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Aggressiveness and production of cell-wall degrading enzymes by *Pythium violae, Pythium sulcatum* and *Pythium ultimum*, responsible for cavity spot on carrots

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Abstract

This study investigated the relationships between pathogenesis, types of symptoms and *in vitro* production of cell-wall degrading enzymes by *P. violae, P. sulcatum* and *P. ultimum*. The three pathogens, considered as the three *Pythium* species principally responsible for cavity spot on carrot roots, secreted only low levels of fatty acid esterases activity, suggesting they have limited ability to degrade suberin in the walls of the outer cell layers of carrot tissues. Among the enzymes that degrade cell-wall polysaccharides, only pectate lyases and cellulases were produced by *P. violae*, and these were produced late and in small amounts: the symptoms caused by *P. violae* were limited and typical of cavity spot. Conversely, *P. ultimum* caused maceration of tissues, and secreted polygalacturonases and β-1,4-glucanases earlier and in larger amounts than *P. violae*. *P. ultimum* also produced a large diversity of proteins and cellulase isoenzymes. Although secreting all the monitored enzymes in higher quantity than the two previous species, *P. sulcatum* was responsible for only typical limited symptoms of cavity spot, with a brown colouring. The role of plant reactions induced in response to early pectinolytic enzyme production by *P. sulcatum* may account for this apparent inconsistency.

Abbreviations: A_{276} – absorbance at 276 nm; CWMCP – cell-wall material containing protopectin; pI – isoelectric point.

Introduction

Cavity spot of carrots, a root disease caused by *Pythium* spp., results in severe yield losses as well as reductions in quality wherever this crop is grown intensively. The significant role of the slow-growing species *P. violae* in the development of this disease was established ten years ago by Groom and Perry (1985), and described more precisely by White (1986) and Montfort and Rouxel (1988). Other *Pythium* species may also be involved in this root disorder. In Europe, White (1986) and more recently Guérin 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* in the etiology of the disease. *P. ultimum*, which has a very wide host range (Plaats-Niterink, 1981), is also commonly isolated from cavity spot lesions on carrots in the USA, and so is considered as the second most important species after *P. violae* (Vivoda et al., 1991). Similarly, Benard and Punja (1995) demonstrated that among eight *Pythium* species aggressive on carrots in British Columbia, the most highly aggressive were *P. violae*, *P. ultimum* and *P. sulcatum*. Finally, another fast-growing species, *P. coloratum*, has been described as responsible for symptoms on carrot roots in Western Australia (El-Tarabily et al., 1996).

It therefore appears that cavity spot may be caused by several *Pythium* species, differing by morphological (Plaats-Niterink, 1981), biological, biochemical

(Guérin et al., 1994), and molecular criteria (Briard et al., 1995). Though their relative importance varies among geographical areas, multiple species may be isolated from a single region, a single field and even a single root lesion (Guérin, 1993). The complex origin of the disease leads to the question of possible differences in the pathogenicity of these different species on carrots, including the type of symptom produced by each species and its colonization potential of root tissue.

Cavity spot symptoms have been widely described in the literature as being translucent and well-delimited elliptical lesions, oriented across the breadth of the root (Groom and Perry, 1985; Montfort and Rouxel, 1988; Vivoda et al., 1991; Zamski and Peretz, 1995). This typical aspect was mainly reported after *P. violae* attacks, in the field or in experiments with artificial inoculation. More progressive, brown root symptoms have been observed after inoculation with fast-growing species, such as *P. coloratum* (El-Tarabily et al., 1996), and on carrots in the field (Montfort and Rouxel, 1988), but no information was given as to the *Pythium* species present or the particular environmental conditions. A specific description of cavity spot symptoms in relation to the causal agent is thus needed to clarify the relationship between *Pythium* sp. and symptomatology on carrot roots.

Most available information on cavity spot concerns *P. violae*, notably regarding the process of lesion formation by collapse of the superficial root tissues (Briard, 1990). Histological and ultrastructural observations have shown degradations of pectic substances and cellulose in infected root tissues (Guérin, 1993), and recent results suggested that this species was able to secrete sufficient enzymic activity to degrade cell-wall polysaccharides *in situ* (Zamski and Peretz, 1996). The only results concerning the other *Pythium* species involved in the cavity spot established a global relation between aggressiveness and *in vitro* production of pectolytic enzymes by *P. violae*, *P. sulcatum*, *P. ultimum*, *P. sylvaticum* and *P. irregulare*; pectolytic activities were not characterized (Benard and Punja, 1995). Consequently, further information is required concerning the ability of *Pythium* species to degrade carrot root tissues.

Penetration of unwounded tissue requires the production by the pathogen of enzymes able to degrade natural barriers (Kolattukudy, 1985) such as the suberized cell-walls present in the outer layer of carrot root tissues (Esau, 1940). It is therefore important to verify whether *Pythium* spp. pathogenic on carrot roots also can secrete enzymes such as fatty acid esterases, as reported in *P. ultimum* by Mozzafar and Weete (1993). The role of cell-wall polysaccharide degrading enzymes also is important in the development of disease symptoms due to *Pythium* species, as observed by Chérif et al. (1991) in the breakdown of pectic and cellulosic material in cell-walls of cucumber infected by *P. ultimum*. Moreover, the production of enzymes that degrade cell-wall polysaccharides may vary with *Pythium* species: *P. aphanidermatum*, responsible for cottony-leak disease on cucumber, and *P. ultimum*, implicated in blighted foliar disease of Highlands bentgrass, display pectinolytic and cellulolytic activities (Winstead and McCombs, 1961; Moore and Couch, 1968), whereas *P. butleri*, responsible for root-rot of Belladonna (*Atropa belladonna* L.), produces polygalacturonase, pectin and pectate lyases (Janardhanan and Husain, 1974). Protopectinase, an enzyme able to solubilize pectic molecules from protopectin (Sakai et al., 1993), is secreted by *P. debaryanum*, pathogenic on potato tubers (Wood and Gupta, 1958). In addition, considering that carrot root cell-walls are particularly rich in polysaccharides (Kirtschev and Kratchanov, 1980) and that their protopectin content is correlated with carrot susceptibility to another root pathogen, *Mycocentrospora acerina* (Le Cam et al., 1994a), it is important to characterize and compare enzymic activities of the *Pythium* spp. involved in cavity spot.

This study investigated the relationships between enzymic activity and pathogenesis in *P. violae*, *P. sulcatum* and *P. ultimum*. The cell-wall degrading enzymic potential was tested *in vitro*, as previously established in several models, including *Pythium* spp. (Janardhanan and Husain, 1974; Carder, 1989; Riou et al., 1991; Lehtinen, 1993; Le Cam et al., 1994b; Degefu et al., 1995). After a biological characterization of typical strains, proteins secreted *in vitro* by the three *Pythium* spp. were quantified and analysed by isoelectric focusing, and fatty acid esterase activity was assessed. Cell-wall polysaccharide degrading activities were identified and quantified and strains were analysed for cellulase isoenzyme diversity. Results are discussed with regard to disease symptoms induced on carrot roots by the different strains.

Material and methods

Fungal strains

Three strains (*P. violae* 20, *P. sulcatum* 199 and *P. ultimum* 185) obtained from the collection of the Station de Pathologie Végétale (Le Rheu, France) were used in this study. All three strains were isolated from cavity spot symptoms on carrot roots grown in the north western region of France and were maintained at 4 °C on malt-agar. Each strain was morphogically representative of its species (Plaats-Niterink, 1981).

Biological characterization

The aggressiveness of each strain was quantified by measuring symptom size on inoculated carrot roots as described by Montfort and Rouxel (1988). Briefly, healthy carrot roots, grown for 4 months in the field, were washed thoroughly, then wounded using an abrasive sheet and inoculated with 4 mm diameter plugs (4 per root) cut from the edge of a 5 day-old fungal colony grown at 20 °C on a carrot juice-agar medium (200 ml commercial carrot juice, clarified by addition of 10 g per litre of $CaCO_3$ then centrifugation, and 20 g agar per litre). Agar plugs were used as controls. Roots were incubated at either 20 °C or 25 °C in darkness in a moist chamber, where humidity was maintained by daily spraying with sterile water. After 4 days, the disease index was calculated as the mean size of 32 inoculation sites per strain and temperature. The size of each elliptical lesion was calculated from its two diameters. The appearance of symptoms also was described for each treatment.

Mycelial growth rate was calculated for each strain. One 4 mm diameter plug was cut from the edge of a 5 day-old fungal culture grown at 20 °C on malt-agar and transferred to a 9 cm Petri dish containing 15 ml of carrot juice-agar medium. Growth rate (mm/24 h) was assessed by measuring two right angle diameters of the fungus colony obtained at 20 °C or 25 °C, at intervals of 8 and 16 h. Results were expressed as the mean of three replications.

Production of culture filtrates

Pythium strains were cultured in a non-shaken liquid medium containing per litre: 2.5 g carboxymethylcellulose (Sigma, C-4888), 2.5 g citrus pectin (Sigma, P-9135), 2.72 g KH_2PO_4, 1.23 g $mgSO_4,7H_2O$ and 1 g casein hydrolysate; pH was adjusted to 5.8 prior to autoclaving. One hundred milliliters of medium were placed in 300 ml Erlenmeyer flasks and inoculated with 10 mycelial plugs (4 mm diameter) from the edge of a 5 day-old fungus colony grown on malt-agar. After 7, 14 or 21 days of incubation at 20 °C for *P. violae* and 25 °C for *P. sulcatum* and *P. ultimum*, cultures were harvested by vacuum filtration through G3 sintered-glass filters, then under pressure through 3 μm and 0.45 μm Sartorius filters. Filtrates were diafiltrated and concentrated (Millipore filters, PLGC11K25) at 4 °C if needed, and conserved at –18 °C.

Protein content and isoelectrofocusing (IEF)

Protein content in the filtrates was measured as described by Bradford (1976). Results were expressed as the mean of two replications.

Concentrated 21 day-old culture filtrates were subjected to isoelectrofocusing on an ultra-thin (0.5 mm) 3–9 pH range polyacrylamid gel (Pharmacia, Phast-Gel IEF 3–9). Samples were loaded and gel was focused on a Phastsystem apparatus at 2000 V, 2.5 mA and 3.5 W until 410 Vh (Accumulated volts $\times$ hours). Proteins were stained automatically by precipitation in a 20% (w/v) trichloracetic acid solution and colouring in a 0.4% (w/v) silver nitrate solution. The experiment was repeated three times, with samples loaded at different sites on the gel. A standard was prepared by loading a calibration kit (Pharmacia) under the same conditions.

Fatty acid esterase activity assay

Fatty acid esterase activity was assayed by following the degradation of the *p*-nitrophenyl ester of butyric acid, as described by Nguyen-The and Chamel (1991). Release of *p*-nitrophenol was measured at 405 nm in a reaction mixture at 30 °C containing 100 μl culture filtrate in 2.5 ml of a solution containing 0.1% (v/v) *p*-nitrophenyl-butyrate (Sigma, N-9876) in 0.1 m Tris-HCl buffer pH 8.0 supplemented with 0.01% (v/v) Triton X-100. Results were expressed in absorbance units per minute per milliliter of culture filtrate (arbitrary unit) and as the mean of two replications.

Assay of cell-wall polysaccharide degrading enzymic activities

Enzymic activities were measured on concentrated culture filtrates and were expressed in pkat/ml of culture filtrate, except for xylanase activity.

Polygalacturonase and β-1,4-glucanase activities were determined by measuring release of reducing groups in a medium containing 450 μl substrate and 50 μl sample at 40 °C. Enzymic reactions were stopped by adding 1 ml of 0.2 m sodium borate buffer pH 9.5. Reducing groups were visualized by reaction with 500 μl of a solution of 2% (w/v) 2-cyanoacetamide at 90 °C during 10 min and A_{276} was measured. Polygalacturonase activity was measured in a solution of 0.25% (w/v) sodium pectate (Sigma, P-1879) in 0.05 m citrate-phosphate buffer pH 5.0 with 0.0005 m EDTA and 0.025 m NaCl. β-1,4-glucanase activity was measured in a mixture containing 0.25% (w/v) carboxymethylcellulose (Sigma, C-4888) in 0.05 m Tris-maleate buffer pH 5.0. A_{276} was measured for galacturonic acid or glucose as standards (0–0.4 μmol ml^{-1}). Results were expressed as the mean of three replications.

Pectin methylesterase activity was determined by automatic titration of the carboxyl groups released from a 0.5% (w/v) solution of 75% esterified pectin (Sanofi) in water. The reaction mixture was kept under nitrogen at 30 °C. Results were expressed as the mean of two replications.

Lyase activities were determined at 25 °C by measuring the release of a reaction product absorbing at 235 nm, in a medium containing 2 ml substrate and 100 μl sample. Pectin lyase activity was measured in a reaction mixture containing 0.25% (w/v) of 75% esterified pectin (Sanofi) in 0.1 m citrate-phosphate buffer pH 6.0 containing 0.16 m KCl. The reaction mixture was centrifuged for 20 min at 21300 g (Voragen, 1972). Pectate lyase activity was measured in a 0.5% (w/v) sodium pectate (Sigma, P-1879) solution in 0.05 m Tris-HCl buffer pH 8.6 containing 3 mm $CaCl_2$. Molar extinction coefficients used to evaluate activities were 5500 $m^{-1}cm^{-1}$ for pectin lyase and 4800 m^{-1} cm^{-1} for pectate lyase. Results were expressed as the mean of two replications.

Xylanase activity was determined at 40 °C in a medium containing 450 μl substrate (0.5% (w/v) xylan-azure in 0.05 m sodium acetate buffer pH 5.0) and 50 μl sample (Massiot, 1992). The reaction was stopped by addition of 1 ml cold ethanol. After centrifugation (500 g), A_{595} was measured in the supernatant. The xylanase activity was expressed as the amount of enzyme which increased the absorbance of 0.1 unit per minute per milliliter of culture medium (arbitrary unit). Results were expressed as the mean of three replications.

Preparation of cell-wall material containing protopectin (CWMCP)

Carrot roots from a hybrid susceptible to cavity spot were grown for 4 months in the greenhouse, in a sterilized soil mixture. Cell-wall material was prepared from peelings of carrot roots (1 mm thick) using the technique described by Massiot et al. (1988) and modified as follows: 200 g of peelings were immersed in 800 ml of aqueous 96% (v/v) ethanol containing 1% (v/v) hydrochloric acid. The mixture was blended for 35 s, boiled for 10 min, cooled, and filtered on a G3 sintered-glass. The material was washed with aqueous 80% (v/v) ethanol until the sulphuric acid-phenol test (Dubois et al., 1956) was negative, rinsed with acetone, then air-dried. Before use, water soluble pectins were removed from the cell-wall material by two successive applications of the following protocol: suspension in 0.025 m sodium acetate buffer pH 4.5 for 1 h, rinsing in 96% ethanol solution and drying in acetone.

Degradation of CWMCP

Twenty milligrams of CWMCP suspended in 5 ml 0.025 m sodium acetate buffer pH 4.5 were incubated overnight at 40 °C with 50 μl of either concentrated filtrates of 21 day-old cultures of the three strains of *Pythium* or a preparation obtained from *Aspergillus niger* and rich in polygalacturonase, provided by Megazyme (North Rocks, Australia). The same quantity of polygalacturonase activity was added in the tubes for *P. sulcatum* and *Aspergillus niger*. Enzymic activities in the tubes corresponding to *P. violae*, *P. ultimum* and *P. sulcatum* were in the same proportions as in the crude filtrates. One tube containing only CWMCP and three tubes containing only culture filtrates in buffer were used as controls. After incubation, tubes were heated at 95 °C for 10 min and centrifuged. The supernatant was filtered on 0.45 μm membranes, then analysed by gel-permeation chromatography on a system composed of a series of four TSK HPLC columns (50 – 40 – 30 – 20 – XL; 300 $\times$ 7.8 mm) thermostated at 40 °C. Samples were eluted with a 0.4 m sodium acetate buffer pH 3.6 at 0.8 ml min^{-1}. The eluate was monitored at 520 nm using the automated colorimetric method with metahydroxydiphenyl as reagent (Thibault, 1979). The quantity of pectic substances solubilized was determined by a measure of the galacturonic acid content using the same automated method with galacturonic acid as the standard (0.05–0.25 μmol ml^{-1}).

Isoelectrofocusing of β-1,4-glucanases

Concentrated 21 day-old culture filtrates were centrifuged at 16000 g. Isoelectrofocusing of β-1,4-glucanases was assayed on an ultra-thin (0.5 mm) polyacrylamide gel prepared by injection between two glass plates (230 × 115 mm) of 15 ml of a acrylamide-bisacrylamide solution (10% (w/v) acrylamide, 0.26% (w/v) bisacrylamide) containing 0.075% (w/v) persulfate ammonium and either 1 ml 3-10 pH range ampholines (Serva, Servalyt 3–10) or 1 ml 3–6 pH range ampholines (Serva, Servalyt 3–6). Samples were loaded and proteins migrated on a Pharmacia FBE 3000 flat bed apparatus at constant power (25 W). After 3.5 h, the gel was equilibrated by immersion in 0.25 m Tris-maleate buffer pH 5.0 for 10 min and blotted for 2.5 h at 37 °C against substrate-containing buffered agar gel (0.5% (w/v) CMC (Sigma, C-4888), 1.5% (w/v) noble agar (Difco) in 0.1 m Tris-maleate buffer pH 5.0), as described by Bertheau et al. (1984). The agar gel was gently soaked for 0.5 h in 1% (w/v) Congo red, rinsed in water and 1 m NaCl, then immersed and soaked in 1 m NaCl for 20 min. After repetition of this colouring protocol, the gel was placed in 1 m NaCl overnight. β-1,4-glucanase activity appeared translucent on a red background. This experiment was repeated three times, with samples loaded at different sites on the gel.

Statistical analysis

Analysis of variance and comparison of means were applied to the experimental data with Newman-Keuls' test.

Results

Biological characterization of the three Pythium *strains*

All three *Pythium* species induced superficial necrosis (less than 1 mm deep) on carrot roots, confirming their potential role in cavity spot disease (Figure 1a). *P. violae* and *P. sulcatum* produced typical symptoms, with delimited elliptical lesions (<200 mm^2) oriented across the breadth of the root. At 20 °C, symptoms caused by *P. violae* were translucent and significantly larger in surface than at 25 °C, whereas those caused by *P. sulcatum* were brown and larger at 25 °C. The largest lesions were due to *P. ultimum*, with sizes of at least 300 mm^2 at 20 °C and of about 500 mm^2 at 25 °C; the symptoms were less typical of cavity spot, however, with maceration of the superficial tissues and development of white mycelium on the root surface.

Mycelial growth rate was influenced to a great extend by temperature. Lesion size of necroses was comparable to the mycelial growth rates at the two temperatures (Figure 1b), with *P. violae* and *P. sulcatum* as slow-growing (<20 mm/24 h), small-lesion species, and *P. ultimum* as a fast-growing (>20 mm/24 h) species that caused large lesions.

Protein content and analysis

Protein content developed differently in the three filtrates (Table 1): content increased with culture duration for *P. sulcatum* and *P. ultimum*, but after 21 days of growth, the *P. sulcatum* filtrate contained twice as much proteins as that of *P. ultimum*. Conversely, the protein content of the *P. violae* filtrate was almost constant at the three times of harvest, indicating that the level of protein secretion decreased after 7 days of culture.

IEF showed that most proteins produced by the three strains presented a pI in an acid pH range (3.0–5.5) (Figure 2). Only the *P. ultimum* filtrate produced proteins with pI between 5.5 and 8.0. The *P. ultimum* filtrate in this pH range also yielded 4 bands at pI higher than 8.0, 2 and 1 bands were revealed in *P. violae* and *P. sulcatum* filtrates, respectively.

Fatty acid esterase activity

Fatty acid esterase activity was detected in the 21 day-old culture filtrates of the three strains studied (Table 1), but production in *P. sulcatum* was twice that in *P. ultimum* and four times that in *P. violae*. This result obtained for *P. sulcatum* was confirmed by the analysis of a filtrate from an independant experiment (48.3 ± 0.9 absorbance unit min^{-1} ml^{-1} culture filtrate).

Cell-wall polysaccharide degrading enzymes

After 7 days of culture (Table 1), no cell-wall polysaccharide degrading enzyme could be detected in the filtrate of *P. violae*. Two enzymes were produced by the two other species; polygalacturonase and pectin methylesterase were secreted by *P. sulcatum* and β-1,4-glucanase and polygalacturonase (two-fold lower than *P. sulcatum*) were secreted by *P. ultimum*.

After 14 days of culture, β-1,4-glucanase and pectate lyase activities were detected in *P. violae* filtrates, whereas all the cell-wall polysaccharide degrad-

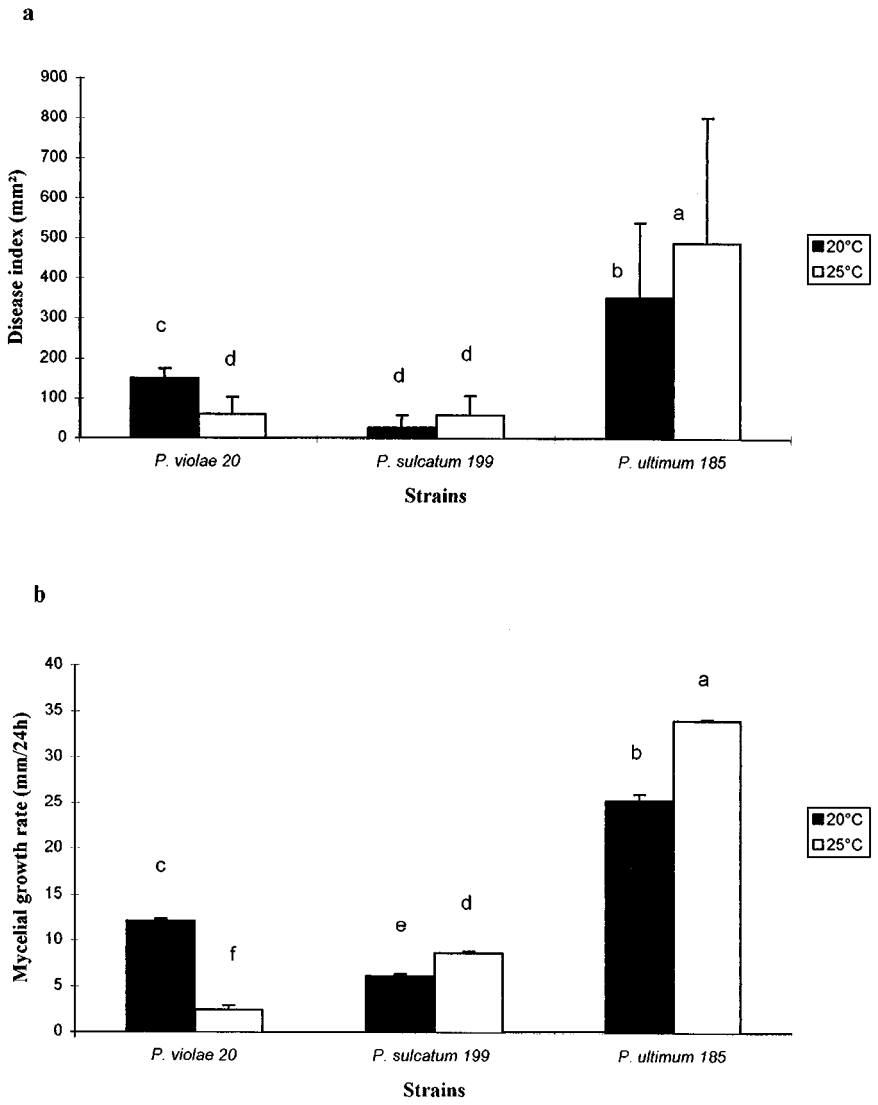

Figure 1. Biological characterization of the three *Pythium* strains, at two temperatures: 20 and 25 °C. (a) Aggressiveness on carrot roots (means of 32 measurements ± SD); (b) Mycelial growth rate (means of 3 replicates ± SD) – Histograms associed to the same letter did not differ significantly according to Newman-Keuls' test ($\alpha = 0.05$).

ing enzymes that were monitored were secreted by *P. sulcatum*, with some of them in large quantities (β-1,4-glucanase, polygalacturonase). After the same period, *P. ultimum* maintained β-1,4-glucanase and polygalacturonase production, but secreted only very low levels of pectin lyase.

After 21 days of culture, the enzymic activity of *P. violae* was stable, compared to 14 days of culture. *P. sulcatum* secreted all the enzymes monitored. β-1,4-glucanase and polygalacturonase were produced in large amounts. The enzymic production of *P. ultimum* was stable, with β-1,4-glucanase and polygalacturonase production being respectively five and fifteen-fold lower than *P. sulcatum*, but without detectable xylanase, pectin methylesterase and pectate lyase. The β-1,4-glucanase and polygalacturonase values obtained by *P. violae*, *P. sulcatum* and *P. ultimum* were confirmed by measure of the activities on two independant filtrates.

Table 1. Enzymic production by the three *Pythium* strains after 7, 14 or 21 days of culture at 20 °C for *P. violae*, 25 °C for *P. sulcatum* and *P. ultimum*

Strains	*P. violae* 20			*P. sulcatum* 199			*P. ultimum* 185		
Days of culture	7	14	21	7	14	21	7	14	21
Protein content (μg ml^{-1} culture medium filtrate)	14.4 (1.3)	15.9 (0.1)	15.1 (0.1)	7.8 (0.6)	16.3 (0.7)	23.5 (0.4)	4.2 (0.1)	10.2 (0.8)	11.6 (0.2)
Fatty acid esterase[b]	N.D.[a]	N.D.[a]	11.9 (0.3)	N.D.[a]	N.D.[a]	48.5 (0.5)	N.D.[a]	N.D.[a]	28.2 (1.1)
Cell wall polysaccharide-degrading activities (pkat ml^{-1} culture medium filtrate)									
Cellulase (experiment 1)	0	78.9 (2.4)	52.4 (1.3)	0	99.8 (5.3)	239.6 (4.4)	32.7 (7.5)	40.0 (1.5)	48.3 (0.2)
Cellulase (experiment 2)	N.D.[a]	N.D.[a]	55.2 (1.3)	N.D.[a]	N.D.[a]	248.7 (46.2)	N.D.[a]	N.D.[a]	32.1 (3.9)
Xylanase[c]	N.D.[a]	N.D.[a]	0	N.D.[a]	N.D.[a]	23^{10-3} (2^{10-3})	N.D.[a]	N.D.[a]	0
Polygalacturonase (experiment 1)	0	0	0	29.3 (2.6)	330.3 (17.6)	444.7 (25.7)	13.5 (0.1)	27.0 (2.4)	30.0 (2.8)
Polygalacturonase (experiment 2)	N.D.[a]	N.D.[a]	0	N.D.[a]	N.D.[a]	143.5 (12.8)	N.D.[a]	N.D.[a]	18.8 (2.7)
Pectin methylesterase	0	0	0	52.5 (3.9)	33.5 (0.5)	33.3 (0.8)	0	0	0
Pectin lyase	0	0	0	0	2	1.4 (0.2)	0	4.3 (0.1)	6.3 (0.2)
Pectate lyase	0	3.0 (0.2)	7.2 (1.4)	0	9.8 (0.3)	14.2 (0.5)	0	0	0
Pectin solubilized from CWMCP[d]	N.D.[a]	N.D.[a]	103[e]	N.D.[a]	N.D.[a]	4378 (244)	N.D.[a]	N.D.[a]	450 (77)

a: not determined
b (arbitrary unit): absorbance unit min^{-1} ml^{-1} culture filtrate
c (arbitrary unit): 0.1 absorbance unit min^{-1} ml^{-1} culture filtrate
d (arbitrary unit): mg uronic acid mg^{-1} MIA ml^{-1} culture filtrate/24 h
e: detection limit of the method
( ): standard deviation

Degradation of CWMCP

The 21 day-old filtrates of all the *Pythium* species degraded the carrot cell-wall material containing protopectin. The most important solubilization of pectic substances was achieved by the *P. sulcatum* filtrate, *P. violae* being the least active (Table 1). Pectic molecules of high molecular weight (> 50000, and notably higher than those present in control samples) (Figure 3) were solubilized by the filtrates. Medium molecular weight molecules were present in the filtrates, probably originating from the culture medium. No uronic acid was detected in the absence of the enzyme. Among the three species, only *P. sulcatum* was responsible for the production of oligomers from pectic substances. However, the degradation of the solubilized pectic substances by this species was limited, compared to that achieved by a pure polygalacturonase preparation. Although the amount of solubilized pectic substances (6440 ± 700 mg uronic acid mg^{-1} MIA ml^{-1} culture filtrate/24 h) was comparable to that solubilized by *P. sulcatum*, pectins were further degraded to a great extent into oligomers by the pure enzyme. The solubilization of high molecular weight pectic molecules and their limited degradation by *Pythium* sp. filtrates could indicate that these pectic molecules were solubilized by the action of a protopectinase present in the filtrates, as defined by Nakamura et al. (1995).

β-1,4-glucanase analysis

IEF of β-1,4-glucanase isoenzymes gave profiles differing among the *Pythium* species (Figure 4). Isoenzymes secreted by *P. violae* focused with difficulty, probably because of the presence in the filtrate of carboxymethylcellulose not degraded by the fungus in the culture medium. As for proteins, *P. violae* and *P. sulcatum* produced isoenzymes in both an acid (4.5–5.5 and 3.0–5.0, respectively) and in a basic (9.0–10.0) pH range. In contrast, *P. ultimum* produced β-1,4-

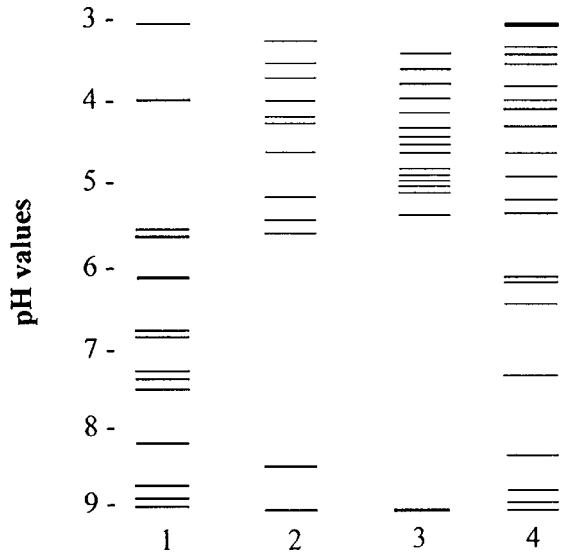

Figure 2. IEF profiles of concentrated culture filtrates of *Pythium* strains on an ultra-thin polyacrylamide gel after detection of proteins. Lane 1: standards, lane 2: *P. violae* 20, lane 3: *P. sulcatum* 199, lane 4: *P. ultimum* 185; standards (from acid to basic pI): amyloglucosidase, soybean trypsin inhibitor, β-lactoglobulin A, bovine carbonic anhydrase B, human carbonic anhydrase B, myoglobin-acidic band, myoglobin-basic band, lentil lectin-acidic band, lentil lectin-middle band, lentil lectin-basic band, trypsinogen.

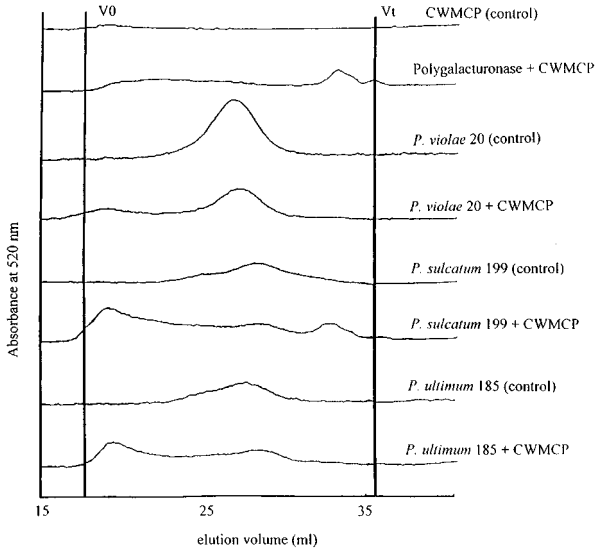

Figure 3. High performance gel-permeation chromatography profiles of pectic molecules solubilised from cell-wall material containing protopectin (CWMCP) after degradation by the three *Pythium* strains and polygalacturonase. V0: exclusion volume of the column; Vt: total volume of the column.

glucanase isoenzymes with pI only in the 5.0-9.5 pH range, with 7 out of 10 bands different from those of *P. violae* and *P. sulcatum*.

Discussion

Results reported here constitute the first report on relationships between aggressiveness and *in vitro* production and quantification of cell-wall degrading enzymes by *P. violae*, *P. sulcatum* and *P. ultimum*, the three major *Pythium* species responsible for cavity spot on carrots.

In our study, *P. violae*, considered to be the predominant species in cavity spot development, produced typical symptoms of the disease, with a translucent appearance and rapid cicatrization of the superficial root tissues. This species induced a weak and late enzymic activity *in vitro*, since cellulase and pectate lyase were detected only after 14 days of culture. The protopectinase activity apparently produced by *P. violae* was weak, leading to a limited solubilization of pectic substances from CWMCP. The presence of protopectinase activity should be confirmed by other experiments. The enzymic secretion after 21 days of culture may be of importance for pathogenicity of *P. violae* on carrot roots, but the weak pectolytic enzyme production by *P. violae*, however, could explain the limited ability of the species to degrade cell-walls and to induce extended symptoms. Its slow growth rate may allow time for a plant to react to invasion by the pathogen, and limit lesion size.

P. ultimum was responsible for less characteristic cavity spot symptoms: root necrosis progressed very quickly, especially at 25 °C, with extensive degradation of the outer root tissues, and mycelial development on the root surface. This type of symptom was comparable to those observed after artificial inoculation of carrots with *P. coloratum*, another fast-growing species (El-Tarabily et al., 1996). *P. ultimum* also produced enzymic activity earlier than *P. violae*. Considering the data obtained for carrot root tissue degradation by Sreenath et al. (1984) with a mixture of β-1,4-glucanases and pectinases, enzymic production by *P. ultimum* is presumed to be qualitatively and quantitatively sufficient to degrade carrot root tissues. This earlier and greater level of secretion may explain the ability of *P. ultimum* to induce more extended and progressive symptoms than *P. violae*. Moreover, the greater number of β-1,4-glucanase isoenzymes secreted by *P. ultimum* may favor an extensive degradation of

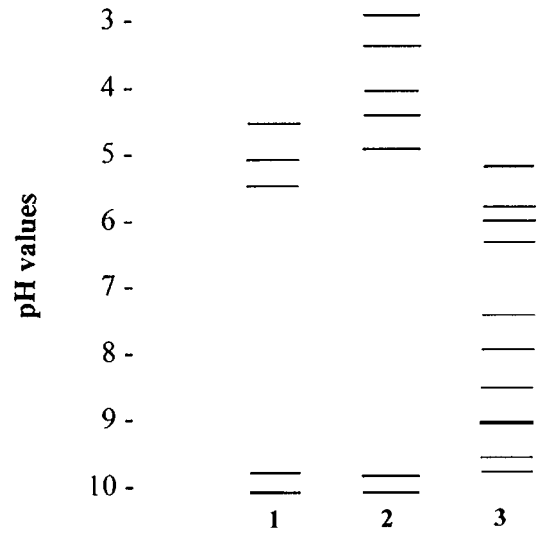

Figure 4. IEF profiles of concentrated culture filtrates of *Pythium* strains on an ultra-thin polyacrylamid gel after detection of cellulases. Lane 1: *P. violae* 20, lane 2: *P. sulcatum* 199, lane 3: *P. ultimum* 185.

cellulose, which requires several kinds of activity such as endo-β-1,4-glucanase, β-glucosidase or cellobiohydrolase (Walton, 1994). Furthermore, considering the probable role of protopectin contents in resistance to another carrot pathogen (Le Cam et al., 1994a), the pectinase (notably protopectinase) activities in *P. ultimum*, higher than in *P. violae*, would account for the more efficient degradation of carrot root tissues. Further studies are needed to investigate the role of the β-1,4-glucanase isoenzymes in cavity spot development.

The third species studied, *P. sulcatum*, produced typical cavity spot symptoms with a brown colouring, especially at the edge of the necrosis. It was unexpected to observe the broad spectrum of enzymes produced by this species. Moreover, pectin methylesterase, which had been looked for but not detected in a number of *Pythium* species (Wood and Gupta, 1958; Winstead and McCombs, 1961; Moore and Couch, 1968; Janardhanan and Husain, 1974) was detected in this genus. Xylanase activity, which may be a reciprocal activity to a β-1,4-glucanase activity (Walton, 1994), also was detected for the first time in a *Pythium* species. After 14 days of culture, all the monitored enzymes were detected, with high levels for polygalacturonase and β-1,4-glucanase. Among the three species, *P. sulcatum* solubilized the largest amount of protopectin, in apparent correlation with the amount of depolymerizing enzymes (polygalacturonase, pectate lyase, pectin lyase and probably protopectinase) present in the filtrates.

Thus, it appears that differences in aggressiveness and types of symptoms due to *P. violae* and *P. ultimum* may be explained, at least partially, by differences in enzymic activity, as shown from *in vitro* studies. The type of symptom produced by *P. sulcatum*, however was apparently not correlated with production of cell-wall degrading enzymes, suggesting that their role in the infection process was not the same as in *P. violae* and *P. ultimum*. Indeed, our results showed clear differences in the kinetics of production among the three species: in contrast to *P. violae* and *P. ultimum*, the first enzymes secreted by *P. sulcatum* were exclusively pectinolytic (polygalacturonase and pectin methylesterase). A similar sequence was observed by Dori et al. (1992) for infection of wheat tissues by another soil fungus, *Gaeumannomyces graminis* var. *tritici*: pectinolytic enzymes were produced one day before symptom appearance on wheat, whereas cellulolytic activity appeared 2 days later. Such a sequence would be related to substrate accessibility (Collmer and Keen, 1986). The limited symptoms caused by *P. sulcatum* might thus be due to plant reactions to the pectic fragments liberated as a consequence of a high and early enzymic (notably pectinolytic) activity (Ryan and Farmer, 1991). Moreover, xylanase activity may also induce defense reactions *per se*, as shown on tobacco leaf tissue (Sharon et al., 1993).

Caution is required in the search for relationships between aggressiveness and enzymic activities as measured *in vitro*, since in these conditions, many factors can act on the enzymic production. It is particularly noticeable that in our study, the production of cell-wall degrading enzymes by *Pythium* spp. was very low, compared to the levels detected in the same conditions with other pathogens such as *Botrytis cinerea* (Wood and Gupta, 1958) or *Mycocentrospora acerina* (Le Cam et al., 1994b). These observations raise the question of ability of some *Pythium* species to express *in vitro* their total potential of cell-wall degrading enzymic activity.

The low level of fatty acid esterase activity, when compared with the secretion observed with *Fusarium solani* and *Rhizopus stolonifer* (Nguyen-The and Chamel, 1991) could be due to the lack of an inducer in the culture medium, even though this enzymic activity was already observed in such conditions by Lehtinen (1993) in *Septoria nodorum*. According to Alghisi and Favaron (1995), the production of this enzyme may be

either entirely constitutive, or due to a basal level of synthesis, sufficient to degrade cell-wall polysaccharides into molecules which further induce synthesis of the enzyme. Further work would be necessary to verify whether the poor secretion of fatty acid esterase by *P. violae*, for example, can explain the limited degradation of suberized cell-walls during the penetration of unwounded carrot roots (Briard, 1990; Guérin, 1993).

The low level of production of cell-wall polysaccharide degrading enzymes by the *Pythium* spp. tested, cannot be explained by absence of an inducer, since pectin and carboxymethylcellulose, known as able to induce enzymic production (Winstead and McCombs, 1961; Moore and Couch, 1968; Janardhanan and Husain, 1974) were present in the culture medium. The low level of enzymic potential could possibly explain the limited development of the cavity spot symptoms, with degradation of the root tissues only to a moderate depth (0.5–1 mm, data not shown); this may be compared with phenomena observed on carrot roots infected by other pathogens such as *Phytophthora megasperma* (Breton and Rouxel, 1985) or *Mycocentrospora acerina* (Davies et al., 1981). It may be postulated that the sequence of enzymic production, in association with a low (*P. violae, P. sulcatum*) or fast (*P. ultimum*) growth rate, affects the plant infection response and thus the type of symptoms characteristic of each *Pythium* species. However, the enzymic production may be more important *in situ*, as shown for *P. ultimum* (Moore and Couch, 1968). This differential induction may be due to the greater diversity of substrates *in planta* (Wood, 1960). Therefore, it would be interesting to compare the enzymic activities of the three *Pythium* spp. *in situ*, as with *Mycocentrospora acerina* on carrots (Le Cam, 1994). However, in the study undertaken on cavity spot by Zamski and Peretz (1996), the enzymic activities detected *in situ* were very poor and apparently difficult to quantify. Cavity spot presents many of the disavantages described by Wood (1960) for this type of study, notably a limited development of symptoms and weak enzymic activities.

To overcome these difficulties, further work in progress in our laboratory is proceeding along two complementary directions: first, a biochemical approach, investigation of the ability of enzymes produced by the three *Pythium* species to degrade cell-wall polysaccharides from carrot roots is being undertaken; secondly, by using microscopic observations combined with the use of specific probes, an analysis of the patterns of tissue colonization and degradation by *Pythium* spp. is underway.

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