European Journal of Plant Pathology **106:** 887–894, 2000.

Molecular and pathogenicity characteristics of *Phytophthora nicotianae* responsible for root necrosis and wilting of pepper (*Capsicum annuum* L.) in Tunisia

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Accepted 26 September 2000

Key words: forma specialis, ITS, *P. capsici*, *P. nicotianae*, solanaceous

Abstract

Isolates of *Phytophthora* from pepper, produced in Tunisia, were characterised according to molecular and pathogenicity criteria. Polymerase chain reaction amplification of the ITS1 region in the ribosomal DNA resulted in different sized fragments. The pepper isolates and *P. nicotianae* yielded a fragment of 310 bp that distinguished it from *P. capsici* with a fragment of 270 bp. The ribosomal RNA gene amplicons of both internal transcribed spacers and the 5.8 S of the pepper *Phytophthora* and *P. nicotianae* were digested with 8 endonucleases. The patterns generated, with the 2 enzymes that cut, were identical for both taxa. This molecular analysis corroborated the morphological and biological characteristics and suggests strongly that the isolates of *Phytophthora* from pepper belong to the species *P. nicotianae*. Inoculation of pepper, tomato, eggplant and tobacco plants with the isolates of *P. nicotianae* from pepper showed they were highly pathogenic on pepper but not on tobacco, while their pathogenicity was weak on tomato and eggplant and was associated with atypical symptoms not observed in the field. These pathogenicity tests suggest that pepper isolates of *P. nicotianae* are particularly adapted to their host and may thus constitute a *forma specialis* of *P. nicotianae*.

Introduction

Davet (1967) reported that symptoms of root rot and wilting of pepper plants (*Capsicum annuum* L.) have been observed by growers from Nabeul (Tunisia) for many years. This syndrome results in severe losses both in fields and greenhouses in the main areas of pepper production. The initial studies concluded that the causative agent of that syndrome belonged to the genus *Phytophthora* (Davet, 1967; Anonymous, 1976; Moens and Ben Aïcha, 1982), but controversy remained about the species involved. According to analyses based on morphological criteria, the isolates of *Phytophthora* from pepper were identified as *Phytophthora nicotianae* Breda de Haan (Allagui et al., 1995; Allagui and Tello-Marquina, 1996). The varieties *nicotianae* and *parasitica* of *P. nicotianae* are no longer recognised (Hall, 1993), since many studies have indicated a lack of genetic differentiation between the types (Förster et al., 1990; Oudemans and Coffey, 1991; Lacourt et al., 1994). Beside identification based on morphological characters, analysis of the internal transcribed spacer (ITS) of ribosomal DNA provides a powerful tool for differentiating groups of *Phytophthora* species (Lee and Taylor, 1992; Crawford et al., 1996; Cooke and Duncan, 1997).

P. nicotianae has a very broad host range as indicated by the numerous hosts in some 298 plant species (Erwin and Ribeiro, 1996), but any one isolate cannot

be considered, especially in natural conditions, to be pathogenic to all the plant species cited. Subgroups showing some specificity were reported on tobacco (Tucker, 1931), petunia (Philips and Baker, 1962; Haasis, 1962), carnation (Tramier and Andréolli, 1969), sesame (Gemawat and Prasad, 1964) and citrus (Erwin, 1964). Advances in molecular genetics have provided the opportunity to examine the mechanisms of host specificity within *Phytophthora* species at a molecular level. A family of small extracellular proteins (termed elicitins) produced by many *Phytophthora* species, which may contribute to host-specificity of these pathogens, have been purified (Ricci et al., 1993) and a gene coding for parasiticein, an elicitor of necrosis and resistance in tobacco, was characterised in *P. nicotianae* (Kamoun et al., 1993).

Despite its wide geographic distribution and numerous hosts, *P. nicotianae* has been infrequently reported from *Capsicum* spp. on which the most severe disease is caused by *P. capsici*. No other *Phytophthora* spp. have been reported causing major damage on the crop. Therefore, our morphologic identification of *P. nicotianae* as a severe fungus, frequently observed on pepper crops in Tunisia, required confirmation based on molecular and pathogenicity characteristics.

Materials and methods

Fungal isolates and mycelia production

The regions in Tunisia prospected for the presence of diseased pepper plants were the Lower Valley of Medjerda, Bizerte, Cap Bon and Sahel. The pathogen was isolated from infected plants using a selective pythiaceous medium (Ponchet et al., 1972) or P_{10}VP medium (Tsao and Ocana, 1969).

Two conventional media were used for growth of the isolates, one based on pea broth (Tello et al., 1991) and the other on potato dextrose agar (PDA) (Difco®). The pea broth was prepared from 100 g mashed canned peas, mixed into 300 ml distilled water and heated to 100 °C for 5–7 min before filtering through hydrophilic cotton. The process of heating and filtration was repeated twice with the residual ground pea tissues and the recovered broth made up to 1000 ml with distilled water before adjusting to pH 5.5.

Isolates were maintained on corn meal agar (CMA) (Merck®) or on PDA at room temperature (20–25 °C) and transferred every 3 months. The isolates of *Phytophthora* used are listed in Table 1.

Mycelium was produced by inoculating 200 ml pea broth with 25 discs of 4 mm diameter excised from the periphery of 7-day-old colonies growing on CMA at 25 °C and in darkness. Inoculated flasks were incubated at 25 °C under 16 h photoperiod for 9 days. The mycelia produced were filtered through cheesecloth and stored in Petri dishes at −20 °C before use.

DNA extraction, polymerase chain reaction (PCR) analysis and agarose gel electrophoresis

The mycelia of each isolate were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. DNA was extracted in 2% CTAB according to the method of Doyle and Doyle (1990). Starting with 25 ng of total genomic DNA per 25 µl mix PCR, the PCR amplification of the ITS1 (flanked by the 18 S and 5.8 S of rDNA) was carried out with ITS_1–ITS_2 universal primer pair in an automated thermal cycler (Biometra, type TRIO-Thermoblock) (White et al., 1990). Cycling PCR parameters were an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. A final extension at 72 °C for 10 min was performed at the end of the amplification. Fifty microliters of the PCR amplification products were analysed (at 120 mV for 75 min) by electrophoresis on 2% agarose gels stained with ethidium bromide and photographed under U.V. lights.

Restriction enzyme digestion and agarose gel electrophoresis

The amplicons generated from isolate 2 (*Pnp* (236-4)) and isolate 13 (*Pnn* (329)) were digested separately with 6 endonucleases of 6 bp (*Acc* I, *EcoR* V, *Pst* I, *Sac* I, *Xba* I and *Xho* I) and 2 endonucleases of 4 bp (*Msp* I and *Taq* I), according to the manufacturers instructions (Pharmacia Biotech). The digestion products were separated (at 120 mV for 50 min) by electrophoresis on 2% agarose gels, stained with ethidium bromide and photographed under U.V. light.

Solanaceous plants: inoculation methods and assessment of root necrosis

The plants used in pathogenicity tests are described in Table 2. The inoculation method (Allagui and Lepoivre, 1996) was based on root inoculation of 30-day-old plantlets. Root inoculation was performed by depositing an inoculum of 280,000 zoospores at the base of

Table 1. Pepper isolates of *Phytophthora* from Tunisia and the isolates of *Phytophthora* species used as reference

Number of the isolate and code	Host, geographic origin and year of isolation	Institution
Pepper isolates of *Phytophthora*		
1 = *Pnp* (148)	*Capsicum annuum*, from Korba, August 1993	INRAT (Tunisia)
2 = *Pnp* (236-4)	*C. annuum*, from Bkalta, February 1994	
3 = *Pnp* (282)	*C. annuum*, from Tebolba, May 1996	
3′ = *Pnp* (283)	*C. annuum*, from Tebolba, May 1996	
4 = *Pnp* (284)	*C. annuum*, from Mraïssa, May 1996	
5 = *Pnp* (285)	*C. annuum*, from Korba, June 1996	
25 = *Pnp* (60)	*C. annuum*, from Soliman, August 1993	
26 = *Pnp* (134)	*C. annuum*, from Jbel Haddad, August 1993	
27 = *Pnp* (255)	*C. annuum*, from Oued Ellil, June 1994	
28 = *Pnp* (260)	*C. annuum*, from Korba, July 1995	
29 = *Pnp* (273)	*C. annuum*, from Ennadour, September 1995	
P. nicotianae		
6 = *Pnp* (A146)	*Lycopersicon esculentum*, Netherlands, 1976	INRA (Antibes)
7 = *Pnp* (A255)	*Solanum melongena*, Guadeloupe, 1983	
8 = *Pnp* (A320)	*Dianthus caryophyllus*, Australia	
9 = *Pnp* (A334)	*Pistacia vera*, Greece	
10 = *Pnp* (A427)	*Citrus*, California (USA)	
P. nicotianae from tobacco		
11 = *Pnn* (308)	*Nicotiana tabacum*, Cuba	INRA (Antibes)
12 = *Pnn* (319)	*N. tabacum*, Australia, 1986	
13 = *Pnn* (329)	*N. tabacum*, Greece, 1989	
14 = *Pnn* (367)	*N. tabacum*, Japan, 1991	
15 = *Pnn* (374)	*N. tabacum*, South Africa, 1992	
30 = *Pnn* (183)	*N. tabacum*, USA	
P. capsici		
16 = *Pc* (15)	*C. annuum*, France	INRA (Montfavet)
17 = *Pc* (101)	*C. annuum*, France	
18 = *Pc* (A266)	*C. annuum*, France, 1973	INRA (Antibes)
19 = *Pc* (A96)	*C. annuum*, France, 1974	
20 = *Pc* (A228)	*C. annuum*, Morocco, 1981	
32 = *Pc* (A281)	*C. annuum*, Mexico	
33 = *Pc* (A346)	*C. annuum*, Vaucluse (France)	
P. citrophthora		
21 = A275	*Citrus*, California (USA)	INRA (Antibes)
P. cinnamomi		
22 = A327	*Geranium rosa*, Reunion, 1987	INRA (Antibes)
P. palmivora		
23 = A392	*Cocus nucifera*, Indonesia, 1990	INRA (Antibes)
P. cryptogea		
24 = A436	*Lycopersicon esculentum*	INRA (Antibes)

each plant, followed by incubation for 10–11 days and evaluation of the root necrosis intensity according to the following scale: 0 (healthy plant), 1 (necrosis only on the lower half of primary roots), 2 (necrosis on all the primary roots), 3 (necrosis reaching the crown and the lateral roots), 4 (hypocotyl rotten) and 5 (whole plant dead).

Results

PCR analysis of the ITS spacers of rDNA

The size of amplicons generated by the ITS_1–ITS_2 universal primers distinguished the 10 pepper isolates of *Phytophthora* (giving rise to an amplicon size of about

Table 2. Solanaceous varieties used in pathogenicity tests

Vegetal species	Varieties	Origin	Characteristics and reference
Capsicum annuum	Beldi	Local variety	Susceptible in the field to the pepper isolates of *Phytophthora*
	Yolo Wonder	Commercial variety	
Lycopersicon esculentum	Marmande verte	INRA (Montfavet)	Susceptible to *P. nicotianae* isolated from tomato (Ravisé, 1970)
Solanum melongena	Black Beauty	Tezier seeds	Susceptible to *P. nicotianae* and *P. capsici* (Polach and Webster, 1972)
Nicotiana tabacum	Samsun	Phytopathology, FUSA of Gembloux	Susceptible to *P. nicotianae* isolated from tobacco

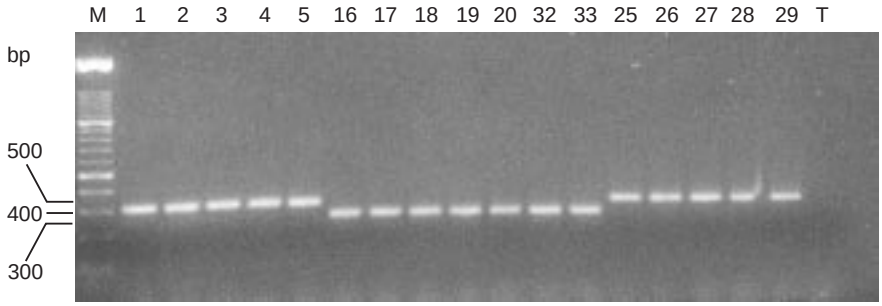

Figure 1. Agarose gel electrophoresis (2%) of amplicons generated with the primer pair ITS_1–ITS_2 flanking the ITS1 region rDNA of 10 pepper isolates of *Phytophthora* (lanes 1, 2, 3, 4, 5, 25, 26, 27, 28 and 29) and 7 isolates of *P. capsici* (lanes 16, 17, 18, 19, 20, 32 and 33). Lane T = negative control (without DNA), lane M = 100 bp lambda DNA ladder markers (Boehringer).

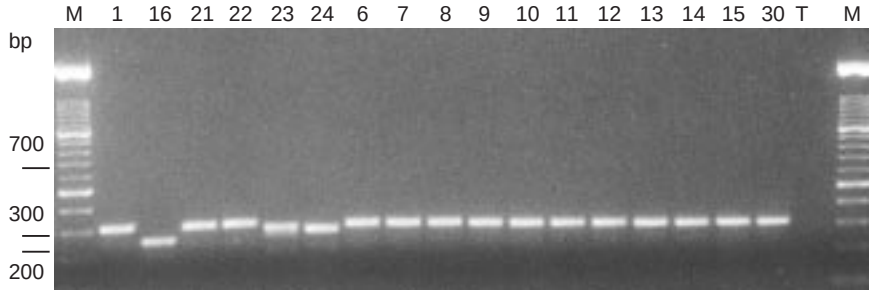

Figure 2. Agarose gel electrophoresis (2%) of amplicons generated with the primer pair ITS_1–ITS_2 of one pepper isolate of *Phytophthora* (lane 1), one isolate of *P. capsici* (lane 16), one isolate of each of the species *P. citrophthora* (lane 21), *P. cinnamomi* (lane 22), *P. palmivora* (lane 23), *P. cryptogea* (lane 24), 5 isolates belonging to *P. nicotianae* (lanes 6, 7, 8, 9 and 10) and 6 isolates of *P. nicotianae* from tobacco (lanes 11, 12, 13, 14, 15 and 30). Lane T = negative control (without DNA), lane M = 100 bp lambda DNA ladder markers (Boehringer).

310 bp) from the 7 reference isolates of *P. capsici* (for which the amplicon size was about 270 bp) (Figure 1). The 10 pepper isolates of *Phytophthora* were *Pnp* (60), *Pnp* (134), *Pnp* (148), *Pnp* (236-4), *Pnp* (255), *Pnp* (260), *Pnp* (273), *Pnp* (282), *Pnp* (284) and *Pnp* (285), while those of *P. capsici* were *Pc* (15), *Pc* (A96), *Pc* (101), *Pc* (A228), *Pc* (A266), *Pc* (A281) and *Pc* (A346).

Sixteen isolates of *Phytophthora* belonging to 5 different *Phytophthora* species were compared with a pepper isolate of *Phytophthora*. The amplicons generated revealed interspecific polymorphism discriminating 3 groups of *Phytophthora* (Figure 2). The pepper isolate of *Phytophthora* (*Pnp* (148)), as well as the 11 isolates of *P. nicotianae* (*Pnp* (A146), *Pnp* (A255), *Pnp* (A320), *Pnp* (A334), *Pnp* (A427), *Pnn* (183),

Pnn (308), *Pnn* (319), *Pnn* (329), *Pnn* (367) and *Pnn* (374)), the isolate of *P. citrophthora* (A275), and the isolate of *P. cinnamomi* (A327) represented a single group generating amplicons of 310 bp. The second group included isolates of *P. palmivora* (A392) and *P. cryptogea* (A436) and gave rise to an amplicon of 300 bp, whereas *P. capsici* (*Pc* (15)) produced an amplicon of about 270 bp. No differences were seen within the isolates of *P. nicotianae* from tobacco, tomato, eggplant, pistachio, carnation, citrus and pepper. The results were constant and reproducible using 2 independent DNA extractions.

Restriction analysis of ITS amplification products

Two strains of*Phytophthora* were submitted to restriction analyses of their ITS amplification products. The first one (*Pnp* (236-4)) was isolated from pepper while the second one (*Pnn* (329)) was obtained from tobacco. The amplicons obtained using an ITS_1–ITS_4 primer pair amplifying the entire ITS region were digested with 8 different endonucleases. Only 2 enzymes (*Msp* I and *Taq* I) cleaved these ITS amplicons. The restriction fragment patterns of the*P. nicotianae* and pepper isolates were, in each case, identical (Figure 3).

Pathogenicity of pepper isolates of Phytophthora *on* Solanaceae *species*

The percentage of mortality observed after the inoculation of the pepper plants (var. Beldi) with a selection of isolates of *Phytophthora* ranged from 80% to 100%. This mortality was associated with generalised root necrosis (necrosis intensity between 4.8 and 5) (Table 3). Reaction of tomato plants (var. Marmande verte) varied with the inoculated isolate. Tomato plant

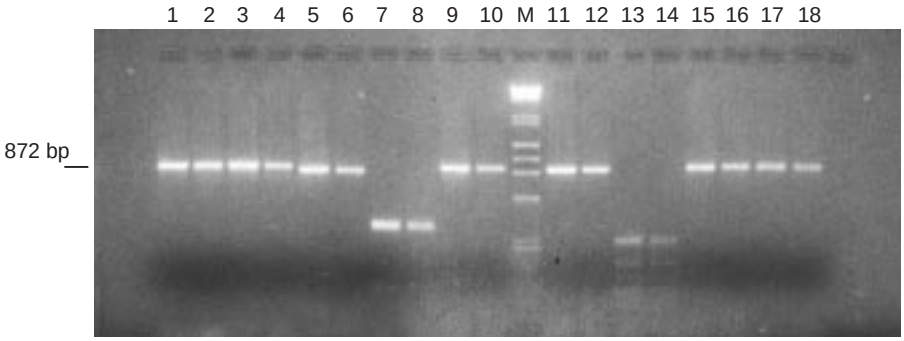

Figure 3. Migration profile on agarose gel (1.5%) of restriction fragments obtained with pepper isolate of *Phytophthora* [*Pnp* (236-4)] and *P. nicotianae* isolated from tobacco [*Pnn* (329)] after PCR analysis with the ITS_1–ITS_4 primer pair and digestion of the amplicons with 8 endonucleases. For *Pnp* (236-4), lane 1 = control (amplicon without digestion), lane 3 = *Acc* I, lane 5 = *EcoR* V, lane 7 = *Msp* I, lane 9 = *Pst* I, lane 11 = *Sac* I, lane 13 = *Taq* I, lane 15 = *Xba* I, lane 17 = *Xho* I. For *Pnn* (329), lane 2 = control (amplicon without digestion), lane 4 = *Acc* I, lane 6 = *EcoR* V, lane 8 = *Msp* I, lane 10 = *Pst* I, lane 12 = *Sac* I, lane 14 = *Taq* I, lane 16 = *Xba* I, lane 18 = *Xho* I. Lane M = DNA marker size *Hind* III – digested λ DNA and *Hae* III – digested Φx 174.

Table 3. Plant mortality percentage and root necrosis intensity in 4 *Solanaceae* species 11 days after plant inoculation by 5 pepper isolates of *Phytophthora*

Isolates	Pepper var. Beldi		Tomato var. Marmande verte		Eggplant var. Black Beauty		Tobacco var. Samsun	
	Plant mortality	Necrosis intensity	Plant mortality	Necrosis intensity	Plant mortality	Necrosis intensity	Plant mortality	Necrosis intensity
Pnp (236-4)	80	4.8 (4–5)	0	3.2 (2–4)	40	1.6 (0–4)	0	0
Pnp (282)	100	5	100	4.2 (3–5)	60	3 (0–5)	0	0
Pnp (283)	100	5	20	1.4 (0–5)	20	1.6 (0–5)	0	0
Pnp (284)	100	5	0	1.5 (0–4)	60	3 (0–5)	0	1.1 (0–3)
Pnp (285)	80	4.8 (4–5)	0	0.6 (0–2)	0	1 (0–3)	0	1.1 (0–3)
Control	0	0.3 (0–1)	0	0	0	0	0	0.5 (0–2)

Average of 5 repetitions and extreme values between parenthesis, notation scale between 0 (healthy plant) and 5 (dead plant).

mortality ranged from 20% (with *Pnp* (283) isolate) to 100% (with *Pnp* (282) isolate). On the other hand, no tomato plant mortality was recorded with the 3 other isolates which induced an average intensity of root necrosis ranging from 0.6 to 4.2.

On eggplant (var. Black Beauty), 2 isolates, *Pnp* (283) and *Pnp* (236-4), caused 20% and 40% plant mortality respectively, while the isolates, *Pnp* (282) and *Pnp* (284), produced 60% plant mortality. The amount of root necrosis recorded on these plants ranged between 1.6 and 3. Isolate *Pnp* (285) did not produce any mortality (average root necrosis intensity = 1).

Tobacco plants (var. Samsun) were not affected by inoculation with the pepper isolates of *Phytophthora*. The mild root necrosis observed on tobacco plants inoculated with *Pnp* (284) or *Pnp* (285) was similar to that observed in the uninoculated controls.

Pathogenicity of the reference isolates of Phytophthora nicotianae *on pepper plants*

The reference strains of *P. nicotianae* isolated from different plant species, did not produce any mortality on pepper plants (var. Yolo Wonder) (root necrosis between 0.1 and 0.4) (Table 4). The 2 isolates *Pnp* (A146) and Pnn (329) which were not pathogenic on pepper plants showed significant pathogenicity on their respective hosts (100% mortality of tomato and tobacco 4 days after inoculation with *Pnp* (A146) or *Pnn* (329) respectively). In the same experiment, the inoculation of pepper plants (var. Yolo Wonder) with the pepper isolate *Pnp* (236-4) or with *P. capsici* (*Pc* (101)) caused 100% pepper plant mortality 6 days after inoculation.

Table 4. Mortality percentage and root necrosis intensity observed on pepper plants (var. Yolo Wonder) 10 days after plant inoculation with reference species, *P. nicotianae* (*Pnp* or *Pnn*) and *P. capsici* (*Pc*)

Origin hosts and isolate code	Mortality percentage	Root necrosis intensity[1]
Pepper: *Pnp* (236-4)	100	5
Pepper: *Pc* (101)	100	5
Tomato: *Pnp* (A146)	0	0.4 (0–2)
Carnation: *Pnp* (A320)	0	0.5 (0–2)
Pistachio: *Pnp* (A334)	0	0.1 (0–0.5)
Tobacco: *Pnn* (329)	0	0.1 (0–0.5)
Citrus: *Pnp* (A427)[2]	0	0.2 (0–0.5)
Eggplant: *Pnp* (A255)	0	0.1 (0–0.5)
Control	0	0.1 (0–0.5)

[1]Average of 5 repetitions and extreme values between parenthesis [intensity scale between 0 (healthy plant) and 5 (dead plant)].

[2]Number of zoospores deposited in plant crown was lower than 60,000 due to poor production of sporangia by this isolate in the conditions indicated (§ Materials and methods).

Discussion

In Tunisia, symptoms of dry necrosis on roots and crown of pepper plants frequently lead to wilt of plantlets and death of adult plant. The foliage, fruits and branches of the infected plants were not attacked. In order to analyse the etiology of these symptoms, pepper plants in pots were inoculated either in roots by zoospores or on decapitated stems or wounded leaves by mycelia discs (Allagui and Lepoivre, 1996). Pepper isolates of *Phytophthora* reproduced the whole syndrome (dry necrosis restricted to the crown, leaves not attacked) observed in field conditions, while reference strains of *P. capsici* produced the typical symptoms of *P. capsici* on pepper (soft rot on roots that progressed up on the stems, generalised necrosis on the decapitated stem, drop of leaves). These results indicated the pathogenicity of the pepper isolates of *Phytophthora* on roots of pepper plants and differentiated the pepper isolates from *P. capsici* on the basis of the symptoms observed in the field and produced by artificial inoculation.

Agarose gel electrophoresis of the ITS1 amplicons obtained by the ITS_1–ITS_2 universal primer pair revealed polymorphisms which discriminated the pepper isolates of *Phytophthora* (generating an amplicon size 310 bp) from the isolates of *P. capsici* (generating an amplicon size 270 bp). Similar interspecific variations have been previously revealed in *Phytophtora* by ITS amplicons (Lee and Taylor, 1992; Crawford et al., 1996; Cooke and Duncan, 1997). These results corroborate our identification based on morphological and biological criteria which also distinguished the pepper isolates of *Phytophthora* from *P. capsici* (Allagui et al., 1995). To date, *P. capsici* has not been isolated from peppers in Tunisia, although the latter has been reported as the major species of *Phytophthora* attacking pepper plants in many other countries (Tucker, 1931; Satour and Butler, 1967; Messiaen et al., 1991).

Enzymatic digestion of the amplified ITS region did not reveal any difference between the isolates of *Phytophthora* from pepper and the standard isolates of *P. nicotianae*. The lack of polymorphism in the restriction sites indicates a close similarity between

pepper isolates of *Phytophthora* and *P. nicotianae*, though restriction analysis only samples a small portion of the ITS region and the complete sequence should be obtained to add further detail. Further genetic analyses using RAPDs, AFLPs or reproductive isolation could be undertaken to test whether pepper isolates of *Phytophthora* represent a genetically distinct population compared to the reference isolates of *P. nicotianae*.

Specific pathogenicity has been used for classifying isolates as formae speciales within species, such as *P. drechsleri* f. sp. *cajani* on pigeon pea (Kannaiyan et al., 1980), *P. sojae* f. sp. *medicaginis* on alfalfa, and *P. sojae* f. sp. *glycinae* on soybean (Faris et al., 1989). Our results on pathogenicity showed that the isolates of *Phytophthora* from pepper were not pathogenic on tobacco plants. On tomato and eggplant plants, they produced symptoms which differed from those observed on pepper plants. Furthermore, field observations indicate the absence of root necrosis and wilting of tomato plants grown in plots where these symptoms have been observed on pepper plants. Moreover, the typical isolates of *P. nicotianae* obtained from tomato, carnation and pistachio either did not produce any symptom or caused only limited root necrosis on pepper plants.

Pathogenicity trials and field observations indicated that the pepper isolates of *Phytophthora*, were particularly adapted to pepper plants. These results would indicate that a new *forma specialis* inside *P. nicotianae* may be appropriate for the pepper isolates of *Phytophthora*. Before giving a new name at the informal rank *forma specialis* to mention the parasitic specificity of the pepper isolates, it would be appropriate to carry out more studies on host selectivity. Elicitins produced by many *Phytophthora* species (including *P. nicotianae*) were reported as microbial signal molecules involved in the host-specificity of these pathogens (Kamoun et al., 1993 ; Panabières et al., 1995). Analyses on such elicitins in the pepper isolates of *Phytophthora* during incompatible interactions would be of significance in order to reinforce the argued specificity of these isolates on pepper.

Acknowledgements

The authors thank Philippe Bonnet and Paul Venard (INRA, Antibes) for the gift of isolates of *Phytophthora* spp. and Mohamed Mahjoub (ESHE, Chott Mariem, Tunisia) for fruitful discussions.

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