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Diversity of the *Pythium* community infecting crown and roots apple in Tunisia

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The genus *Pythium* is important in agriculture, since it contains many plant pathogenic species. Little is known about the diversity of *Pythium* species causing apple dicline. Therefore, the aim of the study was to characterize 21 *Pythium* isolates collected from root and collar rot apple trees in Tunisia from 2006 through to 2009. The isolates were characterized morphologically as well as through sequence analyze of the internal transcribed spacer region (ITS). Three *Pythium* species were identified in this study *P. rostratifingens, P. undulatum* and *P. sterilum*. In virulence assays on excised apple twigs and in the fields, representative isolates of the different *Pythium* species isolated were pathogenic on the Anna, Lorka and Meski varieties and the MM106 rootstock. Results obtained show the great susceptibility of the MM106 rootstock to the infections for the different *Pythium* species tested.

Keywords: *Pythium*, apple, decline, varieties, rootstock, virulence, pathogenicity

Tunisia produces more than 140 mille tonnes of apple fruits, cultivated over 27 000 ha (FAO, 2005). Root and crown rot of orchard apple tree is a destructive and widespread disease in almost all areas of the world where apples are grown on a commercial basis. In recent years, several cases of apple decline have been observed in the apple producing areas in Tunisia namely, Kasserine, Bizerte, Jendouba, Béja and Ben Arous. This disease caused the loss of many trees in many orchards (Boughalleb et al., 2006).

The decline of apple trees is a serious problem, which could be attributed to physiological and/or parasitic factors. In the world, many studies have shown the importance of Pythiaceae as agents of decline of apple trees (Jeffers and Aldwinckle, 1988; Bolay, 1992; Lattore and Rioja, 2001, Mazzola et al., 2002). Tsao (1990) found that many species Pythiaceae are not yet identified as parasites on many host plants and in large parts of the globe. In addition, several types of root and collar rots are attributed to attack by other microorganisms or abiotic factors.

Pythium species are common inhabitants of agricultural soils and many crop plants being susceptible to multiple species (Van der Plaats-Niterink, 1981). Although *Pythium* spp. could be responsible

of root rot of apple, little is known as to which species play a significant role as pathogens of apple. *Pythium irregulare* (Braun, 1995) and *P. sylvaticum* (Mazzola, 1998) were shown to be highly virulent toward apple. More recently, Mazzola *et al*., (2002) reported that the commonly isolated species included *P. intermedium*, *P. irregulare*, *P. heterothallicum*, *P. sylvaticum* and *P. vexans*. These species revealed to be virulent on apple plants in Washington state.

The knowledge of the breadth of interactions and the species associated with apple may provide opportunities for the management and control of pathogenic *Pythium* spp. The objectives of this study were to describe the composition of *Pythium* spp. populations associated with apple trees based on morphological and molecular tools and to determine the relative virulence toward apple plants.

Materials and methods

Orchards study sites and samples collection: Soil and root samples were obtained in 2007-2009 from 23 apple orchards located in five areas in Tunisia (Kasserine, Bizerte, Jendouba, Béja and Ben Arous) (Table 1). To diagnose, remove soil around the crown and roots of declining or dead trees and scrape the bark away along the trunk at the base of the tree and roots. At least five trees in each orchard were sampled and root, crown and soil samples were collected.

Isolation of *Pythium* spp. from apple roots and determination of soil populations: At least twenty pieces (3 to 5 mm in length) of crown or root tissues, selected at the margin of canker lesions, were placed in 1.7 % corn meal agar (CMA) amended with (per liter) 10µg pimaricin, 200µg ampicillin, 10µg rifampicin, 25µg pentachloronitrobenzene and 10µg benomyl (Jeffers and Martin, 1986). Cultures were incubated for 4 to 5 days at 24°C in darkness. Isolates were cultivated on V8 agar (200 mL V8 juice, 2 g CaCO3 and 15 g agar in 800 mL distilled water) at 24°C in the dark to study the morphological features.

Soil samples were collected around the roots or under the dripline of diseased apple trees, 10 to 20 cm beneath the soil surface. The method of Hendrix and Campbell (1970) was used to isolate Pythiaceous from soil. Thus, for each orchard, soil samples were mixed and a small portion placed into three wells cut into an apple. Fruit that developed lesions were cutting and five pieces of tissue from the margin of each necrosis were transferred to PARB medium. Plates were incubated for 4-5 days at 24°C in the darkness. Emerging *Pythium* colonies were purified onto potato dextrose agar (PDA) for colony pattern description and V8 juice agar for identification.

The formation of asexual and sexuel structure were stimulated by growing the mycelium, taken from the margin of V8 cultures, on 10 ml of sterile soil extract. The soil extract was a chemically undefined medium prepared by stirring 100 g of sandy soil in 900 mL distiller water which was exposed to daylight (day light was approx. 24 h) and then 50 mL was retired and added to 950 mL distiller water and finally autoclaved for 20 min at 120° C. The dimensions of oogonia, oospores, chlamydospores and sporangia were measured. For all studied characteristics, 25 measurements were made for each isolate, and the average value was calculated. The maximum, the optimal and the minimum temperature were determinate by incubating the colonies at different temperatures (5 °C to 40°C) with intervals of 5°C.

DNA extraction, PCR amplification, and sequencing: DNA was isolated from 10 days-old cultures of isolates grown in PDA medium. The DNA extraction was carried out according to a protocol of a Commercial Kit (EZNA, Omega Bio-tek-USA). The mycelium was ground in liquid nitrogen

with a mortar and pestle. The DNA extraction was carried out according to a protocol of a Commercial Kit (EZNA, Omega Bio-tek USA) and amplification was performed in a volume of 25 µl reaction tube containing 14.8 µl H2O, 2.5 µl of 10x Buffer, 2 µl of 10 mM désoxyribonucléoside triphosphate (dNTPs), 1 µl of each primers (ITS4 and ITS6, 10 mM), 5 units / µl DNA polymerase (Taq polymerase) and 2.5 µl MgCl2 (25 mM). The products obtained after PCR amplification were purified by centrifugation.

The PCR product ITS6-ITS4 was sequenced by PCR in Technical Analysis of the Institute of Molecular and Cellular Biology of Plants at the Polytechnic University of Valencia (Spain) through fluorochromes ABI (Applied Biosystems, Forster City, Ca). The sequencing was performed by using the Tag Dye Deoxy terminator cycle sequencing kit 3.1. The PCR products purified ITS6-ITS4 were used as matrix by direct PCR sequencing of both ITS regions indicated below.

Bioinformatics Sequence Analysis: The species identity of isolates was determined by first conducting a BLAST analysis for each sequence. The results of the BLAST N 2.2.17 analyses were used to select the sequence/s to which isolate had the highest sequence similarity in the GenBank, EMBL, DDBJ and PDB. The sequence of each isolate was aligned using Clustal X sequence alignement program.

Identification of isolates: Identification of *Pythium* spp. isolated from the field was based on the descriptions and keys of Water-house (1968), Van der Plaats-Niterink (1981), Mugnier and Gosjean (1995) and Lévesque and De Cock (2004). Isolates were identified on the basis of colony morphology, cardinal growth temperatures, and the production, morphology of sporangia, oogonia, and antheridia. Isolates which produced no sexual structures were identified on the basis of the analysis internal transcribed spacer regions of ribosomal DNA.

Pathogenicity test: For pathogenicity tests, two inoculation methods were adopted. For all experiments, the MM106 rootstock, and the apple varieties Anna, Lorka and Meski were used as host material. The pathogenicity test was carried for 3 *Pythium* species, *in vitro* and *in vivo*, namely *P. undulatum, P. sterilum* and *P. rostratifingens.* These isolates were obtained from crown, roots and rhizosphere of declining apple trees.

Inoculation *in vitro*: One year old woody shoots were collected from 6 years-old apple trees and stem of one-year-old MM106 rootstock. The methodology adopted is that described by Kröber and Karnatz in 1979. It is to take portions of 10 cm length branches and 1.5 cm diameter. Before inoculation, the cuttings were disinfected with alcohol 70%. Segments were then rinsed in sterile water. The bark from the basal of each twig was removed on T to expose the cambium. Thereafter, the inocula, consisting of 6 mm diameter Plugs from 6 days-old cultures on PDA, were inserted directly on the cambium of the twig. Then the inoculated site was covered with a strip of Parafilm to protect it from infection and desiccation. Thirty-six cuttings of each block is planted in sterile vermiculite container and incubated for 7 days in darkness at 24°C. Disease severity was determined by measuring the area of lesion that developed around the inoculation sites.

Branch inoculation: Field inoculations were made on two branches of 6 years-old apple trees. Three trees were used for each tested isolate, arranged in a completely randomized design. Branches of tree were wounded by removing a 6 mm disc of bark to expose the cambium. The inocula, consisting of 6 mm diameter Plugs from 6 days-old cultures on PDA, were inserted directly on the cambium of the trees. The wound were covered with a drop of sterile

water and wrapped with adhesive tape to prevent desiccation. Three additional trees served as control and were treated with a sterile plug of PDA. Six weeks after inoculation the lesion area was measured. Inoculations of MM106 rootstock were made on the stem of one-year-old trees in nursery, 15 cm above the soil surface. Three plants for each isolate were inoculated following the procedure described above. Isolations onto PARB were made to confirm that lesions were caused by the inoculated plant pathogens. Analysis of variance (ANOVA) was used to study the effects of cultivars, isolates and their interaction on canker areas.

Results

Crown rot occurs when the infection is below the soil line, and typically affects the rootstock. However, collar rot appears above the soil line on the lower trunk of the scion. In both cases, foliar symptoms were indicative of root or vascular dysfunction. Affected trees exhibited poor terminal growth and became stunted. Upon removal of the periderm, the inner phloem tissue was typically necrotic and orange- to red-brown, and could be dark brown during latter stages of infection (Fig. 1). Infected trees with either of these diseases usually decline over several seasons and eventually die (Fig. 2). Orange to dark reddish brown canker or streaks along the cambium of the collar or crown at ground level or just under the epidermis of the roots; the canker is often limited by a dark or black margin separating it from the white healthy tissue.

Fig. 1. Discolored crown tissue caused by *Pythium* genus.

Fig 2. Rapid collapse of apple trees caused by *Pythium* sp.

Table 1. Morphological characteristics of species identified Pythiacées

Species	Colony patterns (PDA)	Hyphae	Sporangia			Oogonia diameter (µm)	Oospore diameter (µm)	Antheridia	Hyphal swellings	Chlamydo-spores	Cardinale temperature (°C)		
			Form	Prolifera-tion	Persis-tant						T min	T op	T max
P. rost-ratifingens	*Rosette*	*Homo-thallic*	*Spherical*	*absent*	*yes*	*Globose (13.2± 1.27)*	*Plerotic (11.9 ± 1.9)*	*Monoclinous Hypogynous*	*absent*	*absent*	*05-janv*	*16-23*	*35*
P. undilatum	*Rosette*	*Hetero-thallic*	*Irregular*	*absent*	*yes*	-	-	-	*absent*	*globose*	*07-mai*	*26-20-25*	*35-40*
P. sterilum	*Petaloid*	*Hetero thallic*	*Globose*	*absent*	-	*absent*	*absent*	*absent*	*absent*	*absent*	*8*	*23-27*	*33*

Morphological and molecular identification of Pythiaceae species isolated from apple: The morphological feature was recorded for homothallic *Pythium* spp. isolates were presented in Table 1. However, the identification of the heterothallic *Pythium* species was based on the molecular identification given in the absence of reference strains for complementation assays.

Twenty one *Pythium* isolates were identified belonging to three *Pythium* species which are *P. rostratifingens, P. sterilum* and *P. undulatum*. The ITS sequences of isolates obtained from collar, roots trees and from soil was compiled for use in a bioinformatics analysis. Alignment was focused on the ITS sequences of our isolates with other isolates extracted from the gene bank to locate our available cash between them and in relation to other species in the world, at the same time. It is an alignment 'locally' using the 'ClustalX. The selection of *Pythium* isolates extracts from the gene bank is based on the results of alignment 'online' obtained by the 'Blastn 2.2.17'.

Table 2. Canker area in 1-year old shoots of apple and MM106 rootstock 7 days after inoculation *in vitro* with three *Pythium* species (Average of nine replicates).

Rootstock / varieties	Canker area (10^{-6} m^2)		
	P. undulatum	*P. rostratifingens*	*P. sterilum*
MM106	868.78±10.5	725.22±39.4	1464.78±58.7
Anna	0	370.22±16.8	299.22±15.7
Lorca	0	0	893.89±95.81
Meski	363.89±44.2	172.22±21.2	743.89±20.7

Table 3. Canker area in 6-year old trees of apple and 1-year old MM106 rootstock 6 weeks after inoculation in field with three *Pythium* species (average of six replicates).

Rootstock / varieties	Canker area (10^{-6} m^2)		
	P. undulatum	*P. rostratifingens*	*P. sterilum*
MM106	653.3±18.2	558.0±4.5	620.0±14.4
Anna	237.3±11.0	273.3±5.8	248.0±6.8
Lorka	0	0	237.3±11.0
Meski	506.3±15.6	408.0±25.2	356.0±16.3

Pathogenicity tests: The results of *in vitro* and *in situ* inoculation of the majority of isolates tested reproduced typical symptoms observed in the orchards on the diseased apple trees. The results revealed that MM106 rootstock is the most sensitive for all isolates; and showed necrotic areas with the highest response to in vitro and in situ inoculations. However, Anna and Lorka varieties seemed more resistant to the majority of Pythiaceae species (Tables 1 and 2). The classification of the virulence of the isolates in ascending order differs significantly depending on the variety of apple tested. *Pythium sterilum* isolates were the most virulent on the three tested varieties with canker area values ranging from 299.22±15.7 (for Anna) to 1464.78±58.7 mm^2 (for MM106). *Pythium rostratifingens* seemed to be the less virulent causing 725.22±39.4 mm^2 as area of canker developed on 1-year old shoots of apple

and evaluated 7 days after inoculation (Table 1). These results were confirmed by those obtained when we noted the canker are 6 weeks after inoculation (Table 2).

Discussion

The apple decline was attributed in various countries like the United States, Canada, British Columbia and Argentina to *Ph. cactorum* species (Bolay, 1992). In the present study, we characterised three *Pythium* species, *P. rostratifingens, P. undulatum* and *P. sterilum* based on the morphological identification and the sequencing method using the variability of ITS sequences regions of isolates ribosomal genes. Our results confirmed those of Mazzola et al., (2002) who mentioned that in Washington, the apple decline is attributed to attack by *Pythium* species.

P. sterilum fails to form any sexuel organs. This space was isolated from Poland, Spain and France by Belbahri et al. 2006. Its clusters within clade K (McLeod et al. 2009). *In vitro* and *in vivo* branches inoculation of apple varieties and the MM106 rootstock by *Pythium* speices isolated from apple plants could reproduce necrosis symptoms observed in the orchards, at the collar of died trees. The inoculation results showed that the species tested were pathogenic. Only *P. undulatum*, appeared non-pathogenic *in vitro and in vivo*, and did not cause any symptom on the shoots of varieties Anna and Lorka. The *Pythium* species virulence have been shown by other work. Indeed, Mazzola et al. (2002) demonstrated the pathogenicity of *P. irregulare* and *P. rostratum* on apple trees. This last space was revised by Lévesque and De Cock (2004) who had renamed as *P. rostratifingens*. Furthermore, Weber (2004) showed the aggressiveness of *P. undulatum* on roots of *Pinus halepensis*. In addition, Shafizadeh (2005) has also shown the pathogenicity of *P. undulatum* on Aleppo Pine in Ireland. The classification of the isolates virulence in ascending order differs significantly depending on the variety of apple tested. However, we noted that the most aggressive isolates on most apple varieties are *P. sterilum* and the less virulent isolate is *P. undulatum*.

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