilled water) autoclaved for 30 min at 115°C. A mycelial plug was cut from the margin of a 7-day-old culture of *P. violae* growing at 20°C on carrot juice agar and was transferred to the centre of freshly prepared medium in a standard Petri dish (17 mL medium per dish), and was incubated for 7 days in the dark at 20°C. The experiment was set up as a factorial randomized block design with three blocks (three pots)

and four inoculum levels (4·25, 8·5, 17, 34 mL of *P. violae*-colonized CJA per pot, and a non-incubated control). Just before sowing, the contents of each dish were fragmented into small pieces (each about 50 mm^2) and homogenised with soil. In each pot, 13 cm of non-infested soil was covered with 5 cm of infested soil; 2 cm of noninfested soil was added to cover the surface. Carrot roots were harvested, washed and symptoms assessed 16 weeks after sowing.

Method B – primary infection via infected carrot residues to healthy root

The soil was artificially infested with infected carrot residues from a natural CCS epidemic, due mainly to *P. violae*. Typical lesions on 5-month-old carrots were cut to generate small fragments of necrotic tissue, 2 to 3 mm long. These fragments were used to infest soil, by evenly distributing them throughout the volume of soil in the pot just before sowing. The experiment was set up as a factorial randomized block design with three blocks (three pots) and four inoculum levels (0·35, 1·75, 3·5, 7 g of CCS-affected carrot root fragments per pot). Each of these inocula were estimated to provide 30 ± 10, 150 ± 50, 300 ± 100 and 600 ± 200 mm^2 *tda* per pot. Healthy carrot root tissue was added at 3·15 g per pot as a control treatment. Carrot roots were harvested, washed and symptoms scored 12 weeks after sowing.

Method C – secondary infection via diseased whole root to healthy root

Using the same experimental design as above, a PVC tube (32 mm diameter, 230 mm long) was introduced into the centre of each pot at the start of the experiment in place of the seventh carrot seed, as illustrated in Fig. 1. Twelve weeks after sowing, the tube was replaced by a thickened carrot root (cv. Nanco) with CCS lesions (the donor plant). These CCS-affected donor plants were produced by first washing fresh carrots in 0·3% bleach for one minute and then rinsed three times with sterile water. The epidermis of the roots was scarified at a defined location (1–2 cm^2) with abrasive tissue. Four intensities of inoculation were used with infection locations chosen to give a distribution as evenly as possible over the main part of the root. A 5 mm diameter mycelial plug was cut from a 7-day-old culture of *P. violae* grown at 20°C on CJA, and placed with mycelium against the scarified epidermis of the carrot and held in place with a sterilized pin. Inoculated carrot roots were then placed in a box (four carrots per box), which was hermetically sealed to create high humidity conditions. After 48 h incubation, the agar plugs were removed and the carrots were transplanted into the pots. The diameter of the lesions on the donor roots was estimated just before transplantation, and on additional carrots kept in the boxes for a further 24 and 48 hours, to assess the extension of the lesions and the progression of *tda* on the donor plants in soil.

The experiment was set up as a factorial randomized block design with three blocks (three pots) and four inoculum levels with either 2, 4, 6 or 8 CCS lesions per donor root.

The total disease area of the donor carrots inoculated to deliver secondary infection via diseased root to healthy carrot root was assessed prior to transplantation (Fig. 2). The diameter of lesions on donor roots increased little from 48 hours to 96 hours after inoculation (*c.* + 15%) with the maximum *tda* recorded of 140 mm^2. Therefore the *tda* value was estimated on donor roots as 280, 560, 1120 and 1680 mm^2 for 2, 4, 8 and 12 lesions, respectively.

Infectious carrot roots (donor plants) were removed three weeks after transplantation and the resulting hole was filled with sterile reconstituted soil. One week later, the receptor plants were harvested and washed and

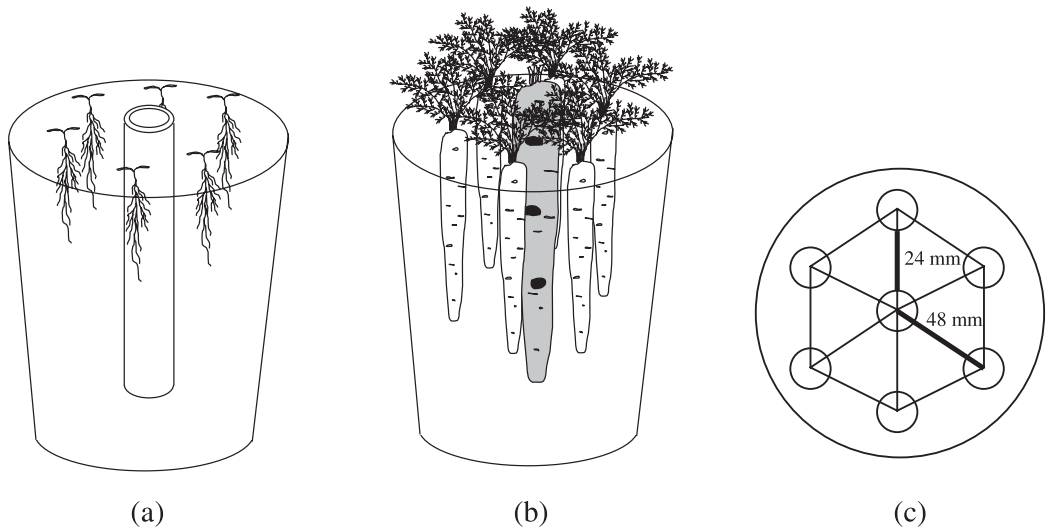

Figure 1 Microcosm design consisting of six receptor carrot plants, (a) before and (b, c) after transplantation of the donor root (method C).

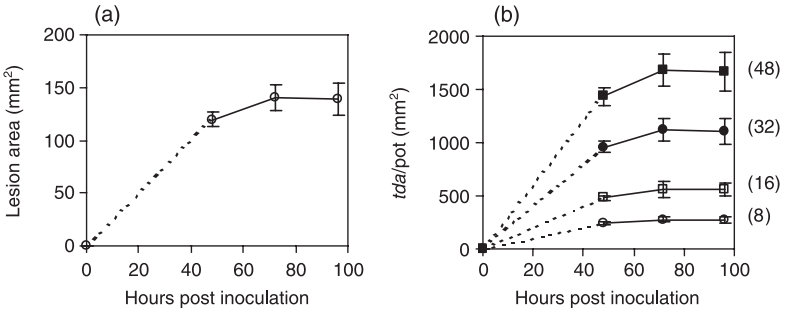

Figure 2 Total disease area *tda* of cavity spot of carrot on the donor roots after inoculation in controlled conditions to provide secondary infection from diseased to healthy carrot roots: a) Mean lesion area on a donor root, b) *tda* on each donor root as a function of dose: ○ two lesions per root, □ four lesions per root, ● eight lesions per root, ■ 12 lesions per root. Numbers in parenthesis indicate number of lesions measured to estimate the mean *tda*.

symptoms scored. Disease assessment therefore took place four weeks after transplantation and 16 weeks after initial sowing.

Results

Effect of different soil infestation methods on CCS

Disease incidence *i* on receptor plants was similar for infestation methods A and C, ranging from 80% to 100% for all doses of both treatments, with no significant dose-dependent effect ($P < 0{\cdot}05$) (Fig. 3). The density of lesions *d* was highest for root-to-root infections: more than 10 lesions per root for method C, from 4 to 8 lesions per root for method A whereas only three or less lesions per root were observed for method B. The highest density of lesions was produced by root to root transfer of inoculum irrespective of inoculum potential with more than 170 mm^2 *tda* per root observed with method C. By comparison 40–80 mm^2 *tda* were recorded for method A, and less than 30 mm^2 for method B. Symptom intensity (*si*) was similar for the three methods (mean lesion diameter ranged from 2 to 4 mm), regardless of the amount of inoculum or number of lesions on the donor plant.

In general, donor plants (method C) induced higher levels of CCS in receptor plants compared to inoculum from infected root fragments (method B). This might have been due to differences in the age and nature of the inoculum used in methods B and C. The total disease area on donor plants following transplantation (method C) did not influence the *tda* (165 to 185 mm^2) recorded on receptor plants ($P < 0{\cdot}05$). Lesions were evenly distributed on receptor plants, and were no more frequent on the side closest to the donor plant and the homogeneity of disease measurements was similar to that for the classical soil infestation method (method A).

Effect of the three soil infestation methods on root weight

Carrot root weight decreased significantly for receptor plants with an increasing amount of inoculum provided by the donor CCS-affected carrot (method C) (Fig. 4). Increasing the inoculum potential to 12 lesions on the donor root led to a significant decrease in root weight of receptor carrots of *c*. 30%. Root weight of receptor carrots in infested soil (method B) was significantly lower than the control in all treatments ($P < 0{\cdot}05$) and increasing the amount of inoculum had no effect on root weight. Soil infestation with mycelium and oospores of *P. violae* (method A) induced the most variable effects. Secondary infection by method C affected receptor-root growth, but later than in the other two treatments.

Discussion

Natural infections from CCS lesions were observed on living carrot roots (simulation of alloinfection *sensu stricto*). Root-to-root infection by *P. violae* provided experimental evidence of the polycyclic nature of cavity spot development in carrots. In addition, diseased carrot residues could also infect healthy carrot tap roots indicating that CCS epidemics also may be polyetic (disease progression over at least two cropping seasons).

Differences in inoculum potential of the donor plants did not affect the total disease area following transplantation probably because the distance between donor and receptor plants (24 mm), and the duration of exposure to the donor plant in soil (3 weeks), were generated for a high disease pressure. Cavity spot of carrot can be caused by more than one species of *Pythium*. Fast-growing species, such as *P. ultimum*, cause water soaked lesions due to the maceration of superficial tissues (Campion *et al*., 1997, 1998) whereas *P. violae* and *P. sulcatum* caused limited root necrosis with the integrity of the diseased tissues conserved due to a lack of host degradation at a distance from hyphae (Groom & Perry, 1985). These previous results suggested that *P. violae* lesions rapidly ceased to be infectious, thus conflicting with the results presented here which showed that CCS lesions caused by *P. violae* were indeed infectious and that the mycelium in the outer root cells was able to grow out of the carrot root tissues. It was also evident that root-to-root infection from a CCS mature lesion is possible and that the latency period is clearly less than 4 weeks.

Some propagules are more efficient than others for initiating infection, but the choice of inoculum in experiments often depends on the *Pythium* species considered (Van der Plaats-Niterink, 1981). Some *Pythium* species do not form sporangia and zoospores in sufficient quantities for reproducible inoculation, thus limiting the choice of inoculum to mycelium. Although soil infestation with

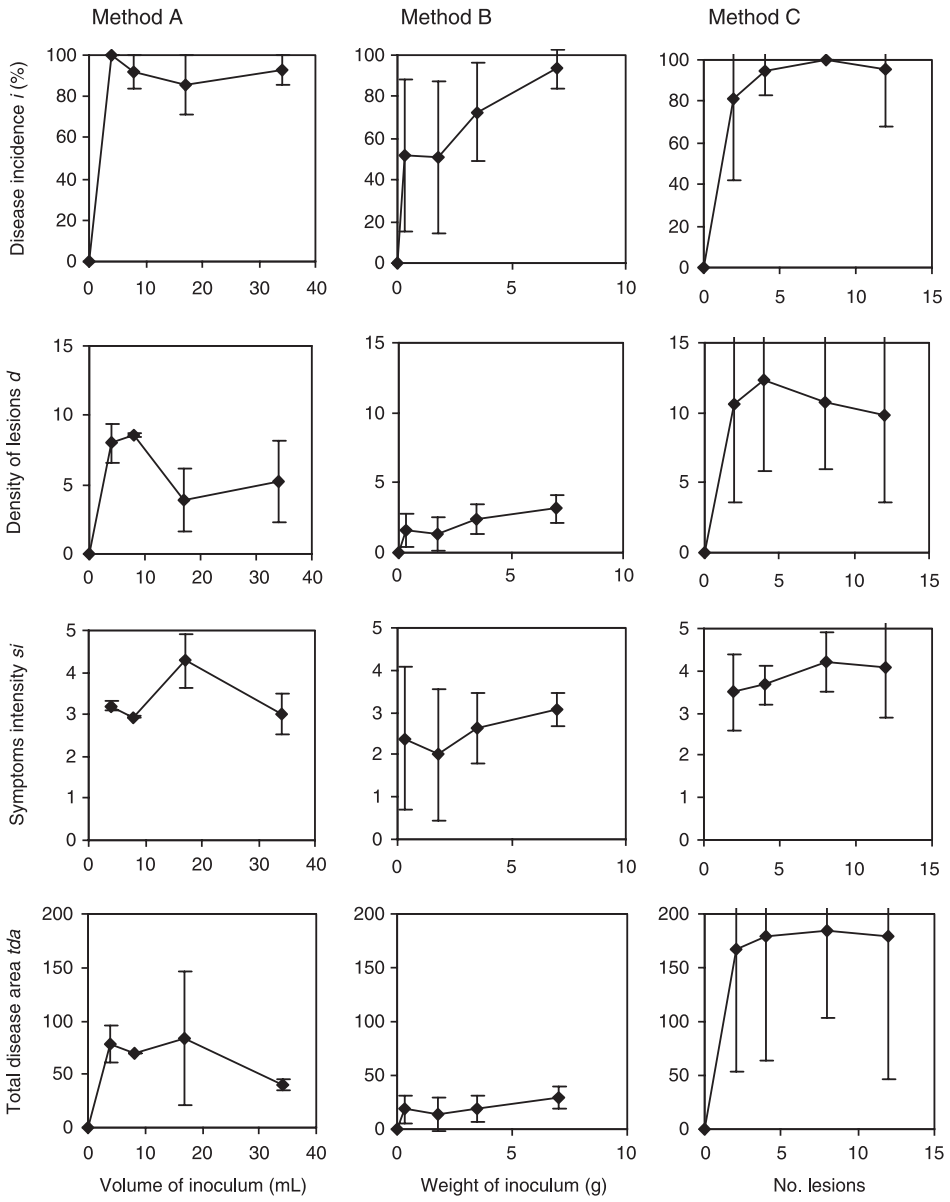

Figure 3 Carrot cavity spot assessment on receptor plants (disease incidence *i*, lesion density *d*, symptom intensity *si*, total disease area *tda*) as a function of the dose of three methods of inoculation. Method A: primary infection via *in vitro* produced inoculum of *Pythium violae* added to soil. Method B: primary infection via infected carrot residues added to soil (weight of infected carrot root fragments per pot). Method C: secondary infection via diseased whole root to healthy carrot root induced by lesions on a living donor root.

colonized carrot juice agar is commonly used for many soilborne pathogens, its reproducibility for direct infection of carrot is low due to the fragility of the inocula (D. Breton, SILEBAN-INRA, personal communication). It can, however, be used to simulate primary infection, although it is difficult to estimate accurately the number of infectious units (oospores, zoospores and mycelial fragments). In contrast the transplantation of a donor plant root is a novel and reproducible method of localized soil infestation for delayed inoculation.

Because the carrot is a biennial plant, donor plants produced large numbers of new radicles (adventitious roots) 2 weeks after transplantation and the leaves re-grew. The transplanted carrot root (method C) was thus living whereas infected carrot residues may be considered to be non-living. Nonetheless, fresh lesions were clearly infectious for adjacent healthy carrot roots indicating that CCS-infected fragments permit survival of *P. violae* among crop residues and therefore a source of inoculum in short rotation carrot cropping.

Theoretically, three types of secondary infection can be distinguished: infection of the taproot of the same plant, infection of a radicle of the same plant, and cross-infection between taproots or radicles from neighbouring plants.

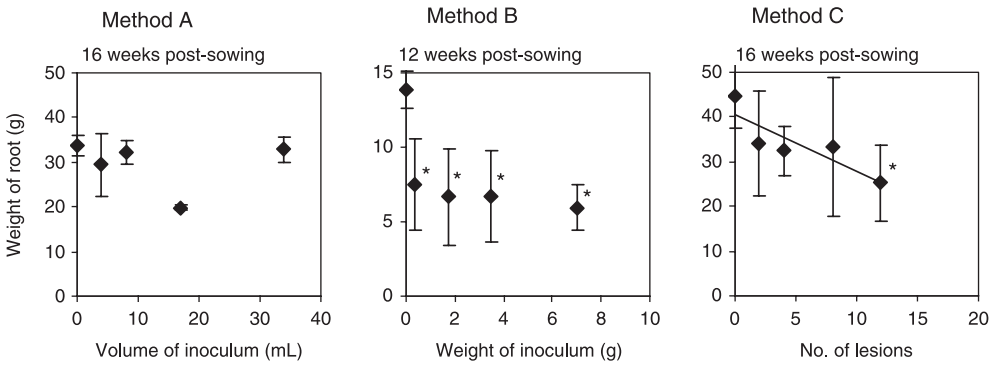

Figure 4 Dose-effect of three forms of *Pythium violae* inoculum on the fresh weight of carrot roots. Method A: primary infection via *in vitro* produced inoculum added to soil. Method B: primary infection via infected carrot residues added to soil (weight of infected carrot root fragments per pot). Method C: secondary infection via diseased whole root to healthy carrot root induced by lesions on a living donor root (*indicates root weight at the dose indicated is significantly different ($P < 0{\cdot}05$) from the control).

Crowe & Hall (1980) showed that *Sclerotium cepivorum* infections more than 2 cm below the stem plate of white rot affected onion bulbs led to extensive plant-to-plant spread, because the pathogen spread to roots of the same and neighbouring plants as the mycelium grew along roots, radiating out from active sites of colonization and infecting other roots up to 2 cm away. Distances between roots were smallest in the zone of highest root density (2–4 cm below stem plates) and roots frequently extended horizontally in this zone, providing a direct route for mycelial growth to other plants. Rekah *et al*. (1999) reported that tomato roots seemed to be essential for movement of the fusarium crown rot and root rot pathogen in soil, and therefore may be regarded as tubes filled with a highly selective and rich habitat protecting the colonising pathogen from the activity of antagonistic micro-organisms. In the CCS pathosystem, the carrot root system was modelled as a single cylinder and possible infections of radicles on the same plant via the taproot were initially ignored (Suffert, 2006). Contact between a healthy taproot and radicles of a neighbouring diseased carrot may increase the spread of pathogens such as *P. violae*, but would require preliminary contamination of the adventitious root from CCS lesions on the diseased taproot. The current data challenges this hypothesis because infections induced by carrot tap root transplantation (method C) occurred within a short time period (less than 4 weeks) and because runner hyphae, which grow along roots, have not been observed in *P. violae*. Previous studies have shown that CCS lesions occasionally coincide with lenticels, but are not consistently associated with anatomical features such as soil ducts or lateral root origins (Perry & Harrison, 1979). The importance of physical contact between taproots and adventitious roots from another plant can be investigated, using a nylon mesh as a barrier to root spread (Crowe & Hall, 1980; Rekah *et al*., 1999; Suffert, 2006) in order to differentiate infections due to mycelium and those occurring from root-to-root contact.

Active growth or dispersal of the fungus in the soil is the most probable explanation for the secondary infections observed. Such growth may result from the growth of hyphae or the dispersion of zoospores. The dispersal of *P. violae* by zoospores is unlikely as zoospores have never been observed in this species (Van der Plaats-Niterink, 1981), but zoospore dispersal may be involved in secondary infections by other *Pythium* species responsible for CCS (Martin & Loper, 1999).

Soilborne infections may arise from two sources: the initial soil inoculum or the active transmission of the pathogen between plant tissues. Several studies on soilborne diseases have asserted that the classical equations describing primary and secondary infections are sufficient to define models. However, as Pfender (1982) pointed out, some investigators have failed to recognise that biological models should be constructed on the basis of knowledge about biological processes rather than biological processes being deduced from disease progression data generated by theoretical models. The fit of a model cannot prove that a particular mechanism operates, but may provide clues as to the underlying mechanism. Experiments designed specifically to investigate the existence of a secondary infection cycle are sometimes more useful than observations of disease progress over time, in which effects of the biological cycle are confounded with those of environmental factors (Stack, 1980; Pfender, 1982). The present biological demonstration of the occurrence of secondary infection and the method for reproducing alloinfection could form the basis of an epidemiological model for CCS, based, in particular, on equations developed by Brassett & Gilligan (1988).

Similar experiments in controlled and field conditions have indicated that the extent of plant-to-plant spread depends on the time and depth of infection, root density, and root distribution (Crowe & Hall, 1980). The first step towards such a simulation model involves the quantification and analysis of transmission rates for soilborne epidemics, as recently proposed by Otten *et al*. (2003), according to inherent factors such as the latent period or the distance between roots.

The separation of the epidemic into monocyclic and polycyclic phases may be used to identify critical stages for integrated approaches to CCS management. Each infected

taproot can cause disease in neighbouring carrot roots. Thus, even an initially low disease intensity may result, in time, in a severe epidemic. Understanding the role of infection processes is crucial when developing holistic approaches including pre-planting control measures such as crop rotation, sanitation, disinfestation and biocontrol with intercrops to limit primary infections, and post-planting curative measures such as applications of fungicide or manipulating harvest date to limit secondary infections.

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