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ORIGINAL ARTICLE

Phytophthora nicotianae and *P. cryptogea* causing gummosis of citrus crops in Tunisia

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Abstract Surveys were conducted in 2013/2014 across seven Tunisian citrus production areas for the identification of pathogens associated with citrus gummosis. Samples were analyzed using PARP-BH selective media. Collected isolates were identified based on morphology and DNA sequences of the internal transcribed spacer (ITS) region analysis. Ten isolates of *P. nicotianae* and five isolates of *P. cryptogea* were obtained. In vitro inoculation of five citrus varieties (Clementine Hernandina, Mandarins Tangerine, Blood Orange Maltese, Valencia Late and Thomson Navel) showed that *P. nicotianae* were pathogenic to cuttings twigs and *P. cryptogea* were pathogenic to leaves. In the case of *P. nicotianae*, the most susceptible variety was C. hernandina, while the most tolerant variety was M. tangerine. For *P. cryptogea* the most susceptible variety was C. Hernandina, while the most tolerant variety was V. late. In greenhouse assays, inoculation of trunks of C. Hernandina with *P. nicotianae* and *P. cryptogea* inocula induced the appearance of necrotic area and gum-exudation when inoculated with *P. nicotianae*. However, inoculation of rootstocks Sour orange with the same isolates induced root necrosis and leaf wilt. This is the first report of *P. cryptogea* causing gummosis in citrus orchards in Tunisia.

Keywords Citrus · Gummosis · *P. nicotianae* · *P. cryptogea*

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Introduction

Citrus is one of the most important group of fruit crops worldwide, raking first in the international fruit trade in terms of value (Cacciola and Magnano Di San Lio 2008). This sector accounts for the production of about 100 million tons with massive area of cultivation spread over 7.2 million hectares (Savita and Avinash 2012). In Tunisia, commercial citrus crop expanded considerbly more recently, being now grown on over 22,000 ha - a 26% growh from 1999 to 2014 (CTA 2014). Citrus is susceptible to soil-borne pathogens which cause serious losses to the crop. Gummosis, caused by *Phytophthora* spp., is one of the most economically important diseases for citrus worldwide (Erwin and Ribeiro 1996; Timmer et al. 2000; Naqvi 2004; Verniere et al. 2004; Mounde et al. 2009; Jagtap et al. 2012; Faldoni et al. 2015). In recent years, gummosis symptoms have been observed in the most grown areas of citrus in Tunisia and became a serious problem for citrus production (Benfradj et al. 2017). The most susceptible variety to gummosis in Tunisia are 'C. hernandina' (Benfradj et al. 2017). The causal agents of this disease are able to survive in soil for long periods as chlamydospores and oospores (Bumbieris 1979). In previous studies, ten *Phytophthora* spp. have been reported to infect citrus around the world. Two species, *Phytophthora citrophthora* and *Phytophthora nicotianae*, are most damaging to roots, trunk and branches (gummosis) and fruits, both before and after harvest of trees (Erwin and Ribeiro 1996). In South-Africa, *P. nicotianae* is one of the most commonly species isolated from affected citrus orchards (Maseko and Coutinho 2002), whereas *P. citrophthora* had a limited occurrence in that region, likely due to local climatic conditions (Ricci et al. 1990). Furthermore, *Phytophthora cryptogea* was also shown to be one of the main pathogens affecting the roots (Benigni and Bompeix 2006) an causing yield losses that ranged from 10%

to 50% (Erwin and Ribeiro 1996; Garibaldi et al. 2000; Ptaszek et al. 2009). The pathogen is easily recovered from irrigation water and from soil (Garibaldi et al. 2003; Stanghellini and Rasmussen 1994). The aims of this study were to 1) identify the agents responsible for citrus gummosis in Tunisia and 2) assess their pathogenicity and incidence.

Materials and methods

Disease surveys and sampling

Surveys were conducted from october 2013 to December 2014 across seven areas of citrus production, Northen Tunisia (Menzel bouzalfa, Benikhaled, Soliman, Bouargoub, Takilsa, Grombalia and Hawaria) (Fig. 1). From each orchard, information on the citrus variety, plant age, the type of irrigation and the type of rootstock were recorded. Diseased trees were firstly examined for gummosis disease symptoms at the lower part of the trunk. A citrus tree was considered infected when symptoms of internal necrosis in tissue of the trunk with or without the presence of a gum-exudation was noticed. At least five diseased trees were sampled from each orchard. From each infected tree, samples were taken from the trunk and the soil with roots of symptomatic trees. The sampling varied according to the type of irrigation (flood or drip system). In flood irrigated orchards, sampling from the trunk was carried out according to the degree of tree's infestation by gummosis. However, in the mildly infested orchards, these samples were taken from the trunks on the line in the direction of the water stream. The soil samples were collected under the canopy of the infected trees to a depth of 15–20 cm. In heavily infested orchards, samples are collected randomly and soil samples were taken in the same way as for the low degree of infestation orchards by gummosis. In drip irrigated orchards, the soil sampling areas located directly under the four emitters arranged in paired rows on either side of the shaft. A total of 30 samples from trunk and 30 samples of soil included roots were transferred to the laboratory in plastic bags for further analysis. Disease incidence per variety was calculated as the percentage of infected trees over the total number of trees per variety.

Isolation of *Phytophthora* species from infected trees and from soil

Trunk samples were washed under running tap water to remove any adhering soil particles and excess water was removed by placed them between steril absorbent filter paper. Then, surface of these samples has been sterilized by ethanol (70%). After, each samples were cut from the edge of the lesions to pieces (about 2–5 mm wide), and when dried, these were plated onto 90 mm Petri dish, containing PARP-BH selective medium (CMA amended with Pimaricin, Ampicilin, Rifampicin, Benomyl, Pentachloronitrobenzene and Hymexazol) as described by Jeffers and Martin (1986). The plates were then incubated at 25 ± 1 °C in the dark and examined within 2–3 days. Pure cultures were obtained by sub-culturing hyphal tips onto potato dextrose agar (PDA) (DIFCO). Samples of soil and roots material were baited using

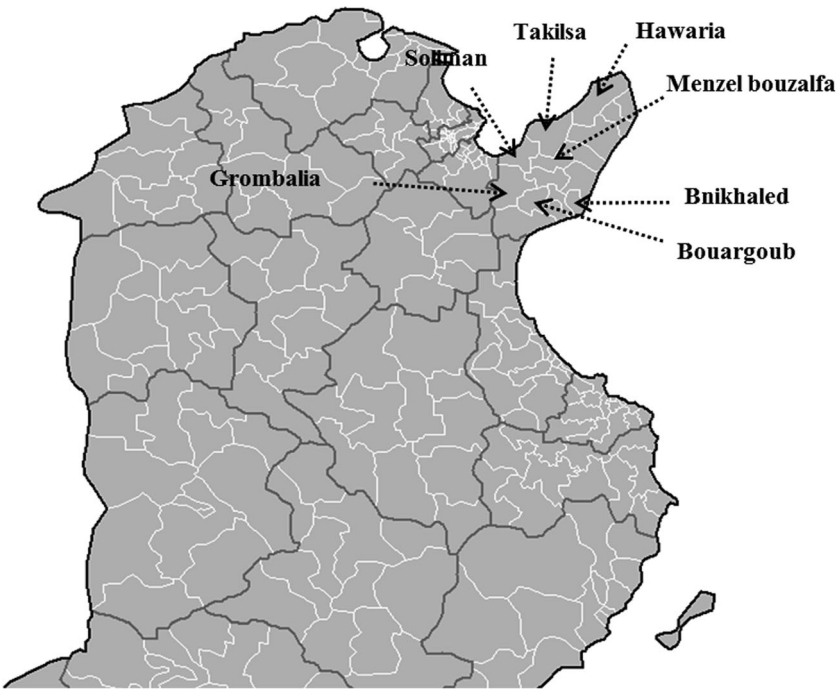

Fig. 1 Citrus growing regions in Tunisia surveyed for gummosis

the technique described by Hendrix and Campbell (1970) for *Phytophthora* isolation. Apples *cv* 'Golden delicious' were used as baits and three holes per fruit and 2 fruits per sample were used. Holes were then filled with sampled soil and incubated at room temperature. After three days, small pieces of tissue were aseptically removed from the inoculated apples fruits at the junction of the healthy and necrotic tissue and placed on PAR-PBH medium. Petri dishes were incubated at 25 ± 1 °C in the dark and checked daily for colony growth. Mycelia resembling to *Phytophthora* species were transferred to PDA plates and the purification was made by the hyphal tip method. Pure mycelium was then placed onto Petri dishes containing V_8 medium (200 ml V_8 juice, 2 g $CaCO_3$ and 15 g agar in 800 ml of distilled water) at 25 ± 1 °C in the dark for morphological features study and stored in 1.5 ml test tubes of sterile soil solution.

Morphological identification

Phytophthora spp. identification was based on the morphology of the colony, mycelial characteristics, cardinal growth temperatures, sporangia, oogonia and antheridia morphology (Erwin and Ribeiro 1996). For this, a 5-mm-diameter of mycelial plug of each isolate was transferred upside down in the center of PDA and on V_8 medium dishes and incubated at 25 °C for 7 days in the dark. For temperature growth studies, a 4-mm-diameter of mycelial plug of each isolate was transferred onto V_8 medium plates and incubated at 5, 10, 15, 20, 25, 30, 35 and 40 °C, using three replicates per isolate. Diameter of colonies was measured after 2, 4 and 6 days, following the onset of linear growth along two lines intersecting the center of the inoculum at right angles (Jung et al. 2000). Sporangia formation was induced by flooding cultures with sterilized soil extract as described below. Sporangia were obtained from 5-mm-agar squares in the advancing margin of a colony grown on V_8 of 5-days-old colonies and floating them with enough sterile soil extract in Petri dishes and incubated under fluorescent light at room temperature. The sterile soil extract was made by mixing 100 g of soil with 1 L of deionized water. Soil extract water was left to settle overnight after which the water was filtered through cheesecloth followed by filter paper. Then 50 ml of the supernatant was taken and added to 950 ml of distilled water and sterilized. Sporangial morphology was examined under a compound Leica microscope (DM 2500) and the shape, size, presence or absence of papilla, proliferation and sporangiophore branching were recorded. The description of sporangial shape, measurement of length and breadth of each isolate were carried out and their length to breadth (l/b) ratios calculated. For all characteristics traits, 25 measurements were taken, per each isolate, and the average value was calculated. The heterothallic species were paired with A1 and A2 tester strains of *P. nicotianae* kindly provided by Pr. P. Abad-Campos (Universidad Politécnica de Valencia). Production of sex organs was cheeked from 2 weeks after the incubation at 24 °C in the dark.

DNA extraction, amplification and sequencing

Isolates obtained has been grouped according to their morphology, and from each group a representative isolate has been used from molecular identification. These representatives isolates were grown on PDA medium covered with sterile cellophane disc and incubated at 25 °C for 5 days. Then, mycelium was scraped from the agar surface and freeze-dried for extended storage at −20 °C. Thereafter, between 50 and 100 mg of mycelia was placed into 2 ml eppendorf tube and the total DNA was extracted, using the kit 'EZNA Plant DNA Kit' (Omega Bio-tek) according to the manufacture's protocol. PCR amplification was carried out using universal primers (ITS1–ITS4) designed on the Internal Transcribed Spacer (ITS) region (Cooke et al. 2000). Polymerase chain reaction was carried out in twenty five microliters of reaction mixture containing: 2.5 μl PCR buffer (10×), 3 μl $MgCl_2$ (2.5 nM), 0.5 μM each dNTP(10 μM), 2.5 μl of ITS4 and ITS1 (10 μM) primers, 5 μl of template DNA, 0.5 μl of Taq polymerase (Genespin) and 11 μl H_2O. PCR amplification was performed with an initial denaturation of 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 40 s at 72 °C with a final elongation step for 10 min at 72 °C. The PCR amplification products were separated by electrophoresis in 1% agarose gel (Genespin) in a 1X TBE (Tris-boric acid-EDTA) and stained with 0.4% ethidium bromide and visualized under UV light. PCR products were purified by using 'mi-PCR Purification kit' (Metabion) and sequenced at Macrogen. The rDNA-ITS sequence of isolates were compared with known sequences of *Phytophthora* spp. obtained from the GenBank (http://www.ncbi.nlm.nih.gov), using the BLAST (Basic Local Alignment Search Tool) program (Table 1). A dendrogram was constructed after cluster analysis of the similarity coefficients by means of the unweighted pair-group method (UPGMA) using MEGA.5 software.

Pathogenicity tests

Pathogenicity tests of all *Phytophthora* isolates collected were conducted in two separate assays (in-vitro and in-vivo inoculations) using mycelium and zoospores as sources of inoculum.

In vitro shoot inoculation

Five citrus varieties (Clementine hernandina, Mandarins tangerine, Blood orange maltese, Valencia late and Thomson navel) collected from citrus orchards of The Technical

Table 1 Species selected from Genbank included in this study showing accession numbers isolate

Species	Strain number	Origin	Host
P. nicotianae	KT148938	Italy	*Citrus*
	KC748453	Australia	*Urban trees*
	KT148942	Italy	*Citrus*
	AB688390	Japan	*Southern cross*
	JF326834	Brazil	*Dracaena sanderiana*
	AY880996	Switzerland	*Salvia officinalis*
	KJ494891	China	*Nicotiana tabacum*
	KT148947	Italy	*Citrus*
	GU111676	Taiwan	*Nicotiana tabacum*
	JX978446	China	*pineapple*
	KF010303	India	*Citrus*
	AF228085	Korea	*Epiphyllum trucatum*
	LT628537	Saudi Arabia	*Washingtonia robusta*
	FN430681	ITALY	*Strelitzia*
	JX978447	China	*Pineapple*
	KC768775	USA	*Catharanthus roseus*
	LN846119	SPAIN	*Persea americana*
	JN135291	Cuba	*Persea americana Mill.*
	HQ287571	Italy	*Chamaerops humilis*
	KR827692	USA	*Dicerandra immaculata*
	HQ711620	New Mexico	*Tomato*
P.cryptogea	EU000096	Switzerland	*Dianthus caryophyllus*
	JN836991	Italy	*Cupressus sempervirens*
	AF087476	Korea	*Gerbera jamesoni*
	JQ071495	Turkey	*Potato*
	KJ744315	Germany	*sugar beet*
	KP070715	Australia	*Gondwana Rainforests*
	KP070717	Australia	*Gondwana Rainforests*
	KP070718	Australia	*Gondwana Rainforests*
	KP070719	Australia	*Gondwana Rainforests*
	AF228099	Korea	*Aster sp.*
	GU111631	Taiwan	*Euphorbia pulcherrima*
	AY995400	USA	*pine*
	KF234764	Serbia	*Quercus petraea*
	HQ697246	Poland	*ornamental container*

Center of Organic Agriculture in Tunisia (CTAB) were chosen for the inoculation test. Isolates were inoculated into one-year-old citrus plants shoots (10 cm × 1 cm) (Kröber and Karnatz 1979). Shoots were cut from fresh branches of citrus trees rinsed in sterile water, and disinfected with ethanol (70%). The bark in the middle of each shoot was removed. Mycelium (4 mm in diameter) plugs obtained from each isolate (7 days-old), were placed onto the wounded area, with the mycelium facing the cambium and covered by Parafilm. Sterile agar plugs were used as a control. All isolates found had been tested and four replicates per isolate and per variety were considered. The inoculated shoots were planted equidistantly in a sterile perlite (autoclaved twice, at 120 °C, for 20 min) imbibed with sterile distilled water, and incubated in the dark at 24 °C. Two weeks after incubation, the outer bark was removed and the inner bark was examined for lesions development. Disease severity was determined by measuring the area of lesion appeared around the inoculation sites. Re-isolation was carried out on PARP-BH medium and the identity of each organism was confirmed by observing colony characteristics and sporangial morphology.

Leaf inoculation

Since some *Phytophthoras* prefers to enter the host by different tissues, isolates used and who did not showing symptoms after the twigs inoculation were used for leaves inoculation assay. Isolates were cultured on V_8 medium and incubated at 25 °C for 14 days to produce sporangia. To obtain zoospores, cultures were flooded with 10 ml of sterile water, until an abundant sporangia production occurred. Sporangia were then dislodged into the water by rubbing the surface of the culture with a sterile bent glass. The liquid was poured off the plates and collected in a sterile beaker which was placed firstly in a refrigerator at 7 °C for 1 h, then returned to room temperature for a further 75 min to induce the zoospores release (Parke et al. 2002). The resulting suspension for each isolate was filtered to remove residual pieces of mycelium, chlamydospores and empty sporangia, to obtain pure zoospores suspensions, which were transferred to sterile beakers. A 0.3 ml of each zoospore suspension was placed in a separate micro-tube and shaken on a vortex mixer (90 s) to initiate zoospore encystment. The concentration of each suspension was adjusted to 2–4 × 10^5 zoospores ml^{-1}. Thirty ml of the adjusted suspension of each isolate was dispensed into individual sterile 50 mL glass beakers and plant material was inoculated as follows. The midpoint of each leaf was marked with a permanent pen. Sixteen leaves of each variety were divided into two treatment groups: i) 'non-wounded leaves': leaves were dipped in the zoospores suspension, apical end first, up to the midway mark, for 1 min. On removal, excess liquid was allowed to drop off; and ii) wounded treatments': two 5-mm-deep V-shaped incisions were made on leaves of each variety. Then the distal end of each leaf was dipped in zoospores solution. Control leaves were dipped in sterile water.

The presence/absence of necrosis was recorded, based on visual inspection after six days. To confirm that lesions were caused by the pathogen, pieces of plant tissue per leaf were plated on PARP-BH. Infection was confirmed by calculating re-isolation percent (number of positive re-isolation out of four to six tissue pieces plated per leaf) of the pathogen.

In-vivo branch inoculation

Pathogenicity of all *Phytophthora* isolates found was tested on 18 months old citrus plants ('C. hernandina' grafted onto Sour orange rootstock), grown in 6 L plastic pots containing potting mix. The inoculation was made in a greenhouse, with three repetitions per plant and per isolate. A 4-mm circular incision was performed in the bark, so as to reach the cambium; the incised portion was then removed and replaced with a same-size plug of mycelium of each isolate of the pathogen, grown in Petri dishes on PDA, for 20 days at 25 °C in the dark. The inoculation site was then sealed with a strip of Parafilm. Five replicates of two plants, for each isolate, were tested and arranged in a complete randomized block design. Five additional trees, used as control, were treated with a sterile plug of PDA medium. The size of the infection spreading from inoculation wound was measured every 2 weeks. At the end of the trial, the bark at the inoculation point was removed and cultured onto PARP-BH medium for re-isolation. When mycelia growth occurred, its culture characteristics were recorded and the isolate was identified. The isolates with similar culture characteristics and disease symptoms on the inoculated plants to those observed originally, were identified as causals agents of citrus gummosis.

Soil infestation test

Soil infestation trials were performed from March to June 2014 in a glasshouse under natural light conditions. Three replicates of 18 months old plants of citrus 'C. hernandina', grown in plastic pots were used for each isolate. For each isolate, a mixture of 400 g of sand-oat (*V*/V) has been putted in a Erlenmeyer flask and autoclaved twice (at 120 °C, for 20 min). Ten mycelium plugs, of 7 days-old, of each isolate were placed in each Erlenmeyer and the mixture has been incubated between 4 to 5 weeks. Then this mixture was added to the substrate of each pot. The soil in the pots was kept moisted by watering when needed. Symptom development (wilting, leaf browning and defoliation) was regularly recorded each week. After 90 days from inoculation, all plants were removed from the potting bags and washed under running water to remove excess potting mix adhering to roots. For each plant, the root fresh weight (g) was recorded. All roots were then excised, weighed and the total root weight has been calculated and expressed as root disease severity. A re isolation has been made from these roots to confirm the presence of pathogens.

Statistical analyses

Morphometric and pathogenicity data were analysed by a one-way analysis of variance (ANOVA) using S-N-K test (Student–Newman–Keuls) as a post-hoc test, emploing Statistical Package for Social Sciences (SPSS) computer software (version 18.0). The percentage data were transformed to angles.

Results

Disease symptoms and affected variety

Conducted surveys have shown that the disease was prevalent in all the orchards with different magnitude of infection. In the same orchards, infected trees exhibited reduced vigor in comparison to healthy ones grown. Mature trees showed symptoms of decline, including leaf chlorosis, dieback of twigs, small and poor colored fruit, off spring fruit production, tip wilting twig dieback and withering of leaves during periods of drought, if the infection affects more than 50% of the circumference of the trunk. Symptoms of the disease usually appear above the soil line on the butt of the plant as narrow bark cracks and cankers of trunks and branches, from which a pale yellow gum-exudation is produced. These symptoms expand upwards affecting the main branches and secondary branches. Necrosis appeared on taproots and crowns, and some roots decayed completely. In case of a severe infection the lesions slowly girdling the tree and died. Gum-exudation proceeds from longitudinal cracks of the bark around necrotic areas, which have a distinct water-soaked discoloration and persistent in dry conditions but disappears after heavy rains. The gum is water-soluble, but even if it is washed away by the rain, the discoloration on the cortex is visible (Fig. 2). The symptoms of gummosis have been observed in the varieties C. hernandina (73%), and T. navel (16%), B. O. maltese (10%) and in the variety M. tangerine (1%). All infected trees were grafted onto sour orange. The infected orchards were mainly watered by drip irrigation (69%), while 31% of them are watered by flood system.

Morphological and molecular characterization of isolates and their distribution

A total of 70 samples has been collected. Twenty *Phytophthora*-like isolates were obtained from trunk (30%) and from soil (70%). Based on morphological characteristics, 15 isolates were identified as *P. nicotianae*-like (75%) and five isolates were identified as *P. cryptogea* (25%). 50% of *P. nicotianae*-like isolates were collected from the region of Takilsa, followed by Bouargoub (10%), Gobba (5%), Soliman (5%) and Bnikhaled (5%). However, 15% of *P. cryptogea*-like isolates were collected from the region of Hawaria, followed by Menzel bouzalfa (5%) and Soliman (5%).

P. nicotianae isolates showed an aerial dense mycelium, immersed in the culture medium in the beginning and stoloniferous to rosaceous on PDA medium, while

Fig. 2 Symptoms of gummosis on citrus trees due to *Phytophthora* spp. infections (**a**: necrotic lesions in the trunk of the tree; **b**: Gum-exudation)

it were submerged with a faint radiation pattern and little or no aerial mycelium on V_8 medium. However, *P. cryptogea* isolates showed, both in V_8 juice agar and PDA medium, a white stellate to slightly radiate colonies and a limited aerial mycelium (Fig. 3).

Sporangia of *P. nicotianae* isolates were formed sparsely in V_8 and abundantly on PDA medium. Shapes of sporangia ranged from ellipsoid, ovoid, pyriform to spherical with a prominent papilla. Measurements of sporangia size were 28.7–54.37 μm in long × 18.67–29.02 μm of wide (average 41.53 μm × 23.84 μm), with a length-breadth ratio of 1.11 μm to 1.71 μm (average 1.35 μm). Sporangia were typically terminal but were often sub-terminal and occasionally intercalary, and weakly deciduous. Chlamydospores were also present,

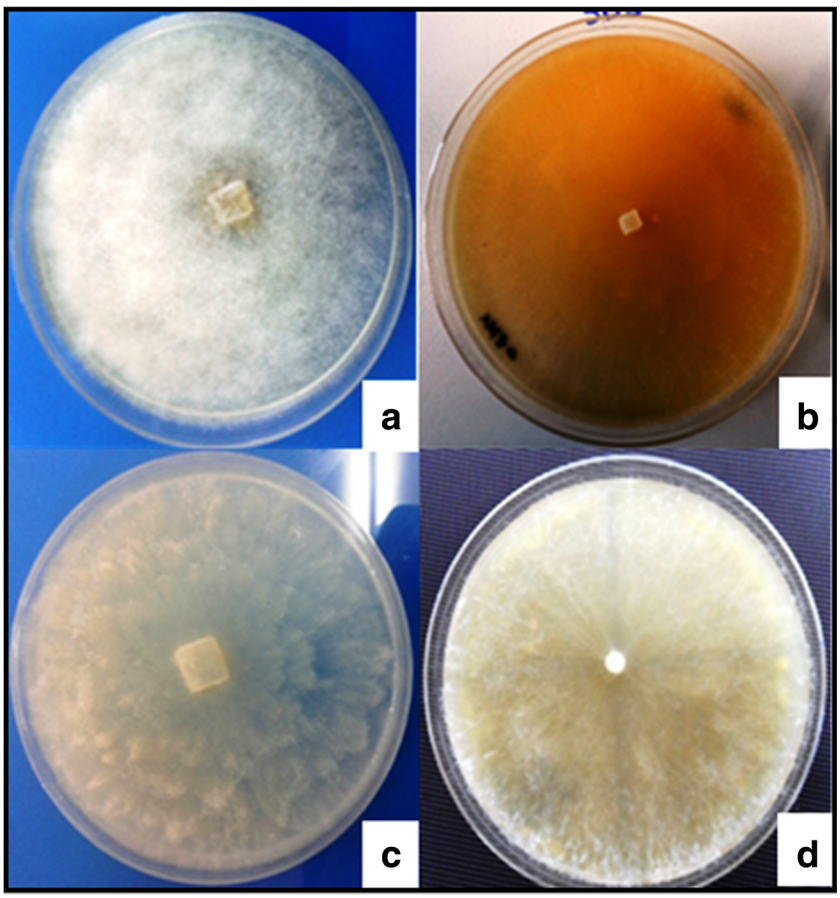

Fig. 3 Colony morphology of *Phytophthora nicotianae* (**a**, **b**) and *Phytophthora cryptogea* (**c**, **d**), respectively, on PDA and V_8 medium, after 6 days of incubation at 25 °C in the dark

and they are abundant in all isolates. They are terminal or intercalary and 17.58 μm to 68.76 μm (average 27.60 μm). Sporangiophore is sympodially branched and hyphal swellings were present. All of the 15 isolates of *P. nicotianae* that were tested for mating types formed sexual structures when paired with the opposite mating type after the 7-day incubation period. Of these, two thirds (10 isolates) were identified as mating type A1 and the remaining 5 isolates were identified as A1. They showed spherical oogonia with amphigynous antheridia. The oospores, are spherical and aplerotic with a diameter ranged from 27.1 μm to 28.6 μm. The growth rates, of *P. nicotianae* isolates, on V_8 were between 15 and 30 °C, with an optimum of 25 °C and no growth at 35 °C (Fig. 4).

The hyphal swelling of *P. cryptogea* isolates was abundant and formed when cultures were submerged in soil extract. Sporangia were not produced on solid media but were abundant in soil-extract medium. Sporangia were non-papillate with rounded bases, ovoid to obpyriform and non-caducous, internally proliferating. Measurements of sporangia size were 25.2 μm −55.5 μm in length and 18.9 μm - 35.1 μm of width (average 40.35 μm × 27 μm). The sporangiophore was generally un-branched. The growth rates of *P. cryptogea* isolates was between 5 and 33 °C, with an optimum at 25 °C.

Identification of all *Phytophthora* isolates obtained from infected citrus trees by gummosis were also confirmed by molecular analysis by rDNA ITS sequences (accessions numbers are given in Table 2). The average of rDNA ITS sequences of *P. cryptogea* isolates identified were all about 750 base pairs (bp) length. BLAST analysis revealed that the nucleotide sequences shared between 97% and 100% identity with nucleotide sequences of 14 isolates of *P. cryptogea* from Genbank .

However, the average of rDNA ITS sequences of the *P. nicotianae* isolates were all about 800 base pairs (bp) length. BLAST analysis revealed that the nucleotide sequences shared identity between 97% and 100% with 12 isolates of *P. nicotianae* from Genbank. Phylogenetic relationship, reconstructed with ITS sequences showed that

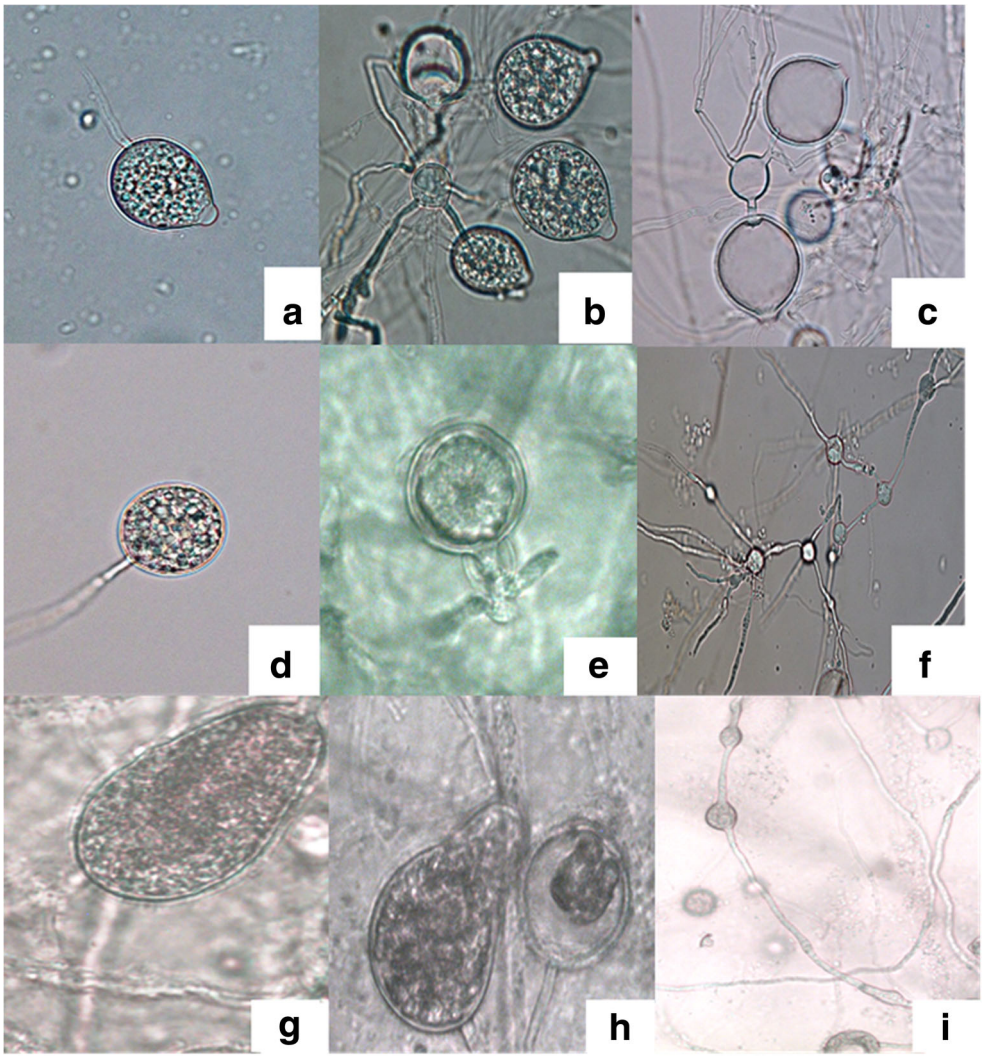

Fig. 4 Microscopic characteristics of *Phytophthora nicotianae* (**a** papillate sporangia; **b**, **c** sporangiophore; **d** Chlamydospores; **e** Representative oogonia; **F** swellings hyphae. Bars: 25 μm (**a**, **b**, **c**, **d**); 40 μm (**e**)) and *Phytophthora cryptogea* ((**g**) and **h** Non-papillate sporangia; **i**: hyphae. Bars: 25 μm)

Table 2 Isolates, strain number, origin and Genbank accession numbers of *Phytophthora* species strains isolated from citrus orchards displaying gummosis in Tunisia

Species	Isolates	Origin	Mating types	Host/habitat	GenBank accession number
P. nicotianae	P.51	Takilsa	A1	Soil	KU248805
	P.52	Takilsa	A1	Soil	KU248806
	P.53	Takilsa	A1	Soil	KU248807
	P.63	Takilsa	A1	Soil	KU248808
	P.64	Bouargoub	A1	Soil	KU248809
	P.65	Bouargoub	A1	Soil	KU248810
	P.66	Bnikhaled	A1	Crown	KU248811
	P.111	Gobba	A2	Crown	KU248812
	P.119	Soliman	A2	Soil	_
	P.122	Takilsa	A1	Soil	_
	P.124	Takilsa	A1	Crown	_
	P.125	Takilsa	A2	Crown	_
	P.126	Takilsa	A1	Crown	_
	P.127	Takilsa	A2	Soil	_
	P.128	Takilsa	A2	Soil	_
P. cryptogea	P.88	Menzel bouzalfa		Crown	_
	P.100	Hawaria		Soil	KU248813
	P.101	Hawaria		Soil	KU248814
	P.102	Hawaria		Soil	_
	P.118	Soliman		Soil	_

P. cryptogea and *P. nicotianae* occurred in two separate clusters (Fig. 5).

Pathogenicity tests

In vitro shoot inoculation

Symtoms of discolored lesions extended from the point of inoculation of the inoculated shoots of citrus has been observed. These symptoms were developed only on shoots inoculated by *P. nicotianae*, while no symptoms appeared when *P. cryptogea* isolates were used. *P. nicotianae* isolates induced brown to reddish, and rotted, necrotic lesions reached into the wood seedlings. *P. nicotianae* was more aggressive on 'C. hernandina' and 'B. O. maltese' varieties than on the other tested varieties. The widest canker area was noted on 'C. hernandina' variety (1543.21 mm^2), while the lowest one was noted on 'M.Tangerine' (912.86 mm^2) (Table 3).

In vitro leaf inoculation

Citrus leaves inoculation exhibited necrosis symptoms with no significant differences among *P. cryptogea* isolates pathogenicity. However, different level of susceptibility to *P. cryptogea* inoculations has been noted among the different varieties of citrus. In fact, the highest levels of necrosis were showed on 'C. hernandina' variety with an average of necrosis between 95% and 100%, for the non-wounded and wounded leaves, respectively, while the lowest levels were on 'V.late' variety with an average of necrosis between 30% and 45% for the non-wounded and wounded leaves, respectively (Table 4). All *P. nicotianae* and *P. cryptogea isolates* were re-isolated from the symptomatic tissues excised from the margin of the lesion of shoots and leaves of citrus varieties.

In-vivo branch inoculation

In greenhouse, branch inoculations with *P. cryptogea* isolates have not cause any disease symptom. On the other hand, Typical symptoms of citrus gummosis were developed in branches of citrus inoculated by *P. nicotianae*. *P. nicotianae* isolates were found to be pathogenic on branches of 'C.hernandina' variety. The inoculated plants showed symptoms of necrotic and lesion. Gum-exudation was observed within. Control plants remained symptomless. 15 days after inoculation, a variation in virulence among *P. nicotianae* isolates has been observed. Results obtained from the first date of evaluation (15 days after inoculation) correlated with the second date of evaluation (30 days after inoculation). The lesion length developed between 17.1 mm and 18.3 mm. After 60 days of inoculation, the lesion measured 30.8 mm and after 90 days, the canker length ranged between 50 and 50.2 mm (Table 5). No significant differences in pathogenicity were noted between the isolates of *P. nicotianae*. Koch's postulates

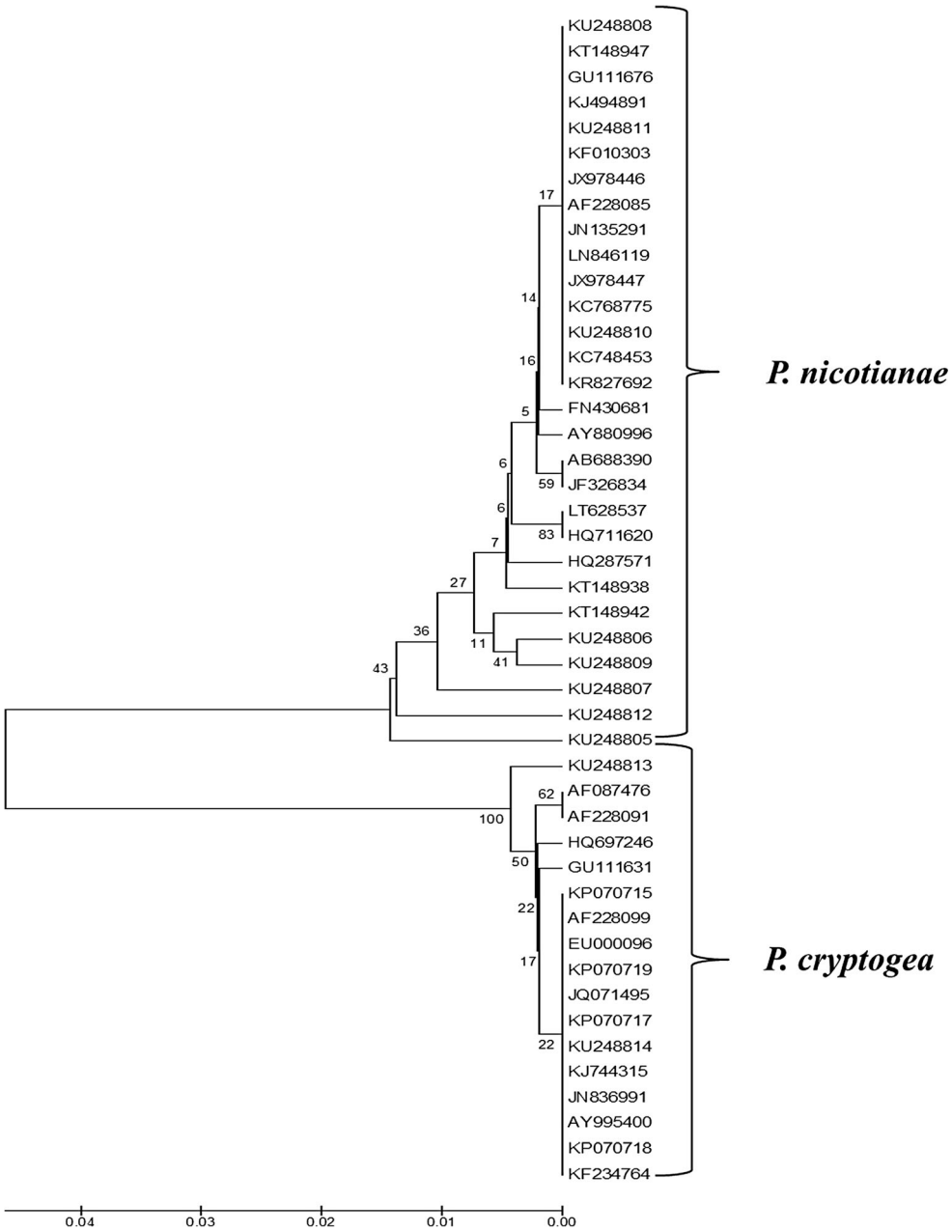

Fig. 5 Evolutionary phylogenetic relationship of *Phytophthora* species based on internal transcribed spacer (ITS) 1-5.8S-ITS2 sequence of nuclear rDNA aligned by ClustalW. Values on the branches are percentages from 1000 bootstrap replicates

Table 3 Mean canker areas and relative standard deviation in 1-year old shoots of citrus after 30 days of inoculation with *Phytophthora nicotianae* in-vitro

Citrus varieties	Canker area (mm^2)
C. Hernandina	$1544.87 \pm 17.48^{a*}$
T. Navel	1498.57 ± 35.05^{b}
B. O. Maltese	1553.85 ± 7.05^{a}
V. Late	1033.21 ± 6.68^{c}
M. Tangerine	912.78 ± 3.74^{d}

(*) means ± standard error in the column followed by the same letter are not significantly different according to S-N-K test at $P < 0.05$

Table 4 Average percent of necrotic leaves of citrus after 6 days from inoculation with *Phytophthora cryptogea* in-vitro

Varieties	Non-wounded (% +/− Standard deviation)	Wounded (% +/− Standard deviation)
C. hernandina	$95 \pm 0^{a*}$	100 ± 0^{a}
T. navel	73 ± 2.37^{b}	81 ± 1.14^{b}
B. O. maltese	70 ± 0^{c}	78 ± 1.14^{c}
V. latevalencia	30 ± 0^{d}	45 ± 0^{d}
M. tangerine	31 ± 0.7^{e}	45 ± 0^{d}

(*) means ± standard error in the column followed by the same letter are not significantly different according to S-N-K test at $P < 0.05$

Table 5 Mean lesion length in trunk of the variety 'C. hernandina' inoculated with isolates of *Phytophthora nicotianae*

Species	Isolates	15 days after inoculation (mm)	30 days after inoculation (mm)
P. nicotianae	P.51	$58 \pm 0.3^{e*}$	63 ± 0.9^{d}
	P.52	70 ± 0^{d}	85 ± 0.2^{c}
	P.53	5 ± 0.1^{f}	18 ± 0.3^{e}
	P.63	80 ± 0.8^{b}	100 ± 0^{a}
	P.64	75 ± 0.1^{c}	92 ± 1.3^{b}
	P.65	$82 \pm 0.\ 8^{a}$	100 ± 0^{a}

(*) means ± standard error in the column followed by the same letter are not significantly different according to S-N-K test at $P < 0.05$

were confirmed by re-isolating the same isolates from diseased plants.

Soil infestation

The results of citrus root inoculation by soil infestated with *P. nicotianae* and *P. cryptogea* isolates showed damaged roots with color varying from brown to completely black and alteration of root architecture changes after 90 days of inoculation. These symptoms were not found in the roots of the un-inoculated control trees. This root infection affects also the development of root system. In fact, *P. nicotianae* isolates (28,68 g) reduced the root weight of infected plants more that *P. cryptogea* isolates (22.47 g) (Table 6). Significant differences in virulence were noted between the isolates of each especie. Koch's postulates were confirmed by re-isolating the same isolates from diseased plants.

Discussion

The present investigation showed that *P. nicotianae* and *P. cryptogera* are involved in this diseases of citrus caused by *Phyytophthora* spp. in Tunisia. The phenotypic and molecular identification showed the existence of *P. nicotianae* in five citrus localities with severe gummosis symptoms. Symptoms observed during the surveys were similar to typical symptoms of gummosis described by others authors (Savita and Avinash 2012).

Morphological and molecular markers revealed also the presence of *P. cryptogea*. ITS sequences of these isolates were identic to those obtained from *P. cryptogea* infected peach trees in Chile (Ampuero et al. 2008). Isolates of *P. cryptogea* were pathogenic on leaves and on roots of citrus, re- producing the symptoms of root rot, foliar chlorosis, lack of vigor and dieback.

Both the A1 and A2 compatibility types for *P. nicotianae* were detected during this study and sometimes occurred the same location. When both mating types are present oospores may also be produced which aid survival of the fungus. Oospores form when the opposite mating types are present, which may occur infrequently in some citrus soils. Although the role of the oospore in the disease cycle is unclear, oospores may serve as an overwintering structure and increase the potential for variation in the fungus (Zltko et al. 1987). The predominance of the Al mating type of *P. nicotianae* has been noted in this study and also has been found in citrus orchards in Taiwan and Texas (Timmer 1973; Timmer et al. 1987) and Florida (Zltko et al. 1987). In-vitro, foliar symptoms appeared after inoculation with zoospores, suggesting that these *P. cryptogea* isolates were pathogen to citrus orchards in Tunisia. In greenhouse, when mycelial fragments were used as inoculum in soil plants developed symptoms. Presumably, mycelium may directly penetrate into root or crown tissues under orchard conditions. Symptoms were similar to those described for diseases caused by this pathogen in other species and include wilt, moderate leaf chlorosis, necrosis, root rot.

Table 6 Canker area and roots weights of 18 months old shoots of citrus variety after inoculation in-vivo with *Phytophthora* spp. (average of five replicates)

P. nicotianae			*P. cryptogea*
Canker area(mm^2)		Fresh root weight (g)	Dry root weight (g)
30 days after inoculation	$17.68 \pm 0.48^{c*}$	28.63 ± 2.17^{a}	22.47 ± 6.17^{b}
60 days after inoculation	30.61 ± 0.51^{b}		
90 days after inoculation	50.05 ± 0.97^{a}		

(*) means ± standard error in the column followed by the same letter are not significantly different according to S-N-K test at $P < 0.05$

P. cryptogea has a host range that includes several trees (Strouts et al. 1985; Erwin and Ribeiro 1996), but as far as we know, it has not been reported as a pathogen on citrus trees. This oomycota was first described in 1919 (Pethybridge and Lafferty 1919) and has since then been reported from several areas around the world, as a pathogen on roots of several cultivated plants (Stamps 1978). In Tunisia, all infested trees observed, were grafted onto sour orange rootstock. This rootstock is widely used in this region, and it has previously been considered tolerant to the disease, especially to root rot caused by *P. nicotianae*, as affirmed by other author (Graham 1995). However, our survey revealed severe attacks on citrus orchards of trees grafted on this rootstock. Thus, the introduction of resistant varieties and rootstocks to gummosis should be enhanced through germplasm exchange and selection programs to avoid further degeneration of the citrus plantations in Tunisia. Recently, the rootstocks 'Citrumelo Swingle-4475' was shown to be tolerant to *P. nicotianae* in Tunisia (Benfradj et al. 2016).

The presence and aggressiveness of *P. nicotianae* may be due to the interactions between the pathogen and other biotic and abiotic factors. The regions are characterized by soils with high salinity level (Trabelsi et al. 2005). This factor could affect the behavior of the rootstock by stressing it, especially because there is a positive correlation between salinity level and *Phytophthora* infections severity (Blaker and MacDonld 1986). Other research mentioned that salinity increased the severity of root rot caused by *P. nicotianae*, when troyer citrange was used as rootstock (Benyahia et al. 2004). Therefore, the emergence of *P. nicotianae* pathogen in Tunisian citrus growing areas could be related to an increasing soil salinity. The rootstock resistance seems to be relative and dependent on environmental factors like soil and quality of irrigation water. For example, *P. nicotianae* has been reportd as the more frequently associated with loamy soils (Taso 1990; Jung et al. 2000).

Our results showed also a dry weight reduction of roots of citrus plants growing on artificially *Phytophthora*-infested soils. A reduction of root system in citrus plantations could bring to lower growth and fruit production resulting in huge economic losses.

P. cryptogea was never successfully re-isolated from in-vitro inoculations and no symptoms were observed in branches. This is probably due to the fact that *P. cryptogea* is a minor pathogen in citrus causing disease only on heavily stressed plants. However, symtoms of necrosis has been observed in leaves of the same variety of citrus inoculated by *P. cryptogea* and necrosis was more severe in the wounded treatments than in the non-wounded treatments. *P. cryptogea* is an emerging one in citrus production areas in Tunisia. The virulence of this pathogen could be the results of the introduction of new virulent strains. In fact, the import of plants, especially from Europe, has been increased in Tunisia (FAOSTAT 2016). The presence of *P. cryptogea* could be the consequence of the introduction and dissemination of non-native *Phytophthora* spp. by the international plant trade (Brasier and Jung 2006). Significant differences in pathogenicity among *P. cryptogea* isolates might explain why this pathogen has not decribed as an agent of citrus gummosis so far (Larsson and Gerhardson 1990). In addition *P. cryptogea*, on some stages of disease development, behaves as a typical vascular wilt disease, hampering the possibility of being isolated from cambial tissues (Larsson and Gerhardson 1990). It is also possible, that, in lack of closed recirculation water system in greenhouses and plastic tunnels, the pathogen can easily spread with surplus water from infected plants to local streams, lakes or rivers (Orlikowski and Ptaszek 2007). The extent to which this pathway of spread is common under ordinary orchard conditions still remains to be ascertained.

Orchards irrigated by submersion generates favorable conditions to infection by *Phytophthora*. Thus, the flooding conditions during the irrigation favors fungal sporulation and allows the fungus to reach the plants (Timmer et al. 2003). The inoculum of *Phytophthora* diseases of citrus could be dispersal in surface water (Ristaino and Gumpertz 2000). So, a good drainage and a good aeration of soil should be maintained to reduce the dissemination and spread of *Phytophthora*; especially that the airborne-disseminated *Phytophthora* species that can be floated to a new host plant. Climate change could affect the behavior of *Phytophthora* tree pathogens (Brasier 2000).

This aspect should be considered as factor of the disease, because Tunisia is characterized by a warm dry summers and cold and wet winters and since *P. nicotianae* is most active during the summer at high temperatures (> 30 °C) in citrus areas (Ricci et al. 1990; Erwin and Ribeiro 1996), an increase of temperatures could hamper the efforts to limit the spread of this pathogen. *Phytophthora* can be also spread by invertebrate from soil to other part of the trees (Taylor and Griffin 1981; El-Hamalawi and Menge 1996; Konam and Guest 2004). Such mechanisms should also be considered in future epidemiological studies to better understand the biology of *P. nicotianae*. Gummosis caused by *P. cryptogea* maybe a potential problem for commercial cultivation of citrus even if no serious losses due to this disease has been reported in Tunisia so far. Different Citrus species displays different susceptibility to this pathogen, leading to a loss of fruit harvest from 10 to 50% (Ptaszek et al. 2009).

The biology and ecology of *P. cryptogea* on citrus must be further investigated because, although the pathogenicity of *P. cryptogea* to a number of host plants has been reported, little information is available about its biology. To our knowledge, this is a first report describing a disease caused by *P. nicotianae* and *P. cryptogea* occurring on gummosis of citrus in Tunisia. Consequently, the Tunisian citrus industry has to consider the risk of a new outbreak due to these

pathogens. The information provided in this paper will be helpful for developing the strategies of management against the disease.

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