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The ecology of a *Pythium* community in relation to the epidemiology of carrot cavity spot

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Abstract

Carrot cavity spot (CCS) is characterised by the appearance of small sunken elliptical lesions on the tap root. It is caused by a complex of *Pythium* species, but the species diversity and interactions within the complex have never been studied for modelling CCS epidemics. The diversity of a pathogenic *Pythium* community was assessed during 3 consecutive years in a field experiment after an initial artificial soil infestation with *P. violae*. 1241 lesions were examined, yielding 728 *Pythium* isolates. Conventional microbiological methods and restriction polymorphism of the internal transcribed spacer regions of the rDNA of 209 representative *Pythium* isolates allowed us to identify 655 isolates as belonging to six *Pythium* species, including *P. violae* and five indigenous species (*P. sulcatum*, *P. intermedium*, *P. sylvaticum/irregulare*, *P. coloratum*, and *P. ultimum*). Biological traits, such as pathogenicity, optimum temperature for mycelial growth and saprophytic survival of the inoculum, explained the fluctuations in the composition of the complex over 17 successive samplings during the 3-year period, most notably the prevalence of first *P. violae* and then *P. sulcatum*. *P. violae* and *P. sulcatum* were occasionally isolated in mixture from single lesions (10.4% and 9.6%, respectively). Other species were more frequently isolated in mixture: 30.8% for *P. intermedium*, 33.8% for *P. sylvaticum/irregulare*, 42.9% for *P. ultimum*, and 66.7% for *P. coloratum*. A contingency analysis allowed us to define 'major' and 'minor' species on both pathological and ecological criteria (frequency of occurrence in the complex, pathogenicity and ability to induce lesions by themselves), and demonstrated that infection by one 'major' pathogen species (*P. violae* or *P. sulcatum*) is not positively correlated with the presence of a second *Pythium* species. The ratio between 'observed' and 'expected' mixed infection frequency under the assumption of independent infection (*mir*) was less than 1 for *P. violae*, *P. sulcatum*, *P. intermedium*, and *P. sylvaticum/irregulare* ($P < 0.05$). For all *Pythium* species, there was a negative linear relationship between *mir* and pathogenicity ($R^2 = 0.638$): the less a *Pythium* species was pathogenic on carrot, the more often it was isolated from a CCS lesion in mixture with at least one other species. The non-significance of interactions between species during the infection phase suggests that CCS epidemics can be analysed as if they were caused by a single *Pythium* species.

Keywords: Epidemiology; Carrot cavity spot; Mixed infection; Pathogen complex; *Pythium sulcatum*; *Pythium violae*

1. Introduction

1.1. The genus Pythium spp.

The genus *Pythium* includes approximately 120 species (Hendrix and Campbell, 1973; Van der Plaats-Niterink, 1981; Dick, 1990). While diseases caused by

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Pythium spp. are often considered as seedling diseases (damping-off), mature plants may also be attacked. In some circumstances, pathogenic species are isolated from healthy looking roots, where their colonisation causes a reduction in plant growth but not typical root rot symptoms (Martin and Loper, 1999). However, non-pathogenic species are sometimes isolated from typical symptoms initially caused by another species. Some overviews of the physiology, etiology of host infection, and life cycle have been published (Hendrix and Campbell, 1973; Ali-Shtayeh et al., 1986). These consisted of specific monographs of given species, compiled for example by Domsch et al. (1980), but with little ecological content as they did not take into account interactions between species. There is a lack of knowledge concerning the effect of the diversity of a species community on the epidemiology of root diseases, particularly during the infection phase.

1.2. Carrot cavity spot

Cavity spot (CCS), one of the most important soil-borne diseases of carrot worldwide, is characterised by the presence of sunken brown elliptical lesions on the surface of the taproot, and is due to a *Pythium* complex (Hiltunen and White, 2002). The involvement of the slow-growing species *P. violae* in the development of CCS was firmly established 20 years ago (Groom and Perry, 1985; White, 1986; Montfort and Rouxel, 1988). However, other *Pythium* species are able to cause the disease. In Europe, White (1986) and Guerin et al. (1994) demonstrated the role of the slow-growing *P. sulcatum* as well as of the fast-growing *P. ultimum*, *P. irregulare*, *P. intermedium*, and *P. sylvaticum*. Another fast-growing species, *P. coloratum*, has been isolated from CCS lesions in Western Australia (El-Tarabily et al., 1996) and more recently in south-western France (Breton, unpubl.).

1.3. Identification of Pythium spp.

In artificial inoculations, symptoms caused by *P. violae* and *P. sulcatum* are well-delimited and oriented across the breadth of the root (Groom and Perry, 1985; Montfort and Rouxel, 1988), while *P. ultimum* and *P. coloratum* cause maceration of tissues and more progressive lesions (Campion et al., 1997; El-Tarabily et al., 1996). However, it is not possible to diagnose visually which *Pythium* species is responsible for a CCS lesion in naturally infected roots. The identification of *Pythium* spp. using keys based on spore and sporangial morphology is also difficult, because several species are asexual or heterothallic and do not readily produce the diagnostic organs in axenic cultures (Van der Plaats-Niterink, 1981; Dick, 1990). Within the last 10 years, molecular techniques have been developed for the identification of *Pythium* spp., especially techniques based on restriction fragment length polymorphism (RFLP) of the rDNA-ITS region (Martin and Kistler, 1990; Wang and White, 1997; Kageyama et al., 1998; Vasseur et al., 2001), which have improved the diagnosis of the *Pythium* complex associated with CCS.

1.4. Ecology and epidemiology of Pythium complexes

Management practices and environmental conditions often have a significant impact on the species diversity in *Pythium* complexes (e.g. pathogens of wheat, alfalfa, and corn). For example, locality, properties of the soil, and land management influenced the composition of *Pythium* communities responsible for root rot of wheat examined in large-scale surveys, defined by order of frequency of species (e.g. *P. abappressorium*, *P. rostratum*, *P. debaryanum*, *P. heterothallicum*, and *P. oligandrum*) (Dick and Ali-Shtayeh, 1986; Paulitz and Adams, 2003). The composition of a *Pythium* complex is usually not homogeneous within an year. Seasonal variations have been reported in the composition of the *Pythium* communities of wheat (Ali-Shtayeh et al., 1986; Hardman and Dick, 1987), of alfalfa (e.g. *P. sylvaticum*, *P. irregulare*, *P. ultimum*, and *P. torulosum*) (Hancock, 1981; Hancock and Grimes, 1990; Larkin et al., 1995a, 1995b), of bean (e.g. R *aphanidermatum*, *P. myriotylum*, and *P. ultimum*) (Lumsden et al., 1976), and of various vegetables such as cucumber, pepper, tomato, and eggplant (11 *Pythium* species including *P. ultimum*, *P. spinosum*, and *P. aphanidermatum*) (Zhang et al., 1990). However, seasonal fluctuations have rarely been described precisely as few studies have investigated entire pathogen communities, including potential interactions between species. This is problematic for a quantitative epidemiological approach because disease progress curves may be influenced by these fluctuations. One or several *Pythium* species are commonly dominant within a complex and may fluctuate in frequency during a season: for example, the population of *P. aphanidermatum* decreased significantly over time and *P. myriotylum* was the most prevalent on infected bean (Lumsden et al., 1976). The development of a soil-borne disease due to several *Pythium* species can be affected by the composition of the pathogen complex, because the biological characteristics such as pathogenicity of each species are different, but also because the factors

usually affecting an epidemic may modify the behaviour of each species. Seasonal fluctuations in *Pythium* spp. in relation to root disease severity were established by Lee and Hoy (1992) and Zhang et al. (1990). Rao et al. (1978) measured the prevalence and pathogenicity of fungi, including *P. graminicola*, *P. torulosum*, *P. dissotocum*, and other genera such as *Fusarium*, associated with the root rot complex of corn. A reduction in *Pythium* population during mid-summer was observed and *P. graminicola* appeared to be the primary cause of root rot early in the season. Pathogenicity tests confirmed that *P. graminicola* was more virulent than *P. dissotocum* or *P. torulosum*.

Little interaction between *Pythium* species (e.g. positive synergy) usually occurs during the infection phase and these complexes behave as independent entities rather than as synergistic communities. To compare the effects of infection by one or several *Pythium* species affecting root rot of sugarcane, pathogenicity tests were conducted by artificial soil infestation with *P. arrhenomanes*, *P. irregulare*, and *P. spinosum* (Lee and Hoy, 1992) and showed that disease severity due to several species in combination was never higher than severity caused by individual species. Comparison of root infection by *P. arrhenomanes* alone and total *Pythium* species with root rot severity in natural field soils have shown that the prevalence of *P. arrhenomanes* is a determinant of disease severity (Lee and Hoy, 1992). Mihail et al. (2002) characterized the diversity of root-colonizing *Pythium* spp. (including pathogenic species) on *Kummerowia stipulata* in one restored prairie site. In contrast to total species richness (15 *Pythium* species identified), a maximum of three *Pythium* species (*P. dissotocum*, *P. diclinum*, and *P. irregulare*) were recovered from any single root system. The occurrence of mixed infections by different species was tested in glasshouse experiments which showed that when *P. dissotocum* and *P. irregulare* were present, infection by each of them was independent of the other.

1.5. Objectives of the study

The present study addressed the question of the relevant scale to analyse a CCS epidemic: a single species or a species complex? The overall objective was to test if a CCS epidemic is specifically affected by the nature of each local pathogen complex and if epidemiological processes occurring (e.g. primary and secondary infection) may be considered identical whatever the nature of this complex. To this end, we investigated the fluctuations of a *Pythium* complex responsible for CCS (a mixture of endogenous *Pythium* spp. and *P. violae* artificially applied) over a 3-year outbreak and related these to the biological characteristics of *Pythium* species (pathogenicity, optimum temperature, and saprophytic survival ability). We also analysed the occurrence of mixed infection (more than one *Pythium* species isolated from a single CCS lesion) and tested by a contingency analysis if the ecological classification into 'major' versus 'minor' species, according to their prevalence in the complex, matched pathogenicity.

2. Materials and methods

2.1. Assessment of the diversity of the pathogen complex from CCS lesions

2.1.1. Inoculum production

P. violae strain PV490 (CBS 102.609), isolated in 1994 from CCS symptoms in a carrot field in Normandy (France), was used as inoculum for field infestation. To produce inoculum, bags containing 240 ml of dry barley grains and 300 ml of distilled water were autoclaved twice at 120 °C for one hour at 24 h intervals, and inoculated with plugs of *P. violae*. They were incubated for 3 weeks at 20 °C in a dark room.

2.1.2. The field experiment

A field experiment was conducted in the INRA Station of Le Rheu (Ille-et-Vilaine, France) to characterize the changes in the diversity of a *Pythium* community responsible for CCS over 3 years after a single initial soil infestation by *P. violae*. This species had not been previously isolated from the soil within the experimental area. The experimental area was sown to carrots (cv. Nanco) on 22 May 2001, on 13 June 2002, and on 20 May 2003. The experiment was set up as a factorial randomised block design with four blocks and four inoculum levels (T: control, D1: 5 g m^{-2}, D10: 50 g m^{-2}, D100: 500 g m^{-2}). Experimental units were 2 m × 6 m plots. The soil was silt loam (with 16.3% clay, 62.5% silt, 21.2% sand, and 2.4% organic matter). In the first year (2001), plots were infested with three levels of barley grain inoculum (D1, D10, D100). Each plot consisted of five rows 50 cm apart with 80 plants per linear meter. Pre-seeded cellulosic paper ribbons (made by ISITOP, Saint-Gilles, Ille-et-Vilaine, France) were used to homogenise carrot emergence.

Cultural practices applied uniformly to all plots were as follow. The fields were ploughed and harrowed 1 week before sowing and the insecticide CURATER (Carbofuran, 1000 g ha^{-1}, BAYER CROPSCIENCE) was applied to control carrot fly (*Psila rosae*) and

wireworm *(Agriotes lineatus)*. The herbicide DOSANEX (Metoxuton, 4000 g ha^{-1}, SYNGENTA AGRO) was sprayed 3 weeks after carrot emergence and weed control was completed by mechanical means. The fungicide SCORE (Diphenoconazole, 200 g ha^{-1}, SYNGENTA AGRO) was applied in August to prevent foliar attacks by *Alternaria dauci* that usually occurr in the area. The application of ammonium nitrogen fertilizer was split (20 kg ha^{-1} N 1 month before sowing and 30 kg ha^{-1} N 1 month after carrot emergence). Phosphate and potassium fertilizers were applied 1 month before sowing (100 kg ha^{-1} P as Superphosphate and 130 kg ha^{-1} K as Patenkali).

The plots were irrigated in 2003 because of the exceptionally dry weather. Air temperature 1 m above soil level and soil temperature at 20 cm depth were measured with a Hobo™ H8 (Prosensor). Data were acquired at 15 min intervals. Daily precipitation was automatically recorded using a tipping bucket type rain gauge. Air and soil temperatures and cumulative precipitations (rainfall and irrigation) are presented in Fig. 1. The difference between maximal and minimal soil temperature was particularly wide in the 2003/2004 season (-4 ± 27 °C). The climate was exceptionally hot and dry in the summer of 2003, with an average mean daily air temperature of 21.5 °C in August 2003, compared with 18.9 °C in 2001 and 17.5 °C in 2002. The average maximum daily air temperature in August was 29.7 °C in 2003, 24.5 °C in 2001 and 23.7 °C in 2002.

2.1.3. Isolation of Pythium from CCS symptoms

Six, five and six samples were harvested in 2001, 2002, and 2003 (S1 to S6 on 21/06/2001, 30/07/2001, 03/09/2001, 05/11/2001, 08/01/2002, and 25/02/2002; S7 to S11 on 26/06/2002, 16/07/2002, 12/08/2002, 08/10/2002, and 02/12/2002; S12 to S17 on 01/07/2003, 21/07/2003, 05/08/2003, 28/08/2003, 30/09/2003, and 24/11/2003). On each sampling date all the roots present along a 50 cm row section were collected. Each sample typically had 30–40 carrot roots. Numbers and size of CCS lesions were measured for each root and the temporal dynamics of the epidemic was monitored by the disease measurements *i* (disease incidence), *d* (lesion density), *si* (symptom intensity), and *tda* (total disease area) (Suffert, 2006).

At each sampling date, microbiological isolations were performed on 3/6 single CCS lesions (according to the disease severity) per plot. Typical CCS lesions were selected, except for samples S1 and S2 in 2001 and 2002 which showed only nonspecific, brown-rust-coloured micro-lesions. The number of isolations for each sampling date ranged from 42 to 81 in 2001, 70–124 in 2002, and 28–81 in 2003 (including control plots in 2001 and 2002, but not in 2003). A corrected data set was defined with an extrapolation to 100 isolations to facilitate graphical illustrations.

Pieces of necrotic tissue (5 mm^2) were cut from the edges of lesions on the taproot, sterilised in 0.5% NaOCl for 15 s, rinsed three times in distilled water and cut into seven small fragments (1–2 mm). These were placed on selective MS2 medium (1 L of water amended with 17.5 g of corn meal agar, 2 mL of pimaricin aqueous suspension 2.5%, and 2 mL of rifampicin alcoholic suspension 0.75%), incubated at 20 °C and examined periodically for up to 7 days for fungal colony development (Breton and Rouxel, 1993). In some cases, more than one isolate was obtained from a single CCS lesion. Subcultures of each *Pythium* isolate were established from the margins of colonies actively growing on MS2 medium on water agar (1 L of water amended with 20 g of agar) and carrot juice agar (800 mL of water amended with 20 g of agar and 200 mL of centrifuged carrot juice CORA©). A total of 1241 CCS lesions were examined, yielding 728 *Pythium* isolates (247 isolates from 509 isolations in 2001, 249 from 409 in 2002, and 232 from 323 in 2003).

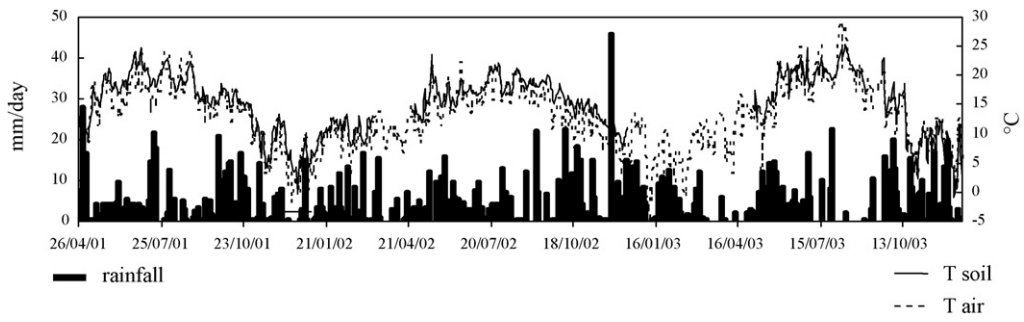

Fig. 1. Meteorological data in the field experiment including cumulative precipitation (rainfall and irrigation), daily mean air and soil temperature.

2.1.4. Identification of Pythium isolates

After mycelial growth on water agar and carrot juice agar, *Pythium* isolates were sorted and pre-identified using the morphological criteria from the key of Van der Plaats-Niterink (1981). Two representative isolates per group of species were selected and characterised by PCR and RFLP. DNA was extracted from mycelium as described by Vasseur et al. (2001), a variation of the standard protocol of Rogers and Bendich (1985). The internal transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA) of 209 representative *Pythium* isolates (76 in 2001, 86 in 2002, and 47 in 2003) from most of the groups pre-identified was amplified by a polymerase chain reaction (PCR) using a standard protocol (Wang and White, 1997; Breton, unpubl.). The primers used amplified ITS1 from 18S rDNA (TCCGTAGGTGAACCTGCGG) and ITS4 from 28S (TCCTCCGCTTATTGATATGC). Restriction banding patterns of PCR-amplified internal transcribed spacer of rDNA after digestion with *Hha*I, *Hae*III, *Hin*fI, *Taq*I, and *Mbo*I, and separation on a 2% agarose gel, were compared with species-specific probes for known *Pythium* spp. using a variation of the standard protocol of Wang and White (1997) and Kageyama et al. (1998). Molecular analyses confirmed the identification of most of the 209 isolates.

2.1.5. Analysis of diversity

The diversity of pathogenic *Pythium* species associated with CCS was assessed by species richness estimated as the number of different species per plot and the normalized Shannon diversity index (H_n), which is calculated as:

$$H_n = \frac{1}{\ln(S)} \sum^{S} P_i \ln(P_i) \quad (1)$$

where P_i is the frequency of the ith species and S the observed number of species (Sheldon, 1969; Begon et al., 1986; Mihail et al., 2002).

For analysis of the *Pythium* community diversity recovered in the field experiment, data were aggregated into two pools: data from infested plots D1, D10, and D100 (2001–2003), and data from control plots (2001–2002). Values of H_n were estimated at each sampling date.

2.2. Association of species on CCS lesions and statistical analysis of mixed infection

Statistical analyses were performed to test the null hypothesis that root infection by the *Pythium* species responsible of CCS occurred independently. The occurrence of association of each *Pythium* spp. with others species on a single CCS lesion (mixed infection or co-infection) was evaluated by a contingency table analysis and the chi-square statistic (χ^2) (Sokal and Rohlf, 1995). This analysis concerned associations of *Pythium* species able to induce CCS lesions, even if the combination is rarely represented. The mixed infection ratio (*mir*) was defined as the ratio between observed mixed infection and expected mixed infection frequency (i.e. the theoretical frequency of mixed infection if infections by a *Pythium* spp. and by another species were independent) to test if mixed infection by two given *Pythium* species was significantly over-represented. Data from the field experiment were firstly analysed year by year (2001, 2002, and 2003), and secondly pooled.

2.3. Biological characterisation of Pythium spp.

Among 728 *Pythium* strains isolated from CCS lesions between 2001 and 2003 in the field experiment, 33 representative isolates belonging to seven groups of species were biologically characterised (FR244, FR89, FR214, FR296B, FR261B, FR10A, FR235A, FR97A, FR57A, FR84, FR292, FR39, FR236, FR6, FR82, PV490 (×2), FR24, FR45, FR100, FR80, FR266, FR60, FR32, FR262, FR297, FR295, FR62 (×2), FR71, FR40, FR232, FR25, FR86, and FR238).

2.4. Optimum temperature of mycelial growth of Pythium spp.

Among the 33 *Pythium* isolates, 13 isolates (PV490, FR24, FR233, FR266, FR297, FR71, FR62, FR262, FR57A, FR296B, FR235A, FR263, and FR39) belonging to four identical groups were selected (4 *P. violae*, 4 *P. sulcatum*, 3 *P. intermedium*, 2 *P. sylvaticum/irregulare*). Response to temperature was studied by determining the radial mycelial growth rates (mm/day) on corn meal agar medium (CMA) in Petri dishes incubated at seven temperatures ranging from 5 °C to 30 °C. The growth of the mycelium was measured on two perpendicular directions, once per day for the slow-growing species *P. violae* and *P. sulcatum* and twice per day for the fast-growing species. The radial mycelial growth rate of each isolate was calculated from the mean of three replicates and the optimum temperature was graphically established with a confidence interval ±1 °C.

2.5. Pathogenicity test

An artificial inoculation of mature roots was performed to measure the pathogenicity of the

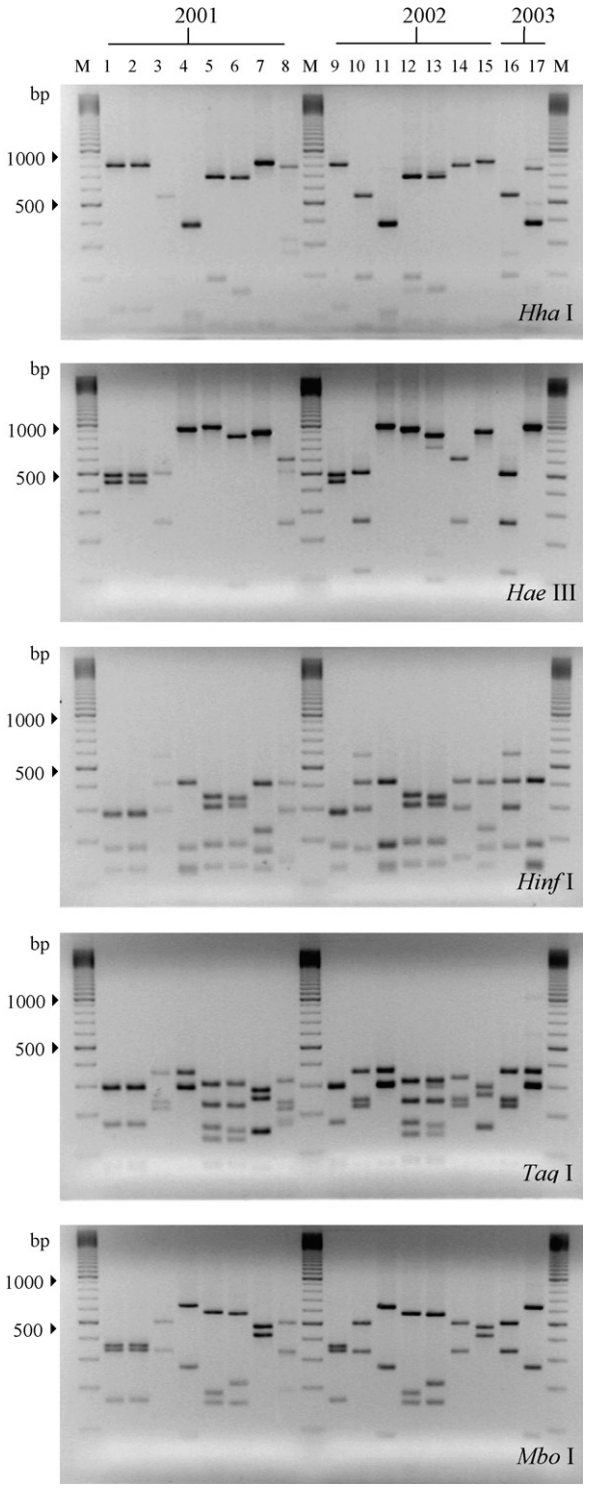

Fig. 2. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) diagnosis of *Pythium* strains isolated from the field experiment (2001–2003). Restriction banding patterns of PCR-amplified internal transcribed spacer of rDNA after digestion with *Hha*I, *Hae*III, *Hin*fI, *Taq*I and *Mbo*I, and separation on a 2% agarose gel. Lanes: M, 100-bp ladder marker; 1: *P. violae* Pv490 (CBS 102.609); 2, 9: *P. violae*; 3, 10, 16: *P. sulcatum*; 4, 11, 17: *P. sylvaticum/irregulare*; 5, 12: *P. intermedium A*; 6, 13: *P. intermedium* B; 7, 15: *P. ultimum*; 8, 14: *P. coloratum*.

representative *Pythium* isolates. Freshly harvested carrot roots (cv. Nanda) were sterilised in water containing 0.3% NaOCl (1 min), rinsed three times in clear water, and cut into 4 cm long root sections. The 33 selected isolates (9 *P. violae*, 10 *P. sulcatum*, 6 *P. intermedium*, 5 *P. sylvaticum/irregulare*, 2 *P. coloratum*, and 1 *P. ultimum*) were grown on CMA for 7 days. Mycelial plugs ($\varnothing = 0.5$ cm) were transferred from the margins of colonies to the surface of root sections (Breton and Rouxel, 1993; Benard and Punja, 1995), previously wounded with a pin forming a small 5 mm hole. For each *Pythium* isolate, 10 root sections were so inoculated and placed on moistened sponge towels in closed plastic containers. The experiment was replicated twice. Root fragments were sprayed with distilled water and incubated at 20 °C.

Two components of pathogenicity, infection efficiency (*e*, estimated by the rate of successful infection) and lesion extension (estimated by the area of a lesion calculated using the ellipse area equation $1/4\pi(a + b)$, where a and b are the minor and major diameter) were assessed after 7 days. These two components were combined into an index φ defined by $\varphi = e \times 1/4\pi(a + b)$.

2.6. Saprophytic behaviour of *P. violae* and *P. sulcatum* in soil

An experiment was performed under controlled conditions to illustrate the decrease over time of the inoculum potential of *P. violae* and *P. sulcatum* in two soils. The strains used were *P. violae* PV490 (CBS 102.609) and *P. sulcatum* FR349 (isolated from CCS lesions in the 2003 field experiment). Microcosms consisted of circular 4 L pots ($\varnothing = 18.7$ cm; $H = 21.8$ cm) containing either a steam sterilised reconstituted soil (1/2 sand, 1/4 compost, 1/4 organic soil), or a natural non-sterilised soil from the field experiment. Soils were infested with either *P. violae* or *P. sulcatum* using a concrete mixer. The method of inoculum production (barley grains) was similar to that described above. After infestation, pots were placed in a climatic chamber under favourable conditions for carrot growth and development of *P. violae* and *P. sulcatum* (14 h of daylight at 20 °C and 8 h of night at 12 °C) (Van der Plaats-Niterink, 1981; Breton and Rouxel, 1993). Pots were watered as needed to adjust soil moisture to the pot water holding capacity. Seven carrot seeds (cv.

Nanco) were sown in each pot on 0, 16, 42, 58, 78, 105, 120, and 140 days after soil infestation. Each treatment was replicated three times (three pots). Carrot roots were harvested and washed 12 weeks after each delayed sowing. Symptoms of CCS were assessed using the variable *tda* (total disease area) and mean root fresh weight was measured to estimate the effect of the inoculum dose on roots growth.

3. Results

3.1. Change in diversity of the species complex in CCS lesions

3.1.1. Identification of Pythium species

1241 CCS lesions were examined from the field experiment, yielding 728 *Pythium* isolates. Of these, 655 isolates (90%) were identified as belonging to six distinct *Pythium* species, including *P. violae* and five indigenous species: *P. sulcatum*, *P. intermedium*, *P. sylvaticum/irregulare*, *P. coloratum*, and *P. ultimum* on the basis of RFLP patterns of amplified ITS regions (Fig. 2). The remaining 73 isolates (10%) could not be unequivocally ascribed to one of these species and were pooled into the group '*Pythium* sp.'

Of the six distinct species, three were not precisely identified. The group *P. intermedium* included two distinct sub-species named A and B, because of small differences in restriction profiles observed after restriction banding patterns and digestion. *P. sylvaticum/irregulare* corresponded to a single species, but we were not able to separate them. *P. coloratum* also corresponded to a single restriction profile, but we were not able to separate *P. coloratum*, *P. dissotocum* and *P. diclinum* because of the similarity of their profiles.

3.1.2. Fluctuations in the composition of the Pythium complex

The number of isolated strains belonging to different species groups, the total number of positive isolations from CCS lesions, and the number of cases in which no isolate was found, were estimated for each of the 17 sampling dates. A distinction was made between data from infested plots in 2001 (pooled whatever the dose of inoculum) and data from

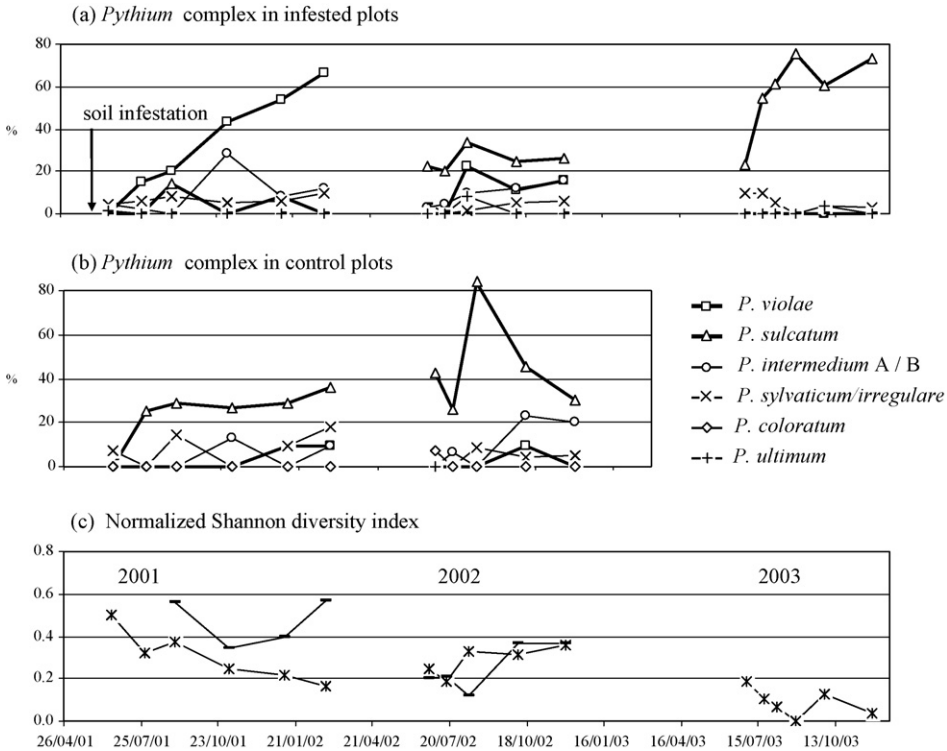

Fig. 3. Fluctuations in the composition of the *Pythium* complex responsible for CCS in the field experiment (2001–2003) after initial soil infestation with *P. violae* in 2001. (a–b) Prevalence of *Pythium* species isolated from lesions. The total 100% corresponds with the number of isolates including non pathogenic *Pythium* sp. Distinction was made between (a) infested plots (D1, D10 and D100) and (b) non-infested control plots. (c) Variations in the normalized Shannon diversity index (H_n) in infested (*) and in control plots (−).

non-infested plots. The pathogen complex was initially composed of 6 *Pythium* species. *P. violae* was increasingly dominant during the first year and responsible for most of the CCS lesions (more than 65% at the end of the epidemic) (Fig. 3a,b). During the second year, *P. violae* was isolated in lower proportions and *P. sulcatum* became the dominant species in the complex. The prevalence of *P. sylvaticum/irregulare*, *P. intermedium*, *P. coloratum*, and other weakly represented species, was low (ranged from 10% to 20%). During the third year, *P. violae* entirely disappeared and *P. sulcatum* dominated the complex (more than 70%). The inter-specific diversity, illustrated by the normalized Shannon diversity index (H_n), decreased with time during the 3-year period (Fig. 3c). Despite the additional presence of *P. violae*, H_n was lower by 28–70% in infested plots than in the control in 2001.

3.2. Occurrence of mixed infections and species interactions

The rate of efficacy of isolation of *Pythium* sp. from a CCS lesion ranged between 45% and 70% during the 3-year period (Table 1). The maximum number of *Pythium* spp. detected from any single CCS lesion was three. The frequency of cases in which a *Pythium* species was isolated in a mixture with other species is less than 5–10% on average. *P. violae* and *P. sulcatum* were rarely isolated in mixture, 10.4% and 9.6%, respectively (Fig. 4). Other species were more frequently isolated in mixture with other *Pythium* spp.: 30.8% for *P. intermedium*, 33.8% for *P. sylvaticum/irregulare*, 42.9% for *P. ultimum* and 66.7% for *P. coloratum*. Ratios between 'observed' and 'expected' mixed infection frequency under the assumption of independent infection (*mir*) were <1 for *P. violae*, *P. sulcatum*, *P. intermedium*, and *P. sylvaticum/irregulare* ($P < 0.05$), within the 3-year data set (Table 2). These four species were also less frequently associated than under the assumption of independent infection. In the case of mixed infections, infections by different *Pythium* species were not positively correlated, in particular for *P. violae* and *P. sulcatum* (see values of χ^2 associated with $P < 0.05$ in Table 2). The number of *P. ultimum* and *P. coloratum* strains was too low for the chi-square test to be significant ($P > 0.05$).

Table 1
Proportion of CCS lesions from which multiple *Pythium* species were isolated, combining infested and control plots in the field experiment over 3 years (2001–2003)

Year	L[a]	N[b]	No. *Pythium* isolates per lesion[c]			
			0	1	2	3
2001	503	247	54.9 (276)	41.2 (207)	4.0 (20)	0.0 (0)
2002	409	249	47.4 (194)	44.7 (183)	7.3 (30)	0.5 (2)
2003	323	232	31.3 (101)	65.6 (212)	3.1 (10)	0.0 (0)
Total	1235	728	46.2 (571)	48.7 (602)	4.9 (60)	0.2 (2)

[a] Total number of CCS lesions examined.
[b] Total number of isolates belonging to *Pythium* species, including multiple isolations of different species.
[c] Percentage of L; brackets indicate the total number of isolates; tabular values ignore species identity of *Pythium* isolates.

3.3. Biological characteristics of Pythium spp.

3.3.1. Optimum temperature

The optimum temperature for saprophytic growth of *P. violae* (19 °C) was less than *P. sulcatum* (25 °C) (Fig. 5). The optimum temperature for *P. intermedium* A/B was 24 °C, and more than 30 °C for *P. sylvaticum/irregulare,* where 30 °C is a lethal temperature for *P. violae* and *P. intermedium*, but not for *P. sulcatum* and *P. sylvaticum/irregulare*.

3.3.2. Pathogenicity

The infection efficiency (rate of successful infection) of *P. violae* and *P. sulcatum* was 95% and 100%,

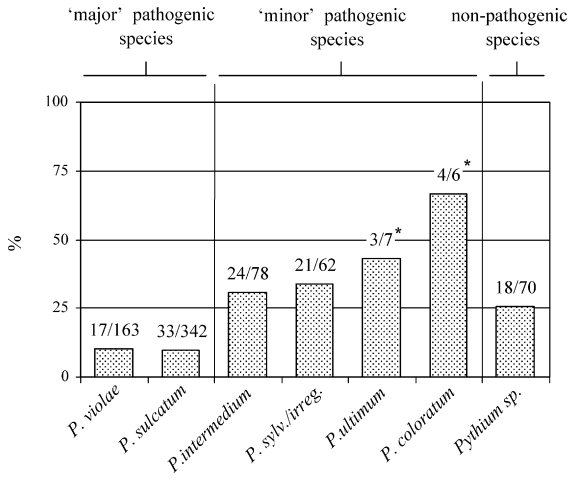

Fig. 4. Proportion of *Pythium* strains isolated with at least one other species from a single CCS lesion in the field experiment (2001–2003). Data were pooled (infested and control plots); the fractions correspond with the ratios between the number of strains (of a given *Pythium* species) isolated in mixture from a single lesion and the total number of strains (of this *Pythium* species); * indicates that the proportion was calculated from a small number of strains.

Table 2
Proportion of CCS lesions from which multiple *Pythium* species were isolated, combining infested and control plots in the field experiment (2001–2003)

Stastistical test[a]	2001			2002			2003			Pooled (2001–2003)		
	χ^2	*P*	*mir*[b]	χ^2	*P*	*mir*[b]	χ^2	*P*	*mir*[b]	χ^2	*P*	*mir*[b]
P. violae	13.06	0.003	0.49	19.60	<0.001	0.20	–	–	–	76.59	<0.001	0.25
P. sulcatum	9.43	0.002	0.33	11.75	<0.001	0.57	31.77	<0.001	0.36	84.21	<0.001	0.34
P. intermedium	4.88	0.027	0.56	1.24	0.266	0.82	–	–	–	11.55	0.007	0.62
P. sylvaticum/irregulare	5.49	0.019	0.50	2.66	0.103	1.45	11.00	<0.001	0.46	7.17	0.007	0.67
P. ultimum	0.82	0.370	–	0.28	0.594	0.77	0.46	0.500	1.46	0.32	0.573	0.80
P. coloratum.	1.22	0.270	2.22	0.13	0.721	1.15	–	–	–	0.41	0.520	1.24
Pythium sp.	0.49	0.486	0.82	1.13	0.288	0.77	33.83	<0.001	0.17	16.87	<0.001	0.52

[a] Distribution of *Pythium* isolates among mixed infection using contingency table analysis and the chi-square statistic.
[b] *mir* is the mixed infection ratio (ratio between 'observed' and 'expected' mixed infection frequency); $mir < 1$ and $P < 0.05$ mean that co-infection was significantly over-represented; 'expected' is the frequency of mixed infection expected if infection by a given *Pythium* species and by another is independent; 'observed' is the frequency of mixed infection observed after *Pythium* isolations.

respectively (Fig. 6). Mean infection efficiency of other pathogenic species was lower and variable between strains belonging to the same species group: *P. sylvaticum/irregulare* ($e = 54\%$), *P. intermedium* A/B ($e = 44\%$ and $e = 18\%$, respectively), *P. coloratum* ($e = 33\%$), and *P. ultimum* ($e = 15\%$). The extension of lesions (area of lesions) induced by *P. sylvaticum/irregulare*, *P. intermedium* B, *P. coloratum*, and *P. ultimum* was limited. Larger lesions resulted from *P. sulcatum*, *P. intermedium* A, and *P. violae* infections. Combination of these measurements explained why upper values of φ were obtained for *P. sulcatum*, *P. violae*, *P. intermedium* A, and to a lesser extent *P. sylvaticum/irregulare*. According to their high pathogenicity *in vitro* and high prevalence in the pathogen complex on lesions, *P. violae* and *P. sulcatum* are considered here as 'major' species. This was confirmed by the decreasing relationship between *mir* and the pathogenicity φ ($R^2 = 0.638$) (Fig. 7). The less a *Pythium* species was evaluated as pathogenic on carrot,

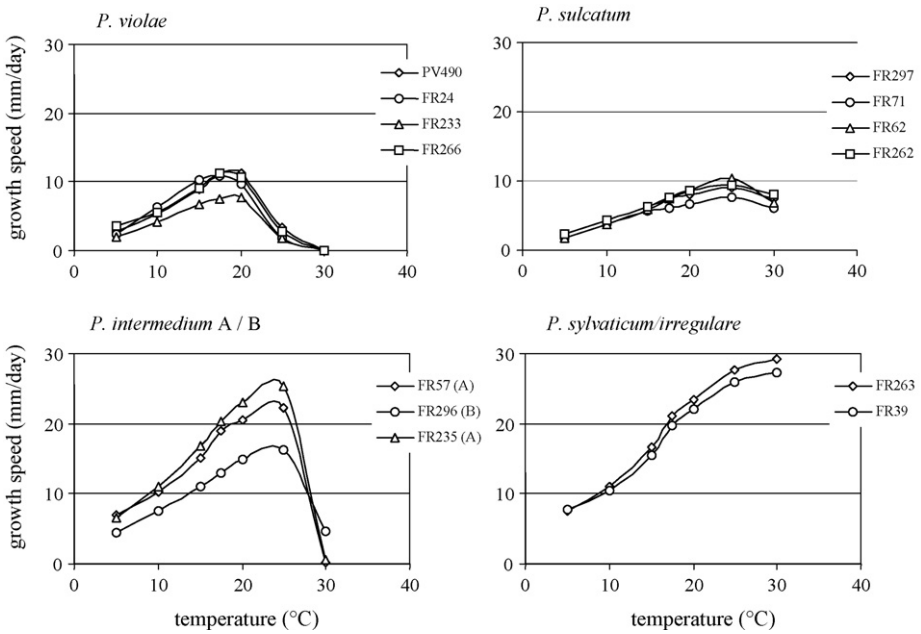

Fig. 5. Radial mycelial growth rates of four pathogenic *Pythium* species after incubation on corn meal agar (CMA) for 96 h. PV490 is the reference strain (CBS 102.609); strains FR24, FR39, FR57, FR62, and FR71 were isolated in 2001 from CSC lesions in the field experiment; strains FR233, FR266, FR262, FR296, FR235, and FR263 were isolated in 2002.

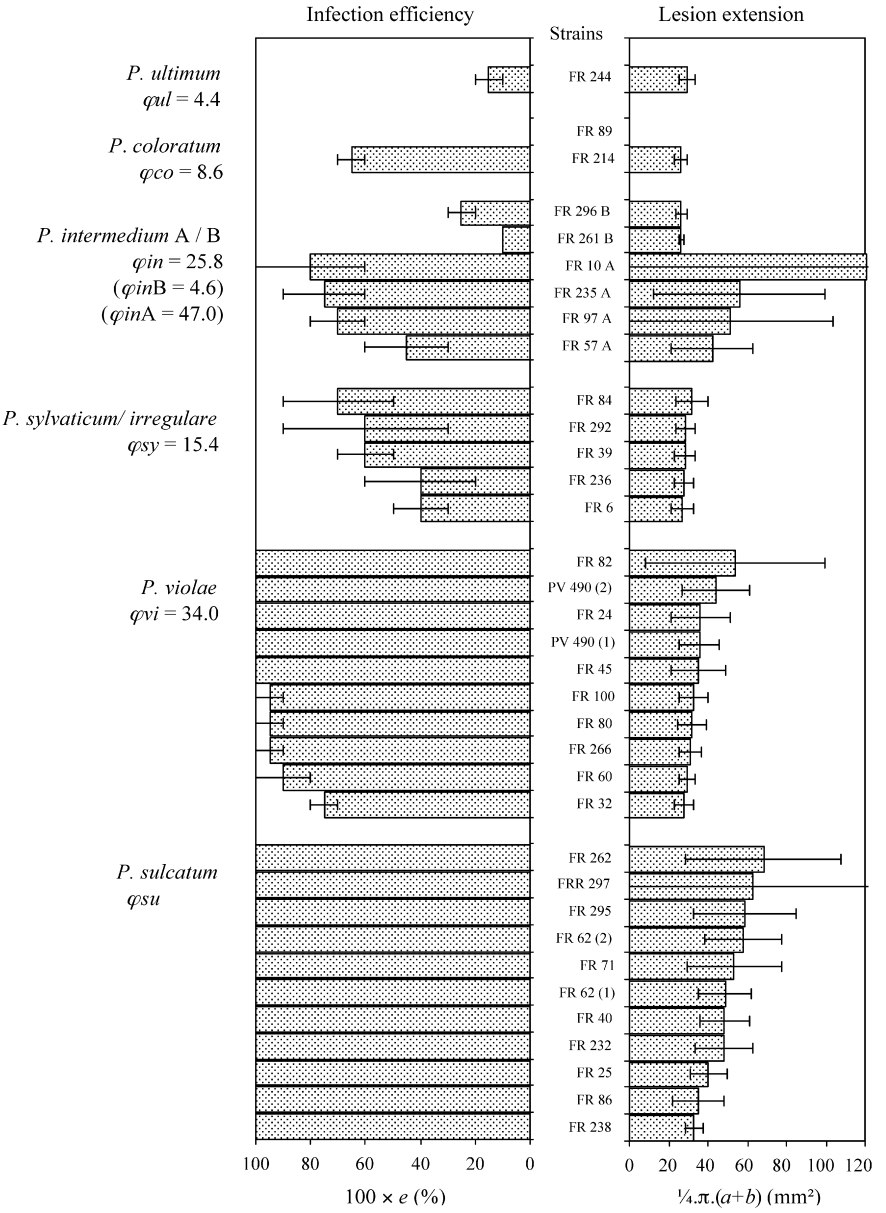

Fig. 6. Pathogenicity of *Pythium* strains isolated from CCS lesions in the field experiment. Infection efficiency e (rate of successful infection) and lesion extension (area of a lesion) were established for each isolate; pathogenicity (mean value of $\varphi = e \times 1/4\pi(a + b)$) was calculated for each *Pythium* sp.

the more it was isolated from a CCS lesion in mixture with at least one other species.

3.3.3. Saprophytic survival and decay of the inoculum potential

The temporal decline of inoculum potential was illustrated by increasing root weight and decreasing disease severity (*tda*) with the time span separating soil inoculation and carrot sowing (Fig. 8). CCS level was relatively high when carrots were sown simultaneously or less than 40 days after soil infestation (*tda* ranged from 0.2 to 0.5 mm^2 for *P. sulcatum* and ranged from 0.2 to 1.5 mm^2 for *P. violae*), but was lower when carrots were sown more than 100 days after soil infestation (*tda* less than 0.1 mm^2 for *P. sulcatum* and less than 0.2 mm^2 for *P. violae*). Although nonsignificant, the temporal decay of the inoculum potential was quite similar between *P. violae* and *P. sulcatum* and was observed in both soil types.

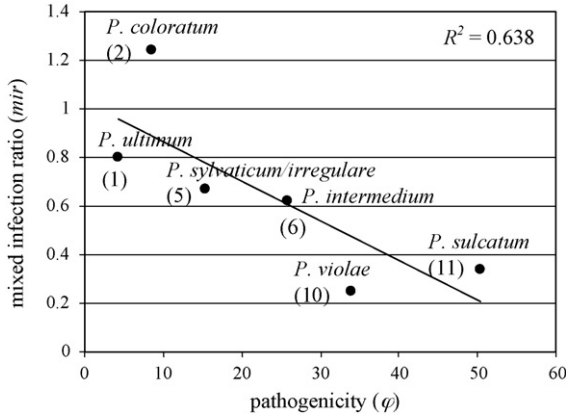

Fig. 7. Relationships between the mixed infection ratio (*mir*) estimated from the field experiment (2001–2003, infested and control plots) and the pathogenicity φ of the *Pythium* spp. estimated *in vitro*. Brackets indicate the number of isolates tested.

4. Discussion

4.1. Diversity and fluctuations in the Pythium complex

The increase in prevalence of *P. violae* during the first year (2001) may have been due to the high artificial soil infestation due to the initial abundance of barley grain inoculum. The decline of inoculum potential was not fundamentally different for *P. violae* and *P. sulcatum* under controlled conditions. Similarly, Hancock (1981) showed a decline in *P. ultimum* in natural soils; the decrease in inoculum densities over the first few months was less evident and the subsequent decrease in densities was more pronounced if soils were sterilized prior to infestation with *P. ultimum*. Similar reasons may explain the differences observed between the rapid inoculum decay of *P. violae* that occurred in the sandy soil, compared with the natural non-sterilised silt soil.

On the other hand, the high temperatures recorded in 2002 and 2003 (when soil temperatures exceed 20 °C) may explain the decrease in the *P. violae* inoculum and the development of the endogenous *P. sulcatum*, since *P. violae* is less adapted to high temperatures than *P. sulcatum* (thermal optimum 19 °C for *P. violae* versus 25 °C for *P. sulcatum*, with a lethal temperature 30 °C for *P. violae*). Soil properties (composition, texture and moisture) may be another reason for the lack of *P. violae* after 2002. Other endogenous species *(P. intermedium*, *P. sylvaticum* or *P. coloratum*) occurred equally but never increased in frequency on lesions, perhaps because they were less pathogenic than *P. violae* and *P. sulcatum*. No significant difference in the prevalence

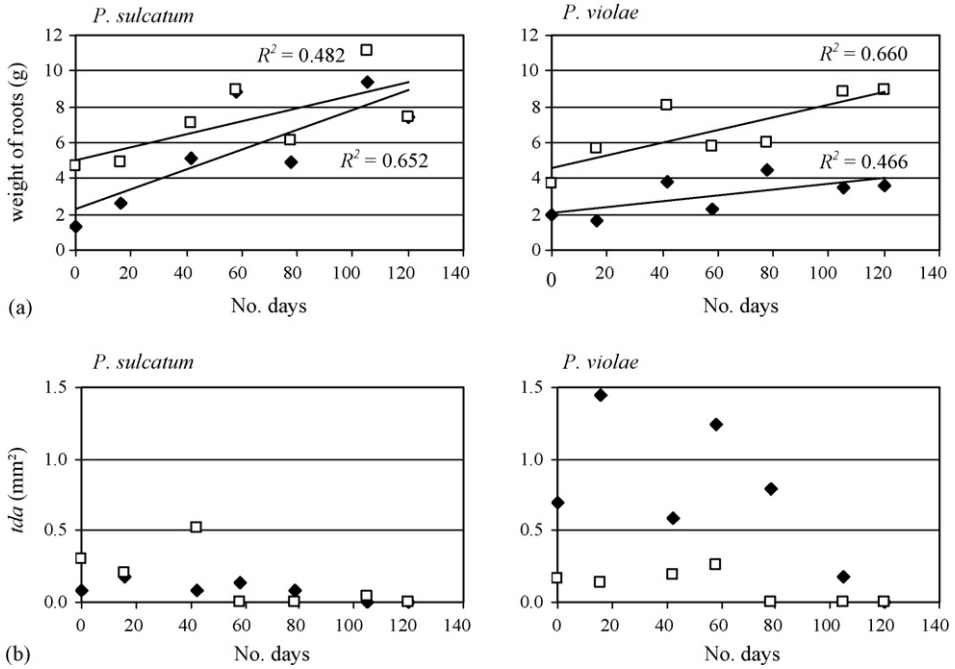

Fig. 8. Variation in the inoculum potential of *P. violae* and *P. sulcatum* in two soils after artificial inoculation with barley grains and different dates of sowing. (a) Effect on the mean weight of carrot roots. (b) Effect on the total disease area (*tda*). The scale on the horizontal axis is the number of days between the soil infestation and the sowing of carrots; white squares (□) correspond with the reconstituted sandy soil, and black diamonds (◆) correspond with the natural non-sterilised silt soil from the field experiment.

of *P. intermedium*, *P. sylvaticum* or *P. coloratum* between infested and control plots was observed.

In our study, the composition of the *Pythium* complex was not representative of communities described from the carrot production areas of Créances (Manche, France), in which *P. violae* is naturally predominant (Breton and Rouxel, 1993). Therefore, results cannot be directly extrapolated to other regions. White (1986), Liddell et al. (1989), and El-Tarabily et al. (1996) described the composition of *Pythium* complexes by isolation from lesions and concluded that the effects of environmental conditions on CCS development differed between *Pythium* species. White (1988) found for example that *P. sulcatum* was less sensitive than *P. violae* to metalaxyl, a standard fungicide for CCS control. Such differences in sensitivity may induce local changes in the diversity of *Pythium* communities.

Serological studies showed that *P. sylvaticum* and *P. irregulare* may be included in the same 'group' of species (Hendrix and Campbell, 1973; White et al., 1994), identified by quite similar PCR profiles (Wang and White, 1997; Vasseur et al., 2001). Distinguishing *P. coloratum* from *P. dissotocum* or from *P. diclinum* (Wang and White, 1997; Breton, pers. comm.) has also proved difficult. Additional molecular tools would be necessary to differentiate the strains belonging to these two groups. Nevertheless, *Pythium* groups defined here were precise enough for our ecological approach. Fast-growing *Pythium* species are more easily isolated from CCS lesions in Petri dishes than slow-growing species (White, 1988). Consequently, the occurrence of the slow-growing *P. violae* and *P. sulcatum* may be theoretically underestimated in case of mixed infections with the fast-growing species *P. intermedium*, *P. sylvaticum* or *P. coloratum*.

4.2. Interactions between Pythium species

We showed that the less a *Pythium* species was pathogenic on carrot, the more often it was isolated in mixture with other species from a single lesion. Based on pathological characteristics of each species, changes over time in the diversity of the *Pythium* community, and contingency analysis of mixed infections, the *Pythium* population may be divided into three 'ecological' groups:

(i) 'Major' pathogenic species such as *P. sulcatum* and *P. violae*, which cause 75–90% of the CCS lesions during a large epidemic, are rarely isolated in mixture with other species; these 'major' species are highly pathogenic and are able to induce CCS lesions by themselves.

(ii) 'Minor' pathogenic species such as *P. intermedium*, *P. sylvaticum/irregulare*, and *P. coloratum*, which induce less than 10–25% of the symptoms and are more frequently present on a lesion in association with a 'major' pathogenic species. These 'minor' species are less pathogenic, are less able to induce CCS lesions by themselves, and may be considered as opportunistic pathogens,

(iii) Non-pathogenic *Pythium* species, which are rarely able to induce typical CCS lesions by themselves *in vitro*.

This classification was suggested for other sympatric species belonging to diverse soil-borne pathogenic communities. The composition of some *Pythium* complexes may fluctuate during (or between) cropping seasons (Mihail et al., 2002; Rao et al., 1978; Larkin et al., 1995a, 1995b; Lumsden et al., 1976) and pathogenic species sometimes occupy the same ecological niche. This is not really the case for the CCS because some species isolated in our study seem to be totally specific to carrots and others not. The fast-growing *Pythium* species, which are less pathogenic on carrots, are less specialised, while the more pathogenic slow-growing species are more specialised.

Infection levels by different pathogenic species were not positively correlated in cases of mixed infections, indicating few interactions between the 'major' species (*P. violae* and *P. sulcatum*) and others. Changes in the *Pythium* complex seemed to be due more to individual dynamics relating to specific biological characteristics (optimum temperature of mycelial growth and inoculum survival in soil), than to interactions between species during the infection processes. Synergistic or antagonistic effects between species in cases of mixed infection have rarely been discussed, except for plant viruses (Zhang et al., 2001) or some pathogens of potato tubers (Pett and Kleinhempel, 1976; Langerfeld, 1981). Our results illustrate a situation where several associated species interact to cause one single plant disease. The degree of mixed infection, found to be more than 10–15%, is not negligible in the case of a CCS epidemic. Nevertheless, few studies on the diversity of *Pythium* communities have focused on the fine spatial scale among the individual lesions and the cohort of lesions on a root. The occurrence of mixed infection has rarely been discussed, excepted by Mihail et al. (2002) who characterized the diversity of a *Pythium* community on *Kummerowia stipulata* at the lesion scale, and by Xu et al. (2005) who studied two- and three-pathogen synergistic

interactions between *Fusarium* species on wheat. In the case of CCS, as for several soil-borne diseases due to a *Pythium* complex (Lumsden et al., 1976; Larkin et al., 1995a; Lee and Hoy, 1992; Rao et al., 1978), the two hypothesis (i) prevalence of a single 'major' species in the complex without interaction with other 'minor' species, and (ii) prevalence of some 'major' species that interact with other 'minor' species, have been rejected. The *Pythium* complex responsible for CCS, spatially diverse and temporally fluctuant, is then characterised by the absence of competition between species during the infection phase. This is a common feature of several other *Pythium* complex-inducing root diseases.

Analyses of mixed infection from field experimental data and comparisons of root infection by total *Pythium* species or one single species with disease severity may be used as an alternative approach to studying interactions of pathogenic *Pythium* species. This combined approach, partially done for CCS, may be generalised to other soil-borne pathogen complexes to characterise the main type of interaction (synergic or not) between species within the complex and potential epidemiological consequences. To this end, knowledge about the ecology of *P. violae* and *P. sulcatum* and other root-infecting species during the growing season and over the course of the crop cycle is needed to gain a better understanding of CCS epidemiology in carrot.

4.3. Consequences for quantitative CCS epidemiology

The absence of major interactions between *Pythium* species during infection has important consequences for quantitative epidemiology. The main issue concerns the species diversity and the time scale relevant to analyse a CCS epidemic due to a *Pythium* complex. Fluctuations in the diversity of the *Pythium* community isolated from lesions showed that the composition of the pathogen complex must be analysed at both the annual and multi-annual scales. Theoretically, it is possible to consider (i) a single, multi-specific epidemic, including the effects due to interactions between species, or (ii) several mono-specific epidemics with a total outcome corresponding to the sum of the effects of each *Pythium* species considered independently. The second hypothesis was here preferred because of the absence of major interactions within the complex during the infection processes: the epidemic results from several major sub-epidemics each due to a single *Pythium* species. It is also necessary to measure some characteristics of the complex (pathogenicity, optimum temperature, and inoculum survival) for reliable CCS predictions and management, even if we do not need to take into account the composition of the complex to model epidemiological processes.

In the absence of interaction between two species, the overall disease progress curve, illustrated by the total disease area *tda*, may be due to the sum of different mono-specific disease progress curves specifically due to single *Pythium* species (tda_i) and the disease progress curves due to mixed infection ($tda_{i,j}$). The Eq. (2) is a mathematical transcription of this formalization and illustrates the case of a double infection (mixed infection by two species):

$$tda = \sum_{i=1}^{S} tda_i + \frac{1}{2}\sum_{i=1}^{S}\left(\sum_{j=1}^{S} tda_{i,j}; i \neq j\right) \quad (2)$$

where *tda* is the total disease area, tda_i the total disease area exclusively due to the species *i* (*i* is isolated alone from a lesion), $tda_{i,j}$ the total disease area due to the species *i* and *j* (*i* is isolated in mixture with *j*), and *S* the total number of species implicated in the epidemic.

Variations within a *Pythium* complex may affect the final disease severity of CCS, through the inoculum density of each species, but would not affect the fundamental form of the disease progress curves. Epidemiological processes (e.g. primary and secondary infections), tested in particular conditions, may also be valid in other conditions. The equation of the model describing disease progress curves does not need adaptation to take into account new hypothetical processes due to synergism or antagonism between species.

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