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Etiology of apple tree dieback in Tunisia and abiotic factors associated with the disease

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An apple tree dieback syndrome causing severe tree losses was observed in the main apple producing regions in Tunisia from 2006-2008. This work aimed at identification of the causal agents and the factors that promote apple tree dieback. The causal agents of the syndrome included the following: *Phytophthora* and *Pythium* species: *Phytophthora parasitica* and *Phytophthora inundata*, *Pythium* sp., *Pythium indigoferae*, *Pythium irregulare*, *Pythium rostratiformis*, *Pythium sterillum* and *Pythium undulatum*. Molecular techniques confirmed the results of the morphological identification. Pathogenicity assays showed the involvement of these pathogens in apple tree dieback. Soil salinity was also shown to be an important factor of disease severity in this study.

Key words: Apple, *Malus domestica*, orchard survey, dieback syndrome, sequencing, pathogenicity.

INTRODUCTION

Over 27000 ha of land is been cultivated for apple (*Malus x domestica* Borkh) in Tunisia. Tunisian apple growing-areas are mainly located along the northeast and northwest of the country. It is an economically important crop in Tunisia, mainly in the Kasserine region. Since 1996, a severe dieback of apple trees was observed at Kasserine, Bizerte, Jendouba, Beja and Ben Arous provinces. This dieback caused severe damages and tree losses in numerous apple orchards. Affected trees showed cankers and necrosis in the collar and roots.

Apple tree dieback could be attributed to numerous physiological or parasitical factors (Mazzola et al., 2002; Latorre et al., 2001; Jeffers and Aldwinckle, 1988;

Matheron et al., 1988; Jeffers et al., 1982), among them are Oomycete pathogens that usually play a significant role in the phenomenon (Jeffers and Aldwinckle, 1988; Bolay, 1992a; Latorre et al., 2001; Mazzola et al., 2002). Tsao (1990) indicated that numerous *Pythiaceae* species have been underestimated as pathogens that cause disease on numerous species world-wide. Moreover, numerous root and collar rots are still attributed to attacks by other microorganisms, or to abiotic factors (Jiménez et al., 2008). Recent studies have highlighted the importance of the genera *Pythium* (Romero et al., 2007) and *Phytophthora* (Moralejo et al., 2008; Moralejo et al., 2009) in natural or managed ecosystems. The present

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Table 1. Regions and number of orchards surveyed in each region.

Species	Locations	Isolates numbers
<i>P. irregulare</i>	Foussana	2
	Sbiba	8
<i>Pythium</i> sp.	Sbiba	6
	Mateur	2
<i>P. rostratiformis</i>	Foussana	2
	Utique	2
<i>P. undulatum</i>	Medjez el Beb	1
<i>P. sterilum</i>	Foussana	2
	Mateur	1
<i>P. indigoferae</i>	Morneg	3
	Foussana	2
	Mateur	2
	Utique	1
<i>P. inundata</i>	Mateur	1
	Jendouba	4
	Morneg	3
	Sbiba	4
<i>P. parasitica</i>	Mateur	
	Beni	2
	Khalled (Agrumes)	2
Total		50

work studied the hypothesis that *Pythium* and *Phytophthora* species were involved in the apple decline recorded in Tunisia.

Therefore, the aims of the present study were: 1) to identify the causal agents of the apple tree decline in Tunisia and to establish the pathogenicity of the isolated agents; 2) to study the involvement of irrigation water, irrigation system and soil salinity as factors that could incite apple tree dieback.

MATERIALS AND METHODS

Survey and isolation

The survey included 23 apple orchards (10 trees per orchard) that were sampled during the 2006-2008 growing seasons. The orchards were located in nine regions, which are the leading areas of apple cropping in Tunisia (Table 1). Disease symptoms were recorded from both aerial and below ground parts of the diseased trees. Collar rot symptoms were recorded following bark removal. These orchards were sampled and root, collar and soil samples were collected.

The soil sampling method varied according to the irrigation system used in each orchard. In the flood irrigation system, the samples were taken from 15 to 20 cm depth under soil level in the direction of water circulation. Sampling was performed under the canopy of the diseased trees and the next one in the irrigation water direction. Whereas, in the case of heavily diseased orchards,

samples were randomly taken. In the case of orchards irrigated by drippers, the soil samples were taken from four quadrants under the dripline of the affected trees from a depth of 15-20 cm. In each orchard, the samples of soil were mixed and a small amount of soil sample was placed in two 10 mm diameter x 15 mm depth wells cut into an apple with a corkborer. Two apples per sample were prepared following this method. Apples were incubated at 25°C for 3 days. Infected fruit tissue from the margin of the necrosis was removed and placed on 1.7% corn meal agar (CMA) amended with (per liter) 10 µg pimaricin, 200 µg ampicillin, 10 µg rifampicin, 25 µg pentachloronitrobenzene and 10 µg benomyl (PARB) (Jaffers and Martin, 1986). The antibiotics were added after sterilization.

To recover and isolate the suspected disease causal agents, collar or root tissues were removed from the margin of cankers. The diseased tissues were cut aseptically into approximately 20 small pieces of 3 to 5 mm in length. Then, they were washed in tap water, surface-disinfested by dipping in 70% ethanol for 30 s, rinsed in sterilized de-ionized water and blotted dry on a filter paper. The infected tissues were placed onto PARB selective medium (Jeffers and Martin, 1986) in 90 mm Petri dishes. The samples were incubated for four to five days at 24°C in darkness. To obtain pure cultures of the suspected pathogens a single hyphal tip from the edge of each colony was transferred by micromanipulation onto potato dextrose agar (PDA: 39 g/L), Biokar Diagnostic) and incubated at 25°C for three days. For morphological identification, the cultures were transferred to V8 juice agar (200 mL of V8 Juice; 2 g CaCO₃ and 15 g agar in 800 mL distilled water) and incubated at 24°C in the dark. Stock cultures were maintained on PDA at 12°C in the dark. All cultures were preserved in the culture collections maintained at the Higher Institute of Agronomy located at Chott Mariem, Tunisia.

Diagnosis of suspected causal agents

Morphological identification

Sexual and asexual structures of the different oomycetes were used for identification. *Phytophthora* isolates were identified on the basis of colony morphology, growth rate, cardinal growth temperatures, and production, morphology and dimensions of sporangia, oogonia and antheridia (Waterhouse, 1963; Erwin and Ribeiro, 1996). Identification of the *Pythium* isolates were carried out by microscopic observations from cultures incubated on PDA medium at 24°C in darkness for 4-6 days. Distinctive structures, such as reproductive structures form and size were observed after transfer to a glass microscope slide using the key of Waterhouse (1968), Van der Plaats (1981) and Mugnier and Grosjean (1995).

DNA extraction, ITS sequencing and molecular phylogeny

Mycelial DNA was extracted from pure cultures grown in potato dextrose broth (PDB, Biokar Diagnostic) (Belbahri et al., 2006). Total DNA was extracted using the EZNA Plant Miniprep Kit (Omega Bio-tek) following the manufacturer's instructions. The amplification of the Internal Transcribed Spacer (ITS) of the ribosomal DNA (rDNA) was carried out using the universal primers ITS4 and ITS6 that target conserved regions in the 18S and 28S rDNA genes (White et al., 1990; Cooke et al., 1996). Amplifications were performed according to Chavarriaga et al. (2007). PCR products were purified and DNA sequencing was performed at the analysis technical service of the institute of Plant Molecular and Cellular Biology of the Polytechnic University of Valencia (Spain). Purified PCR products were cycle-sequenced using ABI Prism™ BigDye™ Terminator chemistry with AmpliTaq® DNA polymerase, and sequenced using a Perkin-Elmer, Applied Biosystems Division, 373 A DNA Sequencer. Sequences were edited using the chromas 1.51 software (Technelysium Pty Ltd,



Figure 1. Affected apple tree showing typical symptoms of dieback.

<http://www.technelysium.com.au/chromas.html>). Consensus sequences were aligned using the CLUSTAL W program. The sequences were subjected to an NCBI BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>) for identification and prior to phylogenetic analyses to identify the closest related sequences and building phylogenetic trees from molecular data with MEGA 5. Trees were constructed based on the test Neighbor-Joining tree.

Study of abiotic factors

To study the effect of irrigation method, water source and soil salinity on the disease, three orchards were selected. One was located in Sbiba, a second in Morneg and the third in Utique. These orchards were irrigated by both drip and flood irrigation and the water source was a well or a reservoir. Apple tree dieback severity was investigated and the assessment of the damage on apple trees was estimated by considering the total number of apple trees by orchard, and the number of diseased and dead trees. The effect of soil salinity on the development of dieback was studied by considering the total salt and sodium chloride concentrations and the dieback rate of dieback recorded at some surveyed orchards.

Pathogenicity tests

Phytophthora and *Pythium* isolates were tested to establish their pathogenicity and virulence over the 3-years growing periods. The following apple cultivars were used: Anna, Lorka and Meski and the rootstock MM106. Pathogenicity assays were performed using six species of *Pythium* (two isolates for each species *Pythium* sp. 1 and sp. 2, *Pythium rostratifingens* 1 and 2, and one isolate of *Pythium irregulare*, *Pythium sterilum*, *Pythium indigoferae* and *Pythium undulatum*), two isolates of *Phytophthora nicotianae* Ph 1 and Ph 2 and one isolate of *Phytophthora inundata* were tested separately. The inoculation was used, *in vitro* and *in vivo*. *In vitro*, the method consisted of the use of detached two-years-old shoots (10 cm length and 2 cm diameter) (Krober and Karnatz, 1979). The detached branches were disinfested with 70% ethyl alcohol, rinsed in sterile distilled water and blotted dry. Sets of 36 shoots of each apple cultivar were planted on containers (4920 cm³) filled with peat. A 7 mm in diameter disc of bark was removed from each inoculation court (2 cm approx. above the substrate) and a transverse T-shaped notch was cut through the cambium to a depth of 1.0-1.5 mm into the sapwood. A 7-mm in diameter disc removed from a 4-days-old culture grown on PDA was inserted into the artificial

wound, placing the mycelium in contact with the exposed tissue. The control plants were inoculated with non-inoculated sterile PDA discs. Inoculation sites were covered with parafilm for 1 month. The containers were maintained at 25°C under cool white light from 30-W fluorescent tubes suspended 30 cm above the chambers (12-h photoperiod). Seven days after inoculation court, the area of the lesions was calculated with an analogical planimeter.

Pathogenicity tests were also carried out *in situ* on six-years-old apple trees. Branches of 20 mm in diameter were randomly selected from the canopy of disease free, non symptomatic trees. The branches were disinfested using 95% sodium hypochlorite for 5 min. A 7-mm-diameter disc of bark was removed from each inoculation court and a transverse T-shaped notch was cut through the cambium to a depth of 1.0-1.5 mm into the sapwood. A 7-mm diameter disc from a 4-day-old PDA culture of each strain was inserted with the mycelium in contact with the host tissue. In the control plants, inoculum consisted of sterile PDA discs. Inoculation sites were covered with a distilled water dipped cotton swab and protected by parafilm and aluminium foil for 1 month.

Twenty seven randomly chosen nursery produced Rootstock MM106 plants were twig-inoculated (three replications). Seven years-old Anna, Lorka and Meski apple cultivars trees were twig-inoculated (2 years-old twigs) with random sites inoculation. Controls were included in all pathogenicity assays and re-isolations were made from the infested tissue by plating onto PARB medium to confirm Koch's postulates.

RESULTS

Apple tree decline symptoms description and isolation

The first symptoms of the syndrome appeared during spring months on the apical parts of the trees. Terminal branches on the canopy dried out or wilted and the symptomatology progressed to lower parts of the plant (Figure 1). At the end of the summer, a severe defoliation was observed on the basal parts of young branches. Additional symptoms included small fruits and premature ripening (Figure 2). Lesion expansion toward the lower parts of the tree caused the formation of cankers on the trunk. A visible reddish brown discoloration of the inner bark was observed by cutting away the outer bark layer on the exposed collars; often, it was possible to see a sharp contrast between the infected and healthy tissues (Figure 3). An unpleasant smell was also noticed.

Isolations made from collar, roots or soil collected in the vicinity of dying back trees allowed the recovery of 50 oomycetes isolates. Six species of *Pythium*: *P. indigoferae*, *P. irregulare*, *P. rostratifingens*, *P. sterilum*, *P. undulatum* and *Pythium* sp., and two species of *Phytophthora* (*P. nicotianae* and *P. inundata*) were recovered (Table 2). *Pythium* species were isolated from the roots and soil whereas *Phytophthora* species were isolated from the soil root and collar.

Morphological and molecular identification of pathogenic isolates

The morphological features recorded for homothallic



Figure 2. Affected apple tree showing typical symptoms of collapse.



Figure 3. Typical lesions observed in the collar area of apple trees in affected orchards.

Pythium spp. and the heterothallic *P. nicotianae* isolates are presented in Table 2. The identification of the heterothallic *Pythium* species however was based on the molecular identification given the absence of the reference strains to do complementation assays.

Conductive abiotic factors

The effect of irrigation water source on the severity of the disease was studied and the results proved that reservoir water was associated with disease severity in the three surveyed regions. Figure 4 shows differences of tree infection using the two water origins that can be explained by texture or structure of the soil. Also, the effect of irrigation system showed a clear correlation

between the irrigation system and the severity of the dieback. The percentage of diseased trees was higher in orchards flood irrigated than in drip irrigated ones. The most significant value was found in Morneg region showing the highest difference between the two irrigation systems (Figure 5).

During this study, two variables were also investigated: total salt concentration and sodium chloride concentration. The results revealed that severity of dieback could be correlated with the total salt concentration (Figure 6 and 7). In fact, in Orchard 1 placed in Foussana region only 21% of diseased trees were noticed where the total salt content was only 0.5 g/l. However, in the orchard located in Oued Mliz region, having 1.8 g/l total salt concentration, 60% dieback was noticed. Orchard 2 in Foussana shows 10% dieback despite a total salt concentration of 1.3 g/l.

This result could be explained by the different salts concentrations. While Figure 8 shows a positive correlation between sodium chloride concentrations and tree apple dieback. In Foussana Orchard 2, the lowest level of infestation was registered in the orchard irrigated with the water having the lowest level of sodium chloride. This result confirms the importance of sodium chloride among total salts.

Molecular identification of Pythiaceae isolates

Molecular identification was performed by sequencing the ITS region of rDNA, using the conserved primers ITS4 and ITS6. The ITS sequences of oomycete isolates recovered from collar roots and soil showed the presence of eight *Pythiaceae* species, confirming the results obtained using morphological criteria. Sequences have been blasted and aligned with sequences from GenBank database (Figures 8 and 9).

Pathogenicity tests

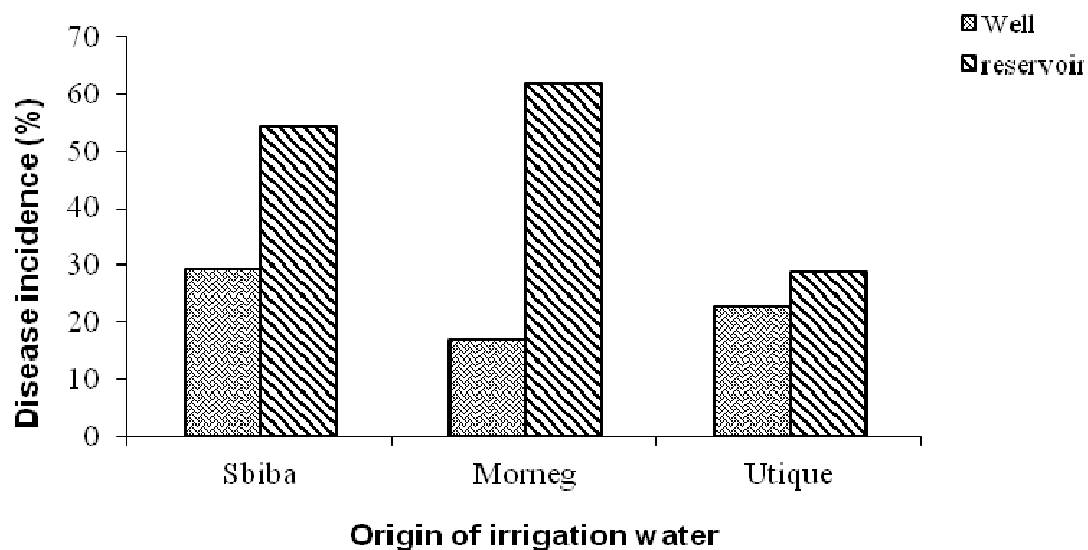
The results of the inoculation tests reproduced the symptoms observed under field conditions. Results showed that the rootstock MM106 was the most susceptible to the different tested isolates. Cultivars Anna and Lorka were the most resistant. The classification of Oomycete isolates differs according to the apple cultivar used. Nevertheless, the most aggressive isolates on the apple cultivars were *Pythium* sp. isolates with *P. undulatum* as the weakest pathogen (Figures 10 and 11). *Pythium* and *Phytophthora* isolates inoculated could be recovered from all the artificially inoculated trees. Control trees did not show any symptoms.

DISCUSSION

The present study show the presence of *P. nicotianae*

Table 2. Morphological characteristic of five Pythiaceae species (sexual structure of *P. inundata* is not determined given the unavailability of reference strains).

Species	Colony patterns (PDA)	Hyphae	Sporangia			Oogonia diameter (μm)	Oospore diameter (μm)	Antheridia	Hyphal swellings	Chlamydo-spores	Cardinal temperature ($^{\circ}\text{C}$)		
			Form	Proliferation	Persistent						T min	T op	T max
<i>P. indigoferae</i>	Petaloid	Homothallic	Filamentous inflated	Absent	Yes	Smooth (19.7 \pm 1.7)	Aplerotic (18.9 \pm 1.4)	Monoclinous Amphigynous	Absent	Absent	11	25-28	35
<i>P. irregulare</i>	Rosette	Homothallic	Globose	Absent	Yes	Smooth (19 \pm 2.6)	Aplerotic (18.7 \pm 2.1)	Monoclinous Hypogynous	Spherical	Absent	5	28-32	40
<i>P. rostratifingens</i>	Rosette	Homothallic	Spherical	Absent	Yes	Globose (13.2 \pm 1.27)	Plerotic (11.9 \pm 1.9)	Monoclinous Hypogynous	Absent	Absent	5-7	16-23	35
<i>Ph. nicotianae</i>	Cotonny	Heterothallic	Spherical Papillate (1 or 2 papilla)	Absent	Yes	Spherical Smooth (20.1 \pm 2.22)	Aplerotic (20 \pm 2.24)	Diclinous Amphigynous	Spherical	Terminal Intercalary (27.9 \pm 4.6)	7-10	26-28	35-39
<i>Ph. inundata</i>	irregular	Heterothallic	Ovoid papillate	Present	yes	-	-	-	Circular	Absent	11	25-29	35

**Figure 4.** Effect of the origin of the water used for irrigation on disease incidence in 3 regions: Sbiba, Morneg and Utique (Average of 6 orchards by region).

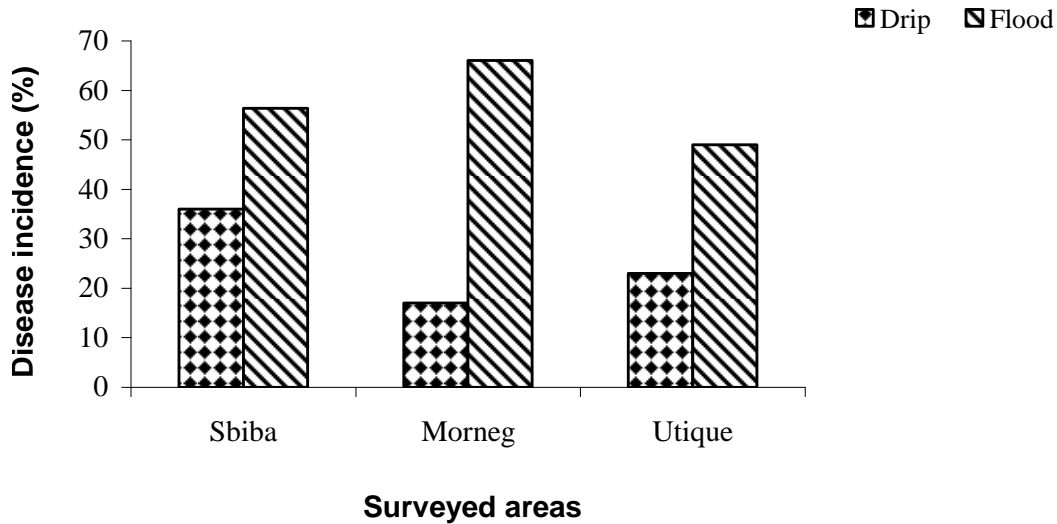


Figure 5. Effect of the irrigation system (drip or flood) on disease incidence in 3 regions: Sbiba, Morneg and Utique (Average of 6 orchards by region).

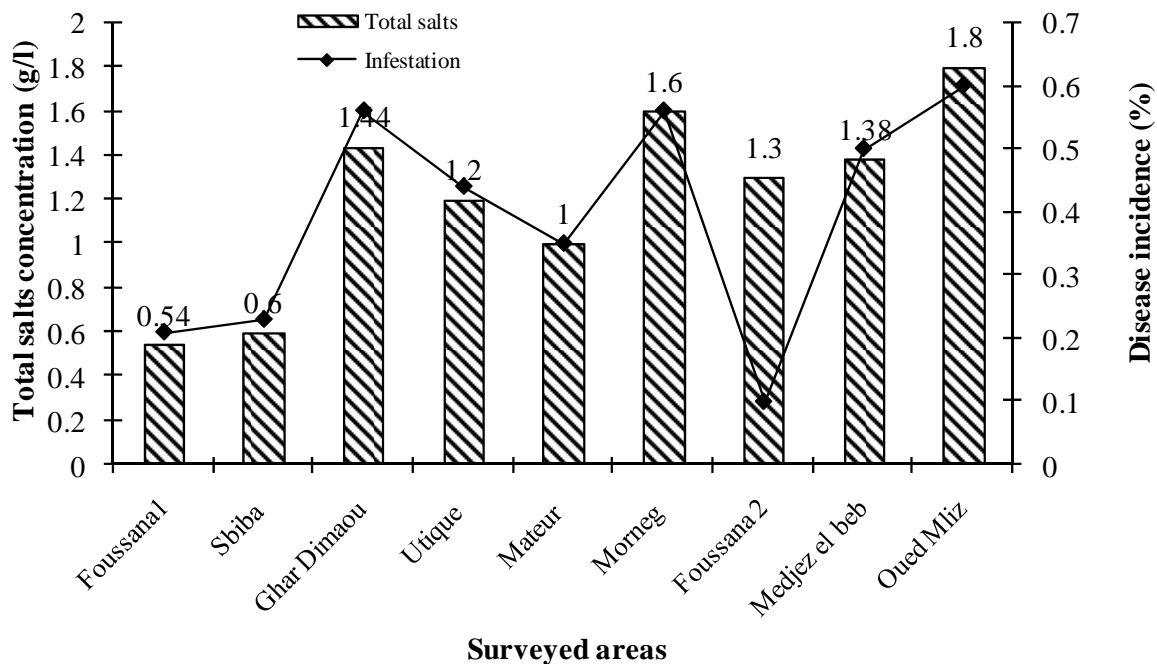


Figure 6. Effect of soil salinity on the incidence of the disease.

associated with apple tree dieback in Tunisia. This pathogen is reported for the first time in Tunisia. In addition, the role of several *Pythium* species on the syndrome was elucidated.

Irrigation increases soil moisture, potentially increasing pathogen activity and promoting development of root, collar rot and other diseases (Ristaino et al., 1993). This is especially the case when irrigation produces patches of

excessive wetness such as in low-lying areas of a field (Larkin et al., 1995) or in areas directly near an irrigation emitter (Café-Filho and Duniway, 1996).

Numerous reports using filtration techniques for recovering fungal spores highlighted the presence of Oomycetes in water. In previous studies, about twelve species in water reservoirs used for irrigation were identified, including *Phytophthora cactorum*, *Ph. cinnamomi*,

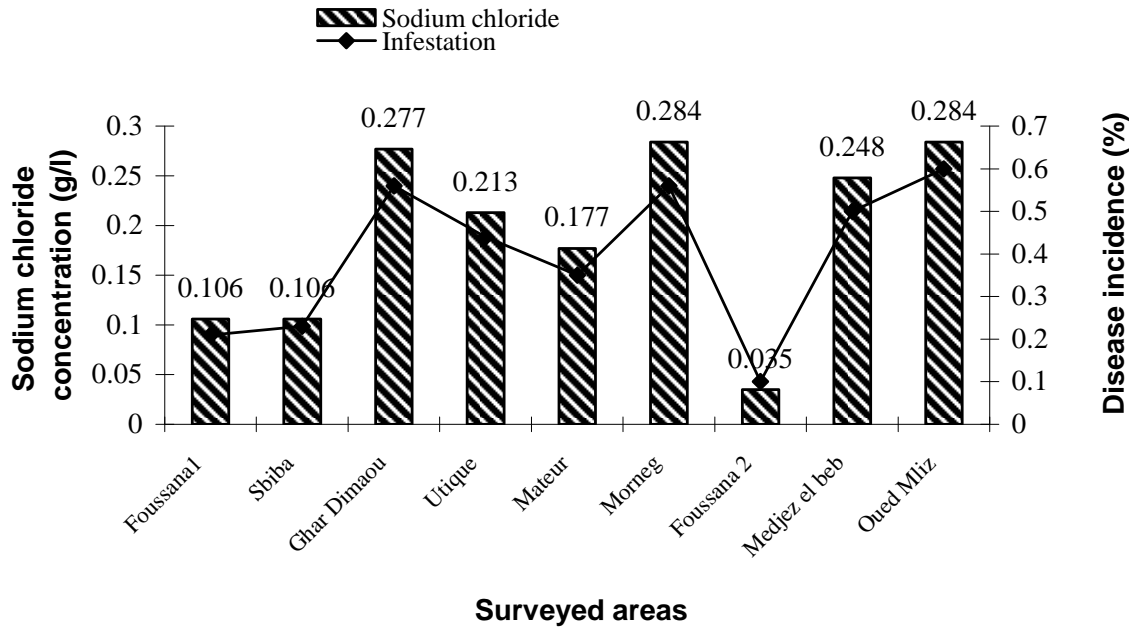


Figure 7. Effect of sodium chloride on the incidence of the disease.

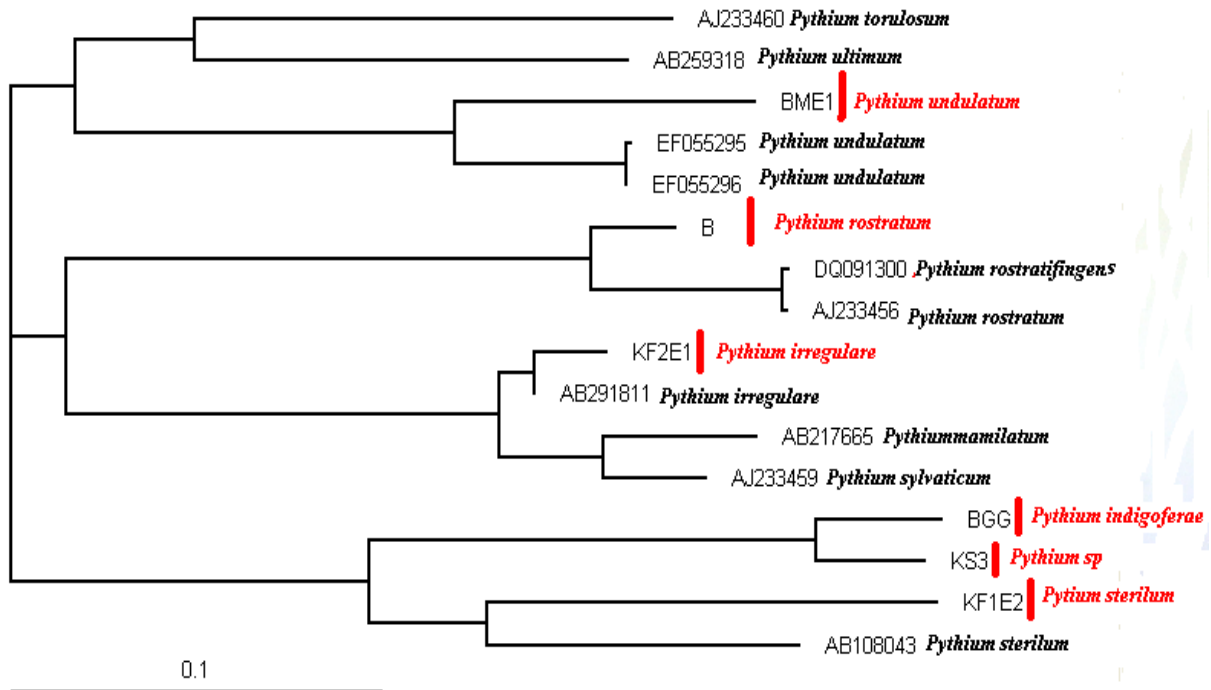


Figure 8. Dendrogram for *Pythium* species based on analysis of ribosomal DNA.

P. citricola, *P. citrophthora*, *P. dreschleri*, *P. nicotianae*, *P. boehmeriae*, *P. gonapodyides*, *P. hibernalis*, *P. megasperma* and *P. syringae* (Ristaino, 1991). Yamak et al. (2002) reported 149 *Phytophthora* sp. isolates from irrigation waters.

The susceptibility of some plant species to Pythiaceae can be enhanced by specific environmental factors. Bolay (1992b) has estimated that some soils are favorable to disease expansion and the annual loss of apple trees dieback ranges from 1 to 5% of the trees. In some

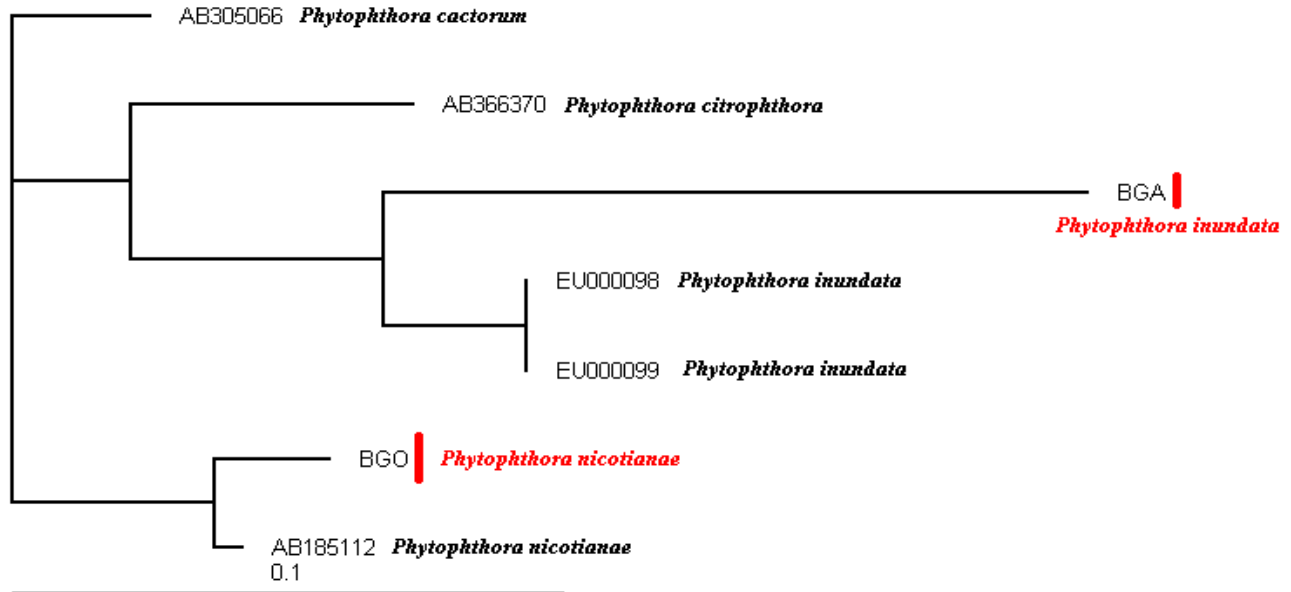


Figure 9. Dendrogram for *Phytophthora* species based on analysis of ribosomal DNA.

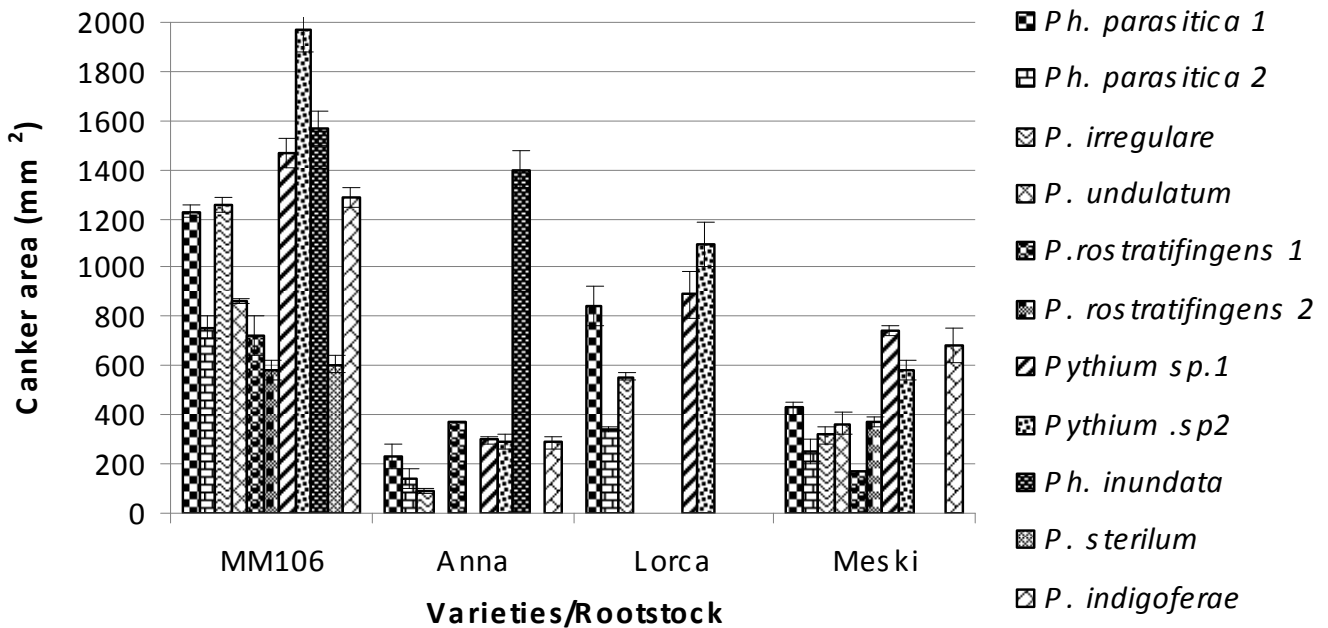


Figure 10. Canker area in 2 years old shoots of apple 7 days after inoculation with 6 *Pythium* species and 2 *Phytophthora* species (average of nine replicates). *Ph.*: *Phytophthora*; *P.*, *Pythium*.

Tunisian apple orchards, the losses could be as severe as 5 to 50%. High soil salinity predisposes crops to infection by Pythiaceae. Soil salinity is normally a problem in arid environments where salt levels fluctuate throughout the year based on water quality and the frequency and duration of irrigation events. During periods of high salinity, diseases caused by Pythiaceae

pathogens can be severe despite high temperature and low relative humidity, which are not favorable for their development. In Morocco, El Guilli et al. (2000) studied the effect of salinity of irrigation water on the severity of *Ph. citrophthora* collar rot on *Citrus* trees and showed a correlation with the severity of infection. Benyahia et al. (2004) studying the effect of salinity on colonization of

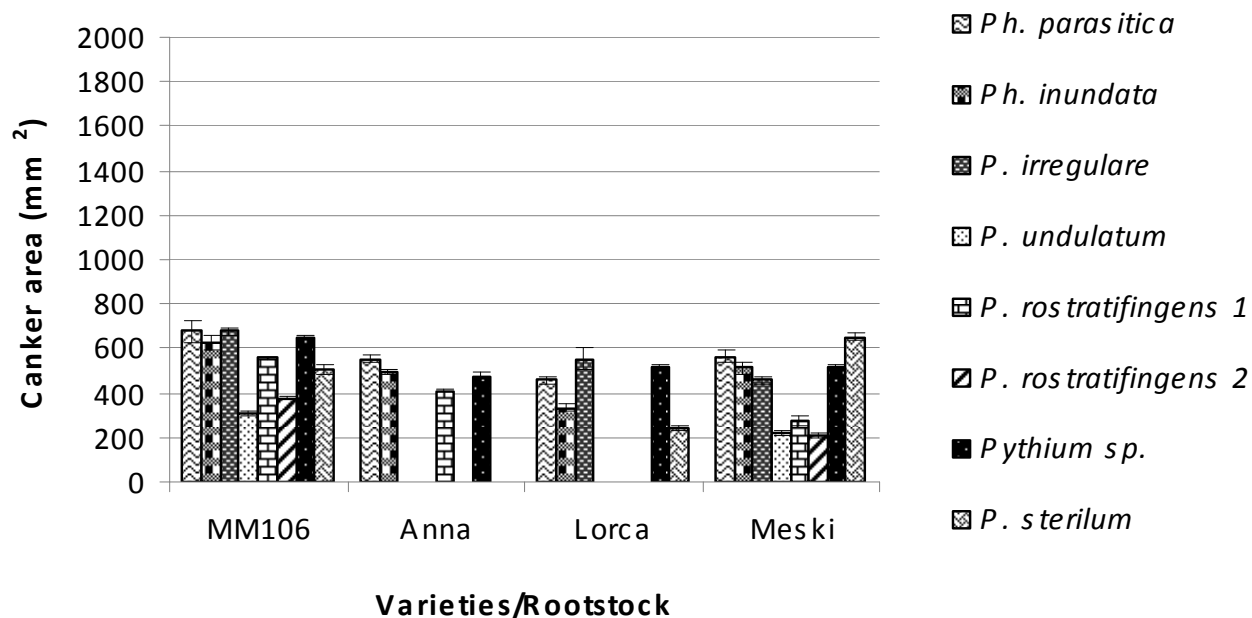


Figure 11. Canker area in 7 years old trees of apple 6 weeks after inoculation with 5 *Pythium* species and 2 *Phytophthora* species (average of six replicates). *Ph.*: *Phytophthora*; *P.*, *Pythium*.

root citrus rootstock (*Citrus aurantium* L.) by *P. nicotianae* showed a clear effect of sodium chloride on disease resistance of the rootstock.

Apple tree dieback has been attributed in many countries such as United States, Canada and Argentina to *Phytophthora* spp., mainly *P. cactorum* (Bolay, 1992a). In Washington State of the USA, apple tree dieback has been mainly attributed to species of the genus *Pythium* spp.: *Pythium* sp., *P. rostratifingens*, *P. indigoferae*, *P. irregulare*, *P. undulatum* and *P. sterilum*. Two *Phytophthora* spp.: *P. nicotianae* and *P. inundata* have also been characterized. This morphological identification was confirmed by sequencing ITS region of rDNA. Such tools proved very useful for identification of Pythiaceus spp.

In vivo inoculation of apple twigs varieties and rootstock MM106 with the *Pythium* and *Phytophthora* spp. reproduced the disease symptoms observed in the orchards, mainly collar rot symptoms. Using controlled inoculations, it was possible to complete Koch's postulates and demonstrate the association of these fungal-like species in apple tree dieback. *P. undulatum* did not show any pathogenicity towards apple trees on cultivars Anna and Lorca. The oomycetes species in the course of our study have been previously described as causal agents of apple trees dieback (Levésque et al., 2004; Mazzola et al., 2002). *P. irregulare* and *P. rostratum* have been shown to be pathogenic on apples (Mazzola et al., 2002). Lévesque et al. (2004) renamed the isolate of *P. rostratum* used in this study as *P. rostratifingens*. The classification of oomycete isolates and species according

to their virulence differs according to the apple cultivar used. Nevertheless, the most aggressive isolates on the majority of apple cultivars are *Pythium* sp.1 and *Ph. nicotianae*

Conflict of Interests

The author(s) have not declared any conflict of interests.

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