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Production of indole-3-acetic acid and tryptophol by *Pythium ultimum* and *Pythium* group F: Possible role in pathogenesis

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Abstract

Pythium group F is a minor pathogen which induces symptomless infections that occur frequently and results in yield losses in tomato soilless cultures. To elucidate the mode of action of this microorganism, the influence of culture filtrates of *Pythium* group F on tomato growth was investigated and compared to that of the pathogen *Pythium ultimum*. Depending on metabolite production by the fungus, marked differences were observed in plant response. *Pythium* group F crude culture filtrates or low molecular weight fractions (<500) caused swelling behind the root tip and reduced root growth; the cohesion and adherence of cells within the cortical area were also affected. These symptoms were similar to those observed on plants treated with indole-3-acetic acid. By contrast, *P. ultimum* filtrates caused a marked distortion of cell shape accompanied with folding of host cell walls, particularly in the cortical area. These symptoms were characteristic of the activity of toxic compound(s) on host cells. Chemical analysis of the filtrates demonstrated that indole-3-acetic acid and tryptophol were produced by *Pythium* group F and *P. ultimum*. However, *Pythium* group F took up and metabolized more indole-3-acetic acid precursors, especially tryptophan, a key amino acid in the pathways leading to indole-3-acetic acid synthesis. The fact that *Pythium* group F and *P. ultimum* transformed tryptamine and indole-3-acetaldehyde into indole-3-acetic acid and tryptophol confirms the existence of a tryptamine pathway within both fungi. These results support the hypothesis that auxins facilitate *Pythium* group F infections. On the other hand, toxin(s) and hydrolytic enzymes are likely involved in *P. ultimum* pathogenesis.

Abbreviations: IAA – indole-3-acetic acid; IAAld – indole-3-acetaldehyde; IAM – indole-3-acetamide; IAN – indole-3-acetonitrile; Ipy – indole-3-pyruvic acid; TNH_2 – tryptamine; Trp – tryptophane; TOL – tryptophol.

Introduction

The colonization of plant root systems by pathogens of the genus *Pythium* causes damping-off on plantlets or severe necrosis and rots on the roots and stems of mature plants. To this end, infections by pathogens such as *Pythium ultimum*, *P. aphanidermatum*, *P. irregulare* have been extensively described (Blancard et al., 1992; Jenkins and Averre, 1983; Kraft et al., 1967; Linde et al., 1994; Martin, 1995). In recent years, scientists have been puzzled by *Pythium* infections that affect plant growth and yield without causing any visible symptoms on roots (Rey et al., 1998; Stanghellini and Rasmussen, 1994). Only a few reports have described this phenomenon on soil-grown plants (Salt, 1979), but it appears to be prevalent in soilless cultures (Stanghellini and Rasmussen, 1994).

Among these intriguing infections, one can cite those by *P. dissotocum* and *Pythium* group F that reduce the yields of hydroponically grown lettuce and tomato,

respectively. For instance, late maturation of lettuce is the only indication of the presence of *P. dissotocum* (Stanghellini and Kronland, 1986), whereas *Pythium* group F is responsible for yield loss in tomato plants even though the roots look macroscopically healthy (Rey et al., 1997). According to Favrin et al. (1988) such infections can be relatively common in soilless cultures, but were previously ignored or overlooked because of the lack of visible root damages.

The factors responsible for pathogenicity are not clearly understood. Stanghellini and Kronland (1986) suggested that *Pythium* may accelerate rootlet maturation and senescence. Over the last few years asymptomatic infections by *Pythium* group F on tomato roots were studied: ultrastructural and cytochemical investigations highlighted a highly complex relationship (Rey et al., 1998). Indeed, *Pythium* group F behaved as a necrotroph in the outer root tissues, whereas it was a potential inducer of plant defense reactions in the inner tissues where colonizing hyphae were destroyed. Finally, plant and pathogen may coexist without evidence of disease. However, the alterations induced, though limited, ultimately results in yield losses.

Among the molecules responsible for *Pythium* group F pathogenicity, several hydrolytic enzymes (e.g. cellulases and pectinases) have been reported (Rey et al., 1996; 1998). However, their destructive activity *in planta* was reduced compared to that of the more pathogenic species such as *P. ultimum* (Chérif et al., 1991) and *P. aphanidermatum* (Rey et al., 1996). In addition, toxin production has been reported *in vitro* and *in planta* with several *Pythium* spp. (Desilets et al., 1994; Ichihara et al., 1985; Rey et al., 1996; Sadik et al., 1982; 1983), but with *Pythium* group F, toxin production has never been detected. It has been assumed that other kinds of metabolites are synthesized by *Pythium* group F during asymptomatic infections. For instance, Hodges and Coleman (1985) reported that a reduction in plant growth may result from impairment of water uptake and translocation and/or production of growth regulators as a way to facilitate pathogenesis without causing rots. Although no evidence for the involvement of plant hormone analogues in *Pythium* group F pathogenesis has been found, it has been suggested from different observations. Desilets (1995) noticed that the root system of geranium plantlets grown on a medium amended with culture filtrates of *Pythium* group F developed proliferating rootlets. Rafin (pers. comm.) observed the same phenomenon on tomato roots infected with zoospores. Disturbance of the physiology of the root system may be attributed to hormones or hormone-like substances produced by the pathogen. Indeed, the ability of *Pythium* species to secrete plant growth hormones was described by Martin (1995). In addition, indole-3-acetic acid (IAA) or IAA derivatives (e.g. tryptophol) were identified in culture filtrates of several oomycete pathogens such as *P. debaryanum* (Yoshii and Hagedorn, 1971), *P. aphanidermatum* (Shimada et al., 1999) and *P. sylvaticum* (Posthumus, 1973).

Our objectives were, (i) to determine the effect of tryptophan-enriched culture filtrates from *Pythium* group F on tomato-growth and root tissue development, and (ii) to evaluate the ability of *Pythium* group F strains to produce IAA and tryptophol (TOL) in liquid cultures amended with different IAA-precursors. In parallel, *P. ultimum* was used as a control throughout the experiments because of its ability to produce toxin(s) (Désilets and Bélanger, 1991) along with its presumed inability to synthesize either IAA or TOL (Furukawa et al., 1996).

Materials and methods

Plant material

Tomato seeds (cv. Prisca) were surface-sterilized by immersion in 70% ethanol for 5 min, soaked in 2% aqueous sodium hypochlorite for 5 min, thoroughly rinsed and soaked overnight in sterile distilled water. Seeds were then placed on sterile filter paper (Whatman no 1) (10 seeds per filter) wetted with 3 ml of different *Pythium* spp. culture filtrates and incubated at 25 °C in the dark.

Pythium *strains*

The four strains of *Pythium* group F identified as 35, 91B8, 317 and 707 had been isolated previously from the roots of tomato plants growing in soilless cultures. Strains 317 and 707 were reported to cause yield losses, but no typical root symptoms (Rey et al., 1997), whereas strains 35 and 91B8 induced root necrosis. Four strains of *P. ultimum* (114, 447, 01 and 02) were also used. Strains 144 and 447, virulent on geranium (Chagnon et Bélanger, 1991) came from RR Bélanger (CRH, Université Laval, Québec, Canada). Strains 01 and 02 were isolated from cucumber exhibiting root rot symptoms. Strain 01 was kindly provided by N Benhamou (Université Laval, Québec, Canada) and

strain 02 was isolated from necrotic roots. All *Pythium* strains were grown on either corn meal agar or malt yeast agar supplemented with antibiotics and incubated at 24 °C in the dark.

Tissue processing for optical microscopy

A minimum of 10 roots from germinated seeds grown on different *Pythium* culture filtrates or roots from seeds grown on sterile water were used in the experiments. Roots were fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, for 2 h at room temperature. All samples were post-fixed for 2 h in 1% osmium tetroxide in the same buffer at 4 °C, dehydrated in a graded series of ethanol, and embedded in Epon 812.

Thick sections (1.0 μm) were cut from the Epon-embedded material using glass knives. Then, they were mounted on glass slides and contrasted with basic fuschin-methylene blue. Observations were made under a BH-2 Olympus microscope. Section from at least five roots from each treatment were studied.

Seed germination and growth on culture filtrates of Pythium *spp.*

Disinfected seeds were placed on sterile Whatman no 1 filter (10 seeds per filter) wetted with culture filtrates. For this purpose, 3 ml of culture filtrates from *Pythium* group F 707 and *P. ultimum* 144 grown in 1 mM Tryptophan (Trp)-amended medium were filtered through a 0.45-μm filter directly onto Whatman filters. In addition, culture filtrates from *Pythium* group F 707 were partitioned into two fractions using an ultrafiltration membrane (Amicon TF2) with a molecular weight cut-off of 500 (Amicon UM-05). Seeds were also placed on these fractions. Controls were prepared with sterile water. Seeds were incubated 5 days at 25 °C in the dark. The length of roots and hypocotyls was measured and symptoms such as root browning, swelling and/or root hair formation were recorded. Thirty seeds were used for each experiment.

Growth of Pythium *spp. in culture media*

Four strains of *Pythium* group F and four of *P. ultimum* were grown in 250-ml Erlenmeyer flasks filled with 125 ml of potato dextrose broth medium (PDB). The medium was supplemented with Trp to a final concentration of 1 mM to evaluate its ability to stimulate the production of auxins.

Media for *Pythium* strains were also supplemented with indole derivatives known to be involved in IAA pathways: indole, indole-3-acetaldehyde (IAAld), indole-3-acetamide (IAM), indole-3-acetonitrile (IAN), indole-3-pyruvic acid (Ipy), tryptamine (TNH_2) and Trp at a final concentration of 0.5 mM. This concentration was chosen because some of these compounds are known to exert an inhibitory effect on mycelial growth at 1 mM. Unamended PDB medium served as control. At least 10 mycelial disks (5-mm diameter) taken from the margin of actively growing colonies on malt-medium were used as inoculum. The cultures were placed for 2 weeks at 28 ± 1 °C on a rotary shaker (120 rpm) in the dark. The mycelium was separated from the culture media, freeze-dried and weighed. For each *Pythium* strain, at least two flasks were used as replicates.

Detection of IAA and TOL by capillary electrophoresis

Fifty millilitre of *Pythium* culture filtrates amended or not with 1 mM Trp or 0.5 mM indole derivatives (IAAld, IAM, IAN, Ppy, TNH_2 and Trp) were collected. They were adjusted to pH 3 and partitioned twice against 75 ml of ethyl acetate. The ethyl acetate extracts that contained acidic and neutral indoles were evaporated to dryness and dissolved in methanol.

The amount of IAA and TOL were measured using a P/ACE 5500 capillary electrophoresis system (Beckman Instruments) with a 470 mm × 50 μm fused-silicon capillary. Detection was performed by on-column measurements of UV absorption (220 nm) at 400 mm from the injection end of the capillary. The separation buffer was prepared with 50 mM sodium tetraborate and 200 mM SDS diluted with distilled water to obtain 12.5 mM sodium tetraborate and 50 mM SDS, and the pH was adjusted to 10. The buffer was filtered through a 0.22-μm membrane prior to use. At the beginning of each experiment, the fused-silicon capillary was activated by three sequential washings: first with NaOH 0.1 M for 5 min, second with H_2O for 2 min and third with the separation buffer for 5 min. Moreover, the capillary was washed with the separation buffer for 2 min before each analysis. The separation parameters were as follows: voltage = 25 kV and temperature = 25 °C. Injections were performed at high pressure for 5 s. At least 2 injections per sample were carried out.

Results

Seed germination and growth on culture filtrates of Pythium *spp.*

Filtrates of *Pythium* group F and *P. ultimum* amended with 1 mM tryptophan caused a significant reduction in the length of roots and hypocotyls (Table 1). Regardless of the experimental conditions, the hypocotyls always looked healthy, whereas roots bore differences with respect to the treatment applied. Control roots were white and had typical root hairs and a healthy appearance. Those grown in contact with *P. ultimum* filtrates were brown with small necrotic lesions and no root hairs. On the other hand, roots grown on *Pythium* group F filtrates were white, hairy and presented a swollen region just behind the root tip (Figures 1 and 2). When *Pythium* group F filtrates were partitioned on the basis of molecular weight, only those containing compounds of molecular weight below 500 caused a reduction of root and hypocotyl length similar to that described for crude filtrates (Table 1). Induction of swelling and root hair proliferation on roots was also similar (Figure 1). Roots from seedlings grown on filtrates containing compounds of molecular weight above 500 looked as healthy as the control ones (Figure 1). Hypocotyl length was equal to that of controls while root length was slightly reduced (Table 1).

Histological observations

Light microscope observations of control roots showed a normal organization of cells and tissues. Within the cortical area, cells were cohesive (Figure 3B, arrows) and regularly shaped; they formed typical rows surrounding the vascular stele (Figure 3B). Root sections from seedlings developed on *P. ultimum* filtrates showed deep alterations within the epidermal and cortical areas; cells were distorted (Figure 3A, arrows) and collapsed (Figure 3A, arrows with doubleheads) and often markedly folded. Roots from seedlings grown on *Pythium* group F filtrates had an intermediate appearance; epidermal and cortical tissues had lost their regular structure. At times, adherence among cells was discontinuous and large intercellular spaces were frequent (Figure 3C, arrows). The same phenomenon was noticed in thin sections of roots grown on *Pythium* group F filtrates containing compounds of MW < 500 (not shown).

Table 1. Root and hypocotyl lengths of tomato seedlings placed on different culture filtrates of *pythium* spp.

Treatment	Root length (mm)	Hypocotyl length (mm)
Control	44.2 c*	28.8 c
Crude *P. ultimum* filtrate	6.1 a	16.9 b
Crude *Pythium* group F filtrate	4.8 a	8.2 a
Pythium group F filtrate with MW < 500	3.4 a	8.5 a
Pythium group F filtrate with MW > 500	33.3 b	28.1 c

*Means followed by a different letter in each column are significantly different at 5% level, determined by Duncan multiple range test.

Production of IAA and TOL by Pythium *group F and* P. ultimum

IAA and TOL were not detected in culture filtrates of all four *Pythium* group F strains grown in PDB alone. On the other hand, both compounds were detected when filtrates were amended with 1 mM Trp; albeit at different concentrations according to the strain (Figure 4). For instance, production of IAA was greater than that of TOL with strains 35 and 317 and less than TOL with strain 707. Equal concentrations of IAA and TOL were found in filtrates from strain 91B8 (Figure 4). Only traces of Trp were detected in the culture medium of *Pythium* group F after two weeks growth. No traces of IAA or TOL were detected in any of the four *P. ultimum* culture filtrates amended or not with Trp, while significant amounts of Trp were detected in Trp-amended solutions (data not shown).

Conversion of various indole derivatives to IAA and TOL by the various strains of Pythium

Addition of IAAld, Indole, TNH_2 and Trp to culture filtrates resulted in their conversion into IAA and TOL two weeks after inoculation by *Pythium* group F-strain 707 (Figure 5). Equal IAA quantities were detected after amendment independently of the four indole derivatives. Except for indole, the conversion of IAAld, TNH_2 and Trp into TOL was much more efficient than that of IAA. Neither IAA nor TOL was detected in the media containing only PDB (control), IAM, Ipy or IAN (Figure 5). When *P. ultimum* strain 144 was inoculated to media amended or not with 0.5 mM of the different indole derivatives, only IAAld and TNH_2 were

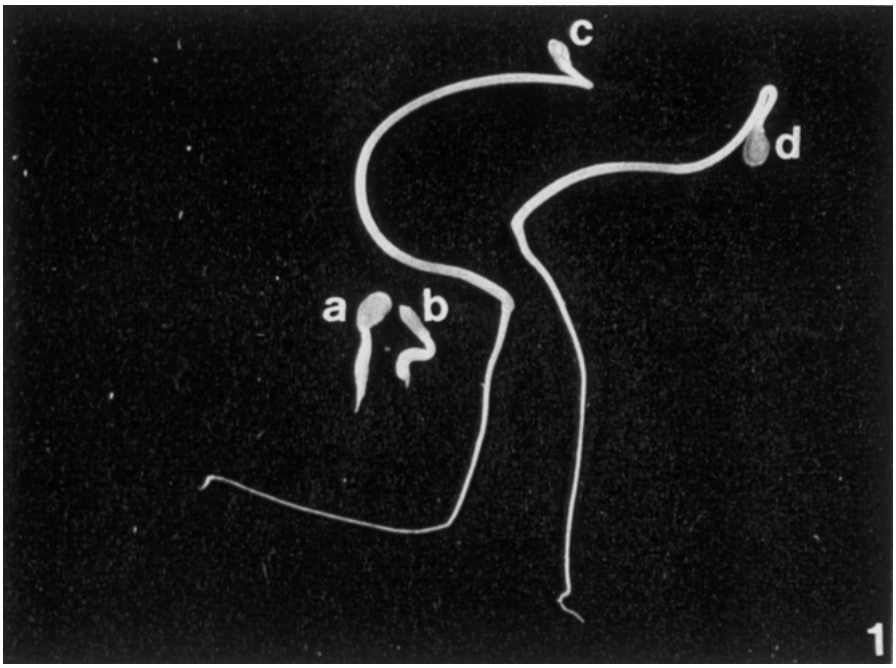

Figure 1. Growth of tomato seedlings on culture filtrates of *Pythium* group F amended with 1 mM tryptophan. Roots grown on *Pythium* group F crude filtrates (a) and on compounds of a culture filtrate with molecular weight below 500 (b) are short, stubby and covered with root hairs. The region just behind the root tip is markedly swollen. Roots grown on water control (c) and compounds of *Pythium* group F culture filtrates with molecular weight above 500 (d) are longer and thinner.

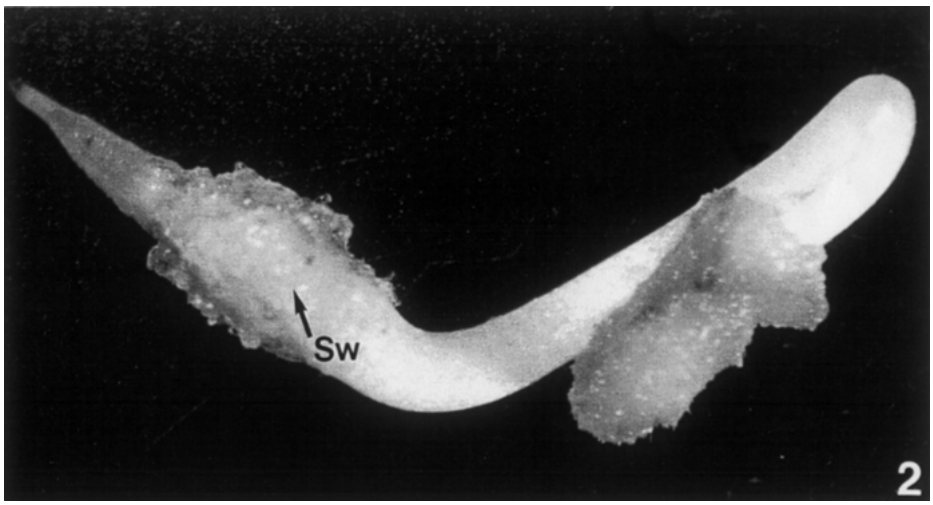

Figure 2. Magnification of roots grown on *Pythium* group F crude filtrates or on compounds of a culture filtrate with molecular weight below 500. Note swelling and root hair formation in the region just behind root tip (arrow). Sw: swollen region.

converted into IAA and TOL (Figure 6). Their conversion into TOL was much higher than that into IAA. Compared to IAAld, conversion of TNH_2 into TOL was relatively low.

Discussion

In recent years, one of the most intriguing phenomena related to plant infections by *Pythium* spp. has been

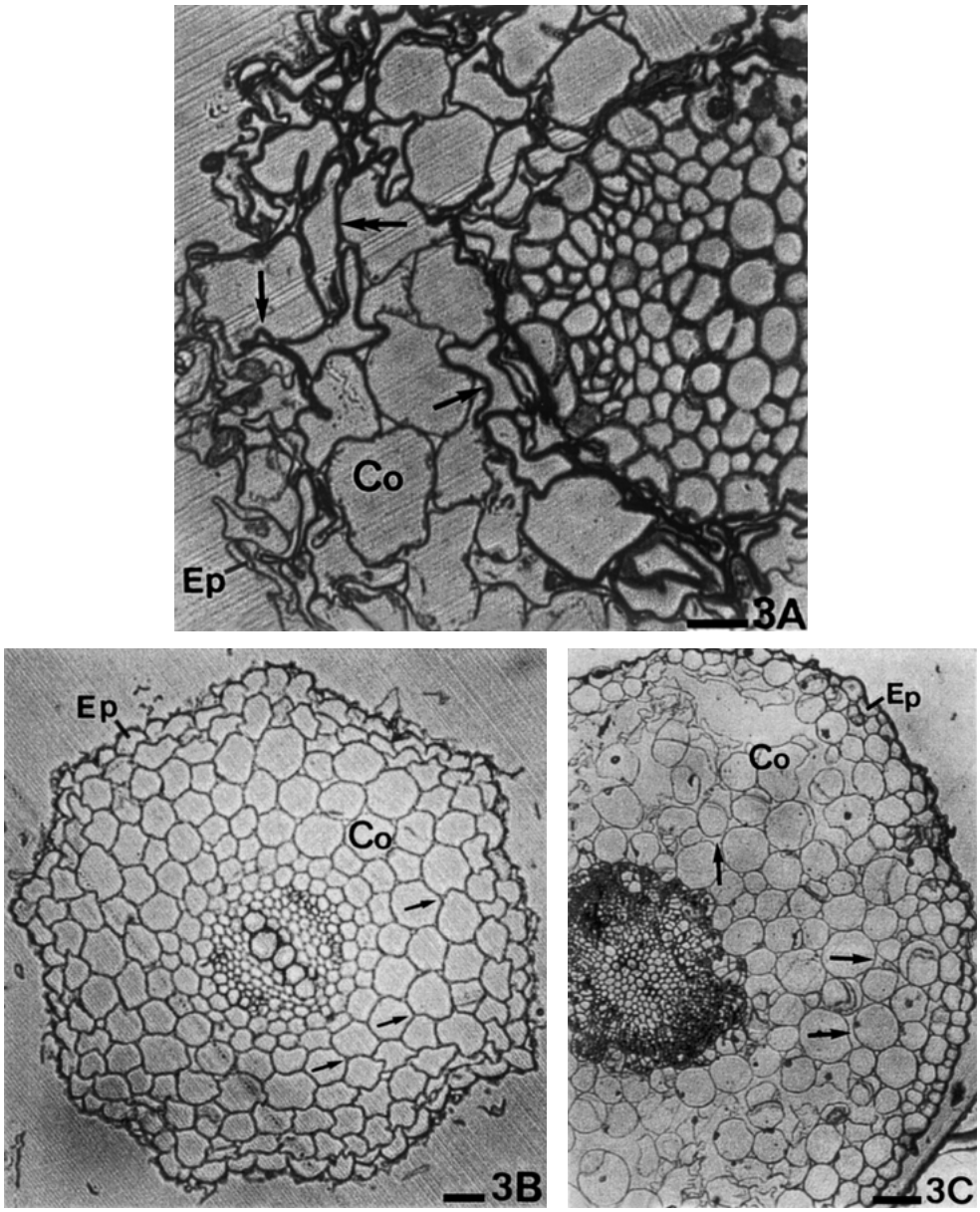

Figure 3. Light micrographs of cross-sections of tomato root tissues stained with toluidine blue. Roots were germinated on different *Pythium* culture filtrates. A: Epidermal and cortical cells from roots placed on *P. ultimum*-amended Trp filtrate exhibit severe alterations. Damage ranged from distortion (arrows) and collapse (arrows with double-heads) of cells to extensive folding of cell walls. Bar = 10 μm. B: Within control roots, epidermal and cortical cells are cohesive (arrows) and regularly shaped. Cells formed evenly organized rows surrounding the vascular stele. Bar = 5 μm. C: Roots grown on *Pythium* F-amended Trp filtrates: in several instances, adherence among cells was discontinued and the presence of large intercellular spaces was frequent (arrows). Bar = 10 μm. Ep: epidermis, Co: Cortical tissue.

the occurrence of attacks reducing plant yield without any apparent symptoms (Rey et al., 1998; Stanghellini and Rasmussen, 1994). Since these infections have an impact on the economy (Rey et al., 1997; Stanghellini and Kronland, 1986), our aim was to expand the overall knowledge of the interaction of one of these pathogens, i.e. *Pythium* group F, with plants. To be more precise, we were interested in the role and production of

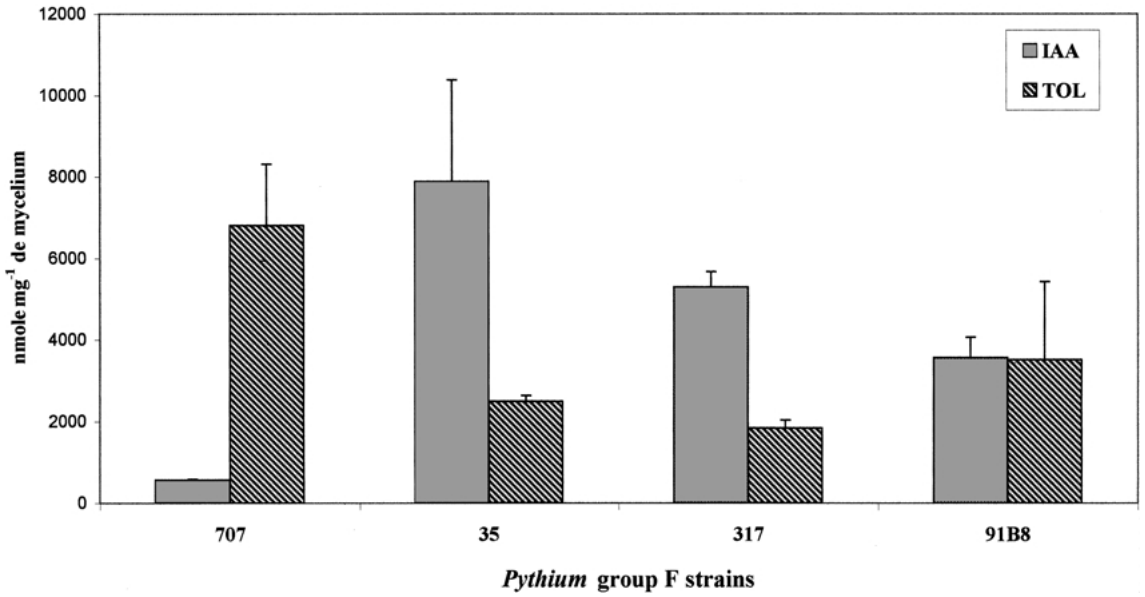

Figure 4. Production of IAA and TOL by four *Pythium* group F strains in culture media amended with Trp 1 mM.

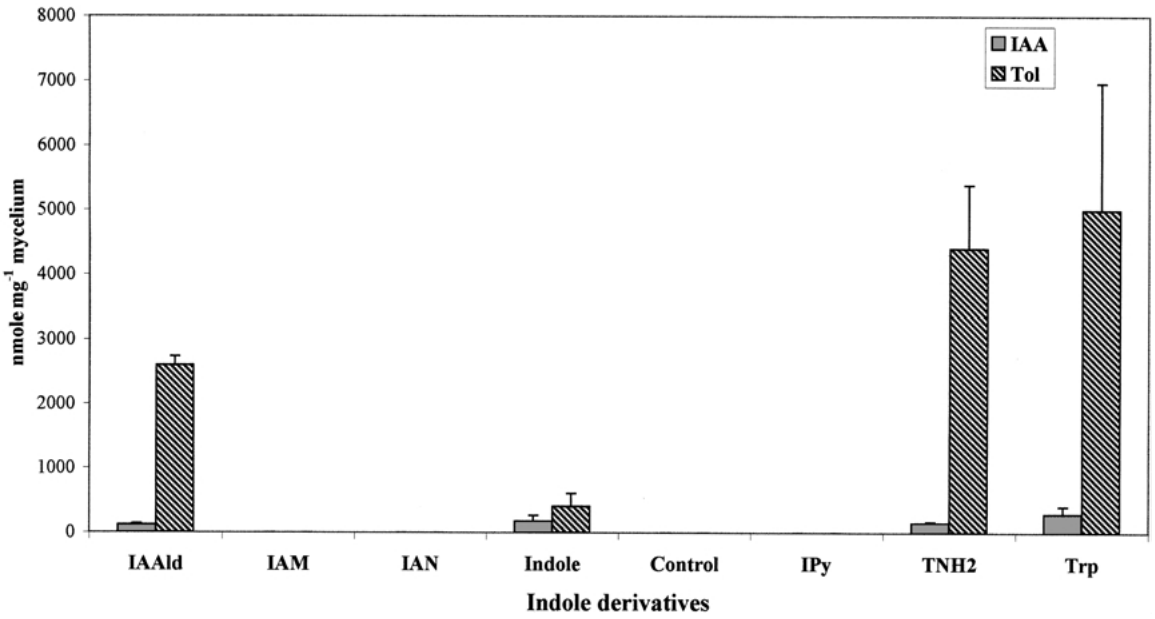

Figure 5. Production of IAA and TOL by *Pythium* group F in culture media amended with indole derivatives 0.5 mM.

auxins since root proliferation and disturbance have been observed further to *Pythium* group F plant colonization (Désilets, 1995; Rafin, pers. comm.).

Results from the present study revealed a marked difference in auxins and auxin analogues produced by *Pythium* spp. and their influence on plant growth. For instance, *Pythium* group F crude culture filtrates or fractions of molecular weight below 500 caused, (i) a reduction in the longitudinal growth of roots, (ii) a swelling behind the root tip and (iii) a proliferation of root hairs.

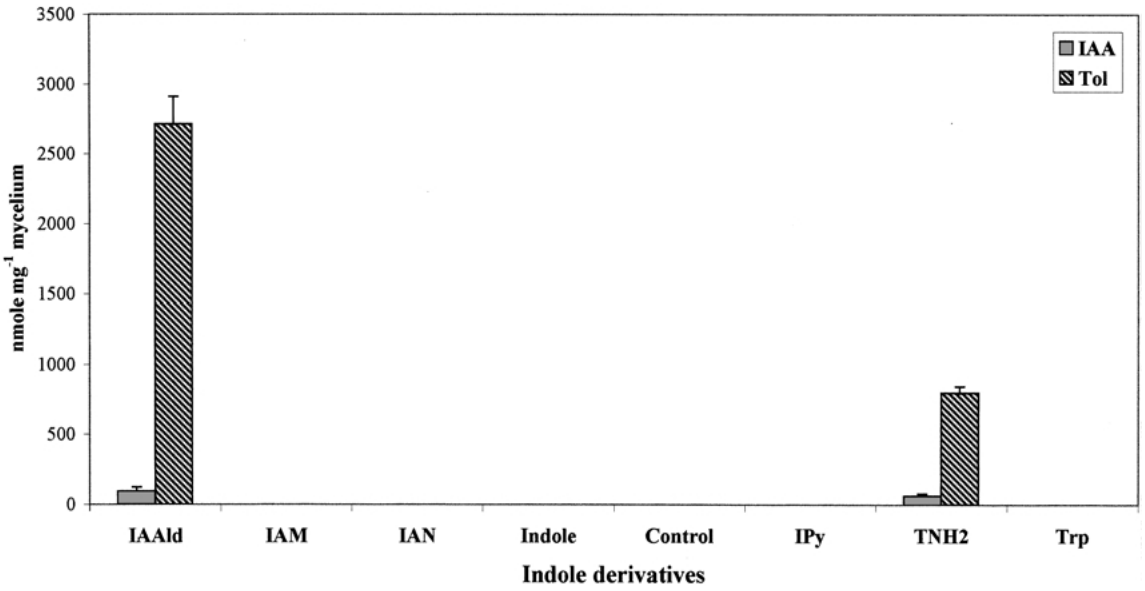

Figure 6. Production of IAA and TOL by *P. ultimum* in culture media amended with indole derivatives 0.5 mM.

All these symptoms are similar to those observed on plants treated with IAA (Hejnowicz, 1961). By contrast, *P. ultimum* culture filtrates provoked root stunting and necrosis; typical symptoms associated with the production of toxic molecule(s) (Désilets and Bélanger, 1991). Histological examination of root tissues highlighted that *P. ultimum* filtrates caused a marked distortion of cell shape accompanied with folding of host cell walls, particularly in the cortical area. These observations corroborate those made by Désilets et al. (1994) following the application of *P. ultimum* or its culture filtrates to geranium roots. These authors concluded that an active principle (toxin) released by *P. ultimum* contributed directly to the expression of these symptoms. In the present study, the treatment of root tissues with *Pythium* group F filtrates induced root cellular changes that differed from what is generally caused by a toxin. For example, root tissues had not collapsed and individual root cells were seemingly undamaged, but the cohesion and adherence of cells within the cortical area were affected. These observations suggest that the metabolites responsible for the induction of an auxinic effect on the growth of roots are key components of *Pythium* group F molecules.

Chemical analyses demonstrated that both IAA and TOL are produced in culture filtrates of *Pythium* group F and *P. ultimum*. To our knowledge, this work presents the first evidence supporting auxin synthesis by these pathogens. However, it is also evident that the culture medium needs to be amended with indole precursors for IAA and TOL to be synthesized. This observation corroborates several *in vitro* studies that had demonstrated that: (i) only some microbial cultures can produce small amounts of IAA in the absence of physiological precursors, and (ii) these organisms release larger amounts of IAA and IAA derivatives in the presence of precursors (Frankerberger and Arshad, 1995). In a previous study, Furukawa et al. (1996) concluded that *P. ultimum* was unable to produce IAA and TOL in Trp-amended liquid cultures. However, they did not include other indole precursors like TNH_2 and IAAld which were efficiently transformed into IAA and TOL by *P. ultimum* in this study. The fact that these two molecules were metabolized confirms the existence of a tryptamine pathway within the microorganism.

The situation is more complex with *Pythium* group F. It did transform IAAld and TNH_2 into IAA and TOL, this would be indicative of a TNH_2 pathway exists. According to Frankerberger and Arshad (1995), the tryptamine pathway is widespread among non-pathogenic species of *Aspergillus*, *Penicillium* and *Rhizopus*; our results support the existence of this pathway among pathogens. In addition, other IAA pathways may exist within *Pythium* group F, because indole was converted into IAA and TOL. For example, a Trp-independent pathway through direct conversion of

indole to IAA was described in plants by Normanly et al. (1995). This point could be confirmed by additional studies into the *Pythium* group F enzymes responsible for the conversion of IAA precursors throughout the different pathways.

Results from this study raise the question concerning the role of auxins in *Pythium*-plant interactions. Usually, no symptoms of hyperauxiny are noticed in *Pythium* group F or *P. ultimum*-infected plants. However, auxins can act indirectly by facilitating plant attacks by other ways. One may postulate three possible indirect roles based on previous observations. (i) The loosening of host walls may facilitate *Pythium* group F ingress in the roots, and then the cellulases and pectinolytic enzymes produced *in planta* (Rey et al., 1996; 1998) by the pathogen could break down wall structures and favour its penetration throughout the root cortex. (ii) Root exudation is correlated with an increasing leakage of tryptophan (Rybicka, 1981) which may serve as source for auxin synthesis. Gay et al. (1989) emphasized that the root system is sensitive to very low auxin concentrations so that even a weak, but continuous, auxin release in the vicinity of host cells could affect their metabolism and consequently their wall structures. This event is supported by the high frequency of *Pythium* group F colonization on tomato roots growing in soilless cultures in which about 40% of plants free of visible necrotic symptoms were invaded by the pathogen, which resulted in yield losses (Rey et al., 1997). (iii) Auxin can promote the formation and elongation of root hairs (Pitts et al., 1998). According to Mojdehi et al. (1990) the stimulation of root hair formation by growth regulator substances produced by some pathogenic *Pythium* species serves their needs to infect plants through root hairs.

Finding an explanation for *P. ultimum* action is more puzzling. Its ability to synthesize auxin from root exudate metabolites (e.g. Trp) is limited; it can produce toxin(s) which destroy root hairs (Désilets and Bélanger, 1991). One may consider that toxin(s) that cause plant cell death in advance of penetration and hydrolytic enzymes are key molecules involved in *P. ultimum* pathogenesis. On the other hand, auxins, which are secondary metabolites, can facilitate infections in altered plant tissues.

In conclusion, by showing that *Pythium* group F takes up and metabolizes IAA precursors, this work presents the first chemical evidence towards elucidating the mode of action of this minor pathogen. A growing body of evidence has implicated auxin synthesis in this kind of *Pythium* group F infections and our results support this hypothesis.

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