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Coniella granati (Saccardo) a New Potential Threat to Pomegranate (*Punica granatum* L.) in Tunisia Causing Twig Dieback and Fruit Rot

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Abstract

The disease caused by *Coniella granati* (Saccardo) (syn. *Pilidiella granati*, Saccardo) is an expanding threat to pomegranate (*Punica granatum* L.) cultivation and industry and is rapidly emerging in almost all pomegranate-producing regions of the world. The current study presents the first attempt to characterize *C. granati* associated with pomegranate dieback, shoot blight and fruit rot in Tunisia. Pathogenic isolates were identified based on their cultural and morphological characteristics and molecular data. Collected *C. granati* isolates were shown able to grow between 10°C and 30°C with an optimal mycelial growth at 20-25°C but they did not grow at 35°C. The fungus was able to grow 4-10 pH with an optimum growth at pH 4-5 for Cg1 and Cg2 isolates. Potato Dextrose Agar (PDA), Carrot Agar (CA) and Oatmeal Agar (OA) followed by pomegranate juice agar (PJA) and Malt Extract Agar (MEA) favored its mycelial growth. Pathogen growth was reduced under continuous light in comparison with the 12 h light/12 h dark regime and was significantly slowest under 24 h dark regime. Inoculated to pomegranate cv. Gabsi fruits, *C. granati* isolates induced soft rot within 9 days following incubation at 25°C and a complete fruit rot after 15 days. Leaves were highly susceptible to *C. granati* infection and completely degenerated 5 days post-inoculation. *C. granati* isolates were found to be pathogenic on pomegranate cv. Gabsi attached shoots and detached branches, giving rise to brown necrotic lesions. Keeping in view the importance of the pomegranate crop and the destructive nature of *Coniella* induced disease, further studies are needed to verify the pathogen host range, its aggressiveness towards the mostly grown Tunisian pomegranate varieties and to search for suitable control methods.

Keywords: Characterization; *Coniella granati*; Dieback; Fruit rot; Pathogenicity; *Punica granatum* L.; Tunisia.

Introduction

Pomegranate (*Punica granatum* L.) is mostly cultivated in the Mediterranean Basin, Southern Asia and several countries in North and South America. It is a temperate climate species requiring high temperatures to mature properly [1]. Tunisia is one of the main regions for pomegranate cultivation and production [2,3] and is considered as a micro-gene center with more than 60 local ecotypes already collected [4]. The main Tunisian producing regions are Gabes and Gafsa oases, Cap Bon, Bizerte and Sousse where pomegranate plays an important ecological and socio-economic role [4,5]. In Tunisia, as well as in many other countries [6], pomegranate has traditionally been considered a hardy tree, suffering from few diseases as compared to most fruit trees. Nevertheless, in the last decade, many fungal diseases were reported in many regions of the globe and were currently classified as one of the most limiting factors for pomegranate cultivation. For instance, wilt due to *Ceratocystis fimbriata* [7], wood canker and branch dieback caused by *Cytospora punicae* [8], and root and crown rot incited by *Phytophthora palmivora* [9] were detected in many countries. Also, *Lasiodiplodia gilanensis*, associated to severe dieback of one to multiple branches in California [6], and *Neofusicoccum parvum*, involved in shoot blight and canker symptoms, are reported to occur on pomegranate trees in Greece [10]. Several fungi are also common pomegranate fruit rot pathogens such as *Alternaria* spp. [11], *Penicillium* spp. [12,13], *Aspergillus* spp. [14], *Botrytis cinerea* [15], *Colletotrichum gloeosporioides*, and *Pestalotia brevista* [16]. They are responsible for substantial losses occurring before and after harvest and are threatening the pomegranate production. Despite this high number of fungi being identified as pathogens causing pomegranate dieback and fruit rots, little information is available about fungal diseases affecting pomegranates in Tunisia. *Cytospora punicae*, involved in canker and severe branch dieback, was the only fungal species identified in Gabes region on June 2014 on cv. Gabsi plants which was associated with a disease incidence of about 8% [17]. However, in a recent survey made in several orchards located in different regions of the governorate of Sousse, twig dieback and fruit rots were observed on several trees. Symptoms noted on most-known cultivars in Tunisia cvs. Gabsi and Kalai, were characterized by the presence of cankers and abundant, black, and solitary pycnidia on diseased twigs and shoots associated with marginal leaf browning, resulting in the dieback of one or multiple branches. On fruits, symptoms of dry rot and fruit mummification were frequently observed and decayed fruits were covered by large black pycnidia. These symptoms have been described in previous works as being associated with the presence of *Coniella granati* Saccardo (an obligate synonym of *Pilidiella granati* according to MycoBank database). This pathogen has been reported to be responsible for dieback and twig blight of pomegranate trees, fruit dry rot and/or crown rot, in many countries including China, India, Iran, Mexico, America, Greece, Turkey, Spain, and Italy [10,18-25] and to cause substantial economic loss to pomegranate industry.

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Although the substantial losses caused and its threat to the expansion of pomegranate cultivation [10,19,24], there are insufficient data on the occurrence of this disease in Tunisia. Furthermore, despite the numerous descriptions of the fungus, little information is available on its biology, including its environmental requirements for growth and inoculum production. Therefore, the aim of the current study was to characterize the pathogen associated with pomegranate dieback and fruit rot symptoms in Tunisia, based on its morphological and cultural traits, rDNA gene sequencing and its pathogenicity on the mostly grown pomegranate cultivar (cv. Gabsi). This study aimed also to provide more information on the effects of various culture media and environmental factors including temperature, pH and light regime on pathogen mycelial growth.

Materials and Methods

Pathogen isolation and morphological identification

Pathogen isolations were made from symptomatic pomegranate twigs and fruits collected during spring and early summer of 2016 from naturally infected orchards located in different regions of the governorate of Sousse, East coast Tunisia. Symptoms included twig necrosis and dieback on Gabsi and Kalai cultivars. On diseased twigs and shoots, abundant black and solitary pycnidia associated with marginal leaf browning were present (Figure 1). On fruits, symptoms of dry rot and fruit mummification were observed where severely decayed fruits were covered by large black pycnidia (Figure 1). Symptomatic twigs and decayed fruits were sectioned in pieces (0.5 cm in length), surface-disinfected in 10% NaOCl for 3 min, rinsed three times with sterile distilled water (SDW), and dried on sterile filter papers. Disinfected pieces were plated onto Potato Dextrose Agar (PDA) medium amended with streptomycin sulphate (300 mg/L) (w/v). Fungal cultures were incubated for 10-15 days at 25°C. Pure cultures were obtained by hyphal tip transfer on freshly poured PDA medium. Three single-spore isolates (namely Cg1, Cg2 and Cg10) were selected and used for all the assays reported in this study. Cg1 and Cg2 were isolated from twigs whereas Cg10 was recovered from fruits sampled from pomegranate orchards located in Chott-Mariem region, East coast Tunisia. Identification of three selected single-spore isolates was carried out based on morphological characteristics of their colonies, pycnidia and pycnidiospores using the description key [26]. For each isolate, the qualitative and quantitative morphological characteristics of 50 pycnidia and 50 conidia were recorded after 6 days of incubation at 25°C using a Nikon microscope and their average length and width were also calculated.

Molecular identification by ITS gene sequencing

In order to identify the pathogenic isolates recovered from pomegranate to species level, total genomic DNA of one representative isolate (Cg10) was extracted using the DNA Mini Kit (Analytik Jena, Biometra) according to manufacturer instructions. Universal primers ITS1 (Forward: 5'-CTTGCTCATTTAGAGGAAGTAA-3'; reverse: 5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (Forward: 5'-TCCTCCGCTTATTGATATGC-3'; reverse: 5'-CAGACTT (G/A)TA(C/T)ATGGTCCAG-3') were used to amplify the internal transcribed spacer (ITS) ITS1-5.8S-ITS4, by polymerase chain reaction (PCR) [19]. The PCR reaction was performed in a total reaction volume of 25 µL containing 5 µL of buffer (5x), 2.5 µL of dNTP (2 mM), 1.5 µL of MgCl2 (25 mM), 0.25 µL Taq polymerase (5 U/µL), 2.5 µL of each primer (6 µM), 5.75 µL of ultra-pure water and 5 µL of genomic DNA templates (10 ng). The amplification program, performed in an OpticonII (Biorad) Thermal Cycle, and the cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. Amplification was terminated by a final extension step of 7 min at 72°C. The resulting PCR products were electrophoresed on a 1% agarose gel (w/v) stained with Ethidium bromide, and visualized under UV light. The homology of the isolate gene sequence was performed using BLAST program from GenBank database (http://www.ncbi.nlm.gov/BLAST/). Sequence was aligned using the Clustal-X (1.81; EMBL, Heidelberg, Germany) and was submitted to Gen-Bank to be assigned an accession number.

Figure 1: Natural infections observed on pomegranate cvs. Kalai and Gabsi in the orchard: (a) Necrotic angular lesions on leaves with abundant gritty black, minute pycnidia; (b) Branch necrosis; (c) Shoot blight and (d) Mummified fruits.

Effect of temperatures on pathogen mycelial growth

To determine the effect of temperature on the mycelial growth of three selected isolates (Cg1, Cg2, and Cg10), mycelial plugs (6 mm in diameter) were cut from 7-day-old cultures on PDA, and placed in the center of 90-mm PDA Petri plates supplemented with streptomycin sulfate (300 mg/L). The inoculated plates were then incubated in the dark at 10°C, 15°C, 20°C, 25°C, 30°C and 35°C for 6 days and the mean diameter of the resulting colony was measured. Five replicate plates were used for each individual treatment. Cultures that did not grow after 6 days of incubation at any tested temperature were replaced at 25°C for 6 days to verify whether the fungus could resume growth or not.

Effect of pH on pathogen mycelial growth

The effect of medium pH on colony growth was determined on PDA supplemented with streptomycin sulfate (300 mg/L). Agar plugs (6 mm in diameter) obtained from actively growing colonies were transferred to Petri plates containing PDA medium adjusted to pH 4, 5, 6, 7, 8, 9 or 10 with the addition of 1 M solutions of HCl and NaOH before sterilization. Just before solidification, pH values were also verified on molten medium. There were five replicate plates for each individual treatment. Plates were incubated at 25°C in the dark and the mean colony diameters were recorded after 6 days of incubation as described above.

Effect of culture media on pathogen mycelial growth and pycnidial production

Pathogen mycelial growth was evaluated on nine culture media: PDA, Malt Extract Agar (MEA), Mathur, Sabouraud, Carrot Agar (CA), Pomegranate Juice Agar (PJA), Apple Juice Agar (AJA), Oatmeal Agar (OA), and Czapeck Agar (CA). These media were prepared, autoclaved for 30 min at 120°C, cooled, and poured into Petri plates. After solidification, plates containing each of the nine media tested were inoculated as previously described and incubated in the dark at 25°C. Five replicate plates were used for each isolate and each tested medium. Mean colony diameter was noted after 6 days of inoculation.

Colony morphology and pycnidia development were examined and the percentage of colony-area showing pycnidia-like structures was visually estimated per plate.

Effect of light on pathogen mycelial growth

To assess the effect of light on pathogen mycelial growth, plates containing PDA medium were inoculated with *C. granati* isolates (Cg1, Cg2, and Cg10) as previously described and incubated at 25°C under three light regimes: 24 h dark, 24 h fluorescent light and 12 h light/12 h dark. Plates under the 24 h dark regime were covered with aluminum foil. Five replicate plates were used for each isolate and each tested light regime. Pathogen colony diameter and pycnidial development were evaluated as detailed above.

Pathogenicity tests

Pathogenicity tests were performed using detached leaves, fruits and branches collected from visibly symptomless pomegranate cv. Gabsi trees grown in the experimental station of the Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem. Three isolates (Cg1, Cg2, and Cg10) were used for this test.

Detached leaf test

Apparently healthy, green and well developed cv. Gabsi pomegranate leaves were collected on April 2017 and immediately brought to the laboratory in plastic bags. They were washed under running tap water to remove any adhering dust. Sampled leaves were then surface-sterilized with 10% NaOCl (5 min), rinsed thrice with SDW and placed into plastic boxes (with five leaves per box) containing humidified sterile filter paper. For each tested *C. granati* isolate, an agar plug (6 mm in diameter) cut from 7-day-old cultures on PDA was deposited in the center of each leaf upper side. Control leaves were inoculated with pathogen-free agar plugs. Ten leaves were used for each isolate. All treated leaves were incubated at 25°C and regularly observed for development of any symptoms. Isolations from necrotic tissues were conducted to fulfill Koch's postulate.

Detached fruit test

Ripe middle size pomegranate cv. Gabsi fruits were surface-sterilized by immersion in 10% NaOCl (5 min), washed thrice with SDW and allowed to dry at ambient temperature on sterilized filter papers. Fruits were wounded with a sterile cork borer (3 mm in depth and 6 mm in diameter) and a mycelial plug obtained from 7-day-old culture on PDA was transferred into each wound (one plug per fruit, ten fruits per isolate). Similarly wounded fruits inoculated with non-colonized agar plug served as controls. All fruits were placed in plastic boxes and maintained at 70% relative humidity at 25°C for 9 days. After this incubation period, the external lesion diameter was measured. Fruits were cut longitudinally through the inoculation site and the mean rot penetration was calculated as the average of width and depth of the occasioned rot. Isolations from necrotic tissues were conducted to fulfill Koch's postulate.

Detached branch test

Apparently healthy segments of pomegranate cv. Gabsi branches (15 cm long and 1 to 1.6 cm in diameter) were removed from symptomless pomegranate trees cv. Gabsi, sealed at both ends with parafilm to avoid desiccation and directly brought to laboratory and disinfected with 10% NaOCl (3 min) and 70% ethanol (1 min). Disinfected branch segments were wounded (3 mm in diameter and in depth) each at three alternate sites using a sterile cork borer. A 3 mm diameter mycelium plug, cut from an actively growing colony on PDA, was inserted into each wound and the inoculated area was wrapped with parafilm. Twelve replicate branch segments were used for each individual treatment. Inoculated and control (inoculated with non-colonized PDA plugs) branches were incubated in humid chambers as described above and maintained at 25°C for 30 days. Segments with the outer bark or the outer and inner bark removed were also used in this assay and treated as described above to verify if isolated pathogen could develop on old detached branches left in the orchards. For each branch segment, the length of the induced necrosis or lesion was measured after 30 days of incubation and the presence of pycnidia was visually examined. Isolations from necrotic tissues were conducted to fulfill Koch's postulate.

Attached shoot test

Intact shoot inoculations were performed on one-year-old potted pomegranate cv. Gabsi plants maintained in greenhouse conditions. Five plants were used per individual treatment. Three green shoots (~5-7 mm in diameter) per plant, emerging from the main stem were wounded at 5 cm from their bases as previously described for detached branch test. The wound was covered with humidified cotton and wrapped with parafilm to prevent desiccation. Plants whose shoots were inoculated with non-colonized PDA plugs served as controls.

All pomegranate plants were grown under greenhouse conditions (temperatures ranging between 15 and 30°C) in the Regional Research Centre on Horticulture and Organic Agriculture, Chott-Mariem, Tunisia. Plants were regularly watered to avoid abiotic stress. The length of the induced necrosis developing above and below the inoculation points was recorded 60 days after inoculation. Small pieces cut from the developing lesions were placed on PDA in an attempt to recover the inoculated fungus and fulfill Koch's postulate.

Statistical Analysis

For all the *in vitro* trials, statistical analyses were performed following a completely randomized factorial design where fungal treatments (isolates and control) and tested factors (temperatures, pH, or culture media) were the two fixed factors. Five replicates were used per individual treatment and means were separated using Fisher's protected LSD or Students Neuman Keuls tests (at $p \leq 0.05$). Pathogenicity tests were conducted according to a completely randomized design where fungal treatments were the only fixed factor. All the experiments were repeated twice and for each test, the mean data is presented in the current study. Statistical analyses were performed using SPSS software version 16.

Results

Isolation and identification of *Coniella granati*

After 7-10 days of incubation at 25°C, dark greenish globose pycnidia were formed on the majority of the diseased pomegranate tissues (i.e., dry rotten fruits, twigs showing necrosis and dieback and from necrotic leaves) and consistent fungal colonies with white to pale green aerial mycelia were developed. A total of 15 isolates with resembling colony and conidial morphology was obtained and morphological characterization was performed on three selected isolates. On PDA medium, selected pathogenic isolates developed light yellow colonies with leathery mycelia and irregular concentric rings of abundant black, globose, solitary pycnidia (75 to 225 µm in diameter) developing throughout the Petri plate within 15 days of incubation at 25°C (Figure 2). Hyphae were septate and conidia were one-celled, hyaline to olivaceous brown, ellipsoid to fusiform, straight,

apex obtuse, base truncate, with mucoid appendage along the side of the conidium, averaging 11.4 to 17.2 µm × 3.6 to 4.15 µm in size (Figure 2). These morphological features matched those described earlier for *C. granati* or *P. granati* [26]. One representative isolate (Cg10) was used for further identification based on ITS sequence data. In fact, BLAST analysis of the ITS sequence obtained and the phylogenetic analysis based on neighbor joining (NJ) method with 1000 bootstrap sampling, showed 99% identity with *Coniella granati* (= *Pilidiella granati*) (i.e., GenBank Accession Nos. KX507098 and KX833578). Consequently, the representative isolate (Cg10) was ascribed to *C. granati* and it's ITS sequence deposited in GenBank, MG256184 (Figure 3). Accordingly, fungal isolates from pomegranate dry rotten fruits, twigs showing necrosis and dieback and from necrotic leaves were identified in this study as *C. granati* based on morphological characteristics and molecular data. The current study reports for the first time *C. granati* to occur on Tunisian pomegranate.

Effect of temperature on *Coniella granati* mycelial growth

Mean diameter of *C. granati* colonies formed after 6 days of incubation on PDA medium, varied significantly (at $p \leq 0.05$) depending on tested temperatures and isolates. A significant interaction was also noted between these two factors. As shown in Figure 4, *C. granati* isolates were able to grow from 10 to 30°C. Optimal growth occurred at 25°C for Cg2 and Cg10, whereas Cg1 showed maximum mycelial extension at 20 and 25°C. The mycelial growth of Cg10 was significantly similar at 20 and 30°C, while that of Cg1 and Cg2 was better at 20 than at 30°C. The Cg1 isolate exhibited significantly comparable mycelial growth at 30 and 15°C. At 35°C, mycelial extension of *C. granati* isolates was totally inhibited, but growth resumed when cultures were re-incubated at 25°C. For all the temperatures pooled, the mycelia growth of the isolate Cg10 was significantly higher than that of Cg2 and Cg1 isolates.

Effect of pH on *Coniella granati* mycelial growth

All tested *C. granati* isolates were able to grow on pH-adjusted PDA, from pH 4 to pH 10, and their mycelial growth varied significantly depending on isolates and pH values; a significant interaction ($p \leq 0.05$) was noted between these two factors. As shown in Figure 5, Cg10 isolate exhibited significantly similar radial growth in pH range 4-10, with a colony average of 77.93 mm. However, Cg1 and Cg2 growth was favored by acid conditions as maximum colony diameter of 79 mm and 77.07 mm was noted after 6 days of incubation at 25°C at pH 4-5 and pH 4-6, respectively. Increasing pH level from 7 to 10 and from 6 to 10 lead to a decline in Cg1 and Cg2 growth, respectively. Whatever the pH of PDA medium, mycelial growth of Cg10 was the highest as compared to the two other isolates.

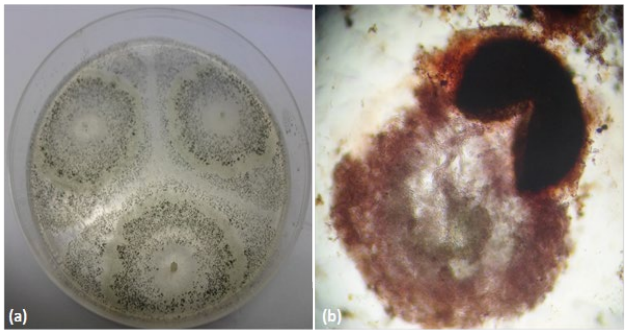

Figure 2: *Coniella granati* Cg10 isolate colonies formed on PDA medium after 15 days of incubation at 25°C (a); Pycnidium of *C. granati* from PDA culture, with conidia oozing out (G×100) (b).

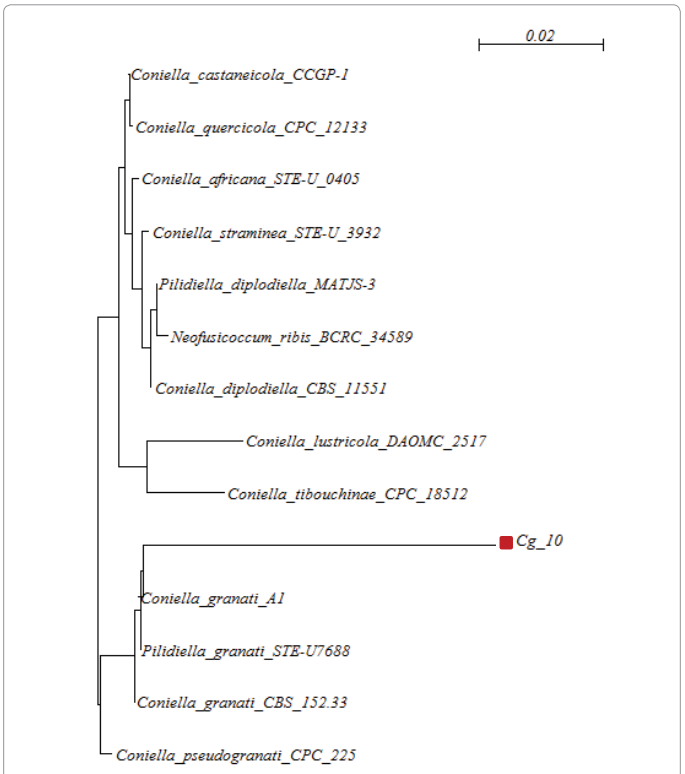

Figure 3: Neighbor-joining phylogenetic tree of rDNA sequence of Cg10 isolate recovered from rotten pomegranate cv. Gabsi fruits and its closest phylogenetic relatives. The nucleotide sequences used of representative isolates were obtained from Genbank database under the following accession numbers: KX507098 (*C. granati* strain A1); KU666470 (*P. granati* strain STE-U7688); KX833578 (*C. granati* CBS:152.33); KJ869132 (*C. pseudograna*ti strain CPC 22545); NR_111706 (*C. tibouchinae* CPC 18512); MF631781 (*C. lustricola* strain DAOMC 251734); KX833527 (*C. diplodiella* CBS:115514); GU905992 (*Neofusicoccum ribis* strain BCRC 34589); KC771899 (*P. diplodiella* strain MATJS-3.7); AY339348.1 (*C. straminea* strain STE-U 3932); Y339344 (*C. africana* strain STE-U 0405); KX833596 (*C. quercicola* CPC:12133); KF564280 (*C. castaneicola* CCGP-1), and for the tested isolate MG256184 (Cg10). The tree topology was constructed using Clustal-X (1.81).

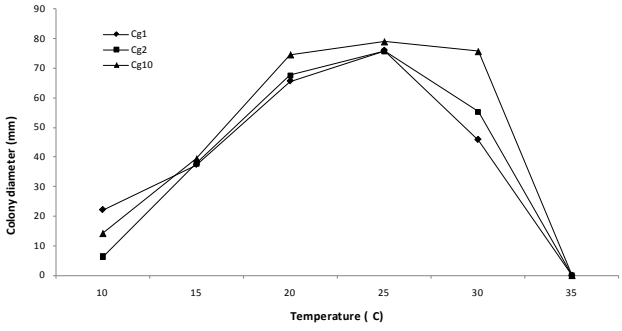

Figure 4: Effect of temperature on the mycelial growth (mm) of three *Coniella granati* isolates recorded after 6 days of incubation on PDA medium. LSD (Temperature**s** × *Coniella granati* isolates) = 9.97 mm at $p \leq 0.05$.

Effect of different culture media on *Coniella granati* mycelial growth and pycnidial production

Radial growth of *C. granati*, noted after 6 days of incubation at 25°C, varied significantly depending on tested isolates and culture

media; a significant interaction was also noted between both fixed factors ($p \leq 0.05$). As shown in Table 1, all *C. granati* isolates were able to grow on the different agar media tested except for Czapeck agar (CzA) medium. For Cg1 isolate, optimal mycelial growth was recorded on CA followed by PDA, while that of Cg2 was noted on PDA and OA followed by CA. However, PDA and CA media were the most favorable for Cg10 radial growth. When grown on OA, PJA and MEA media, Cg10 grew also well where its colony diameter ranged between 72 and 74.7 mm. The poorest mycelial growth was observed on cultures grown on Mathur and Sabouraud, Mathur and AJA, and Mathur media, for Cg1, Cg2 and Cg10 where the average colony diameter, noted after 6 days of incubation at 25°C, did not exceed 28.1, 35.4 and 34.9 mm, respectively.

Regardless the tested culture medium (pooled data of all media), Cg10 was the fastest growing isolate. After 6 days of incubation at 25°C, pycnidia were observed in the central area of colonies on four (MEA, PJA, CA and OA) out of the nine media tested (Table 1). The colony area yielding pycnidia ranged between 28.58-89.11, 9.87-78.46, and 51.61-82.69% when Cg1, Cg2 and Cg10 were respectively grown on PJA and CA, PJA and OA, and OA and CA media. For all the tested culture media (pooled data of all media), Cg1 produced more pycnidia than the other *C. granati* isolates. The morphology of fungus colonies formed after 6 days of incubation varied depending on culture media (Figure 6). In fact, on PDA medium, tested isolates formed light yellow and compact colonies with few or no aerial hyphae that appear in irregular concentric rings. However, on Mathur, PJA and AJA media, the fungus formed white circular colonies with thin mycelium. When grown on MEA and OA, *C. granati* isolates developed colonies with an aerial and dense mycelium in the center which turned thin and scanty at the edge. On CA, developing colonies were initially creamy with thin mycelium and later turned grey to blackish in the central area due to pycnidia formation. On Sabouraud, fungus colony grew in irregular concentric rings of white to cream relatively dense mycelium.

Effect of light regime on *Coniella granati* mycelial growth

C. granati mycelial growth on PDA medium, noted after 6 days of incubation at 25°C, varied significantly ($p \leq 0.05$) depending on tested isolates and light regimes. As no significant interaction was recorded between both fixed factors, pooled data of each factor was presented and commented as shown in Figures 7a and 7b. In fact, for all tested *C. granati* isolates, mycelial growth was significantly the slowest under 24 h dark regime while the optimum mycelial extension occurred under the 12 h light/12 h dark regime (Figure 7a). For all tested light regimes, the radial growth of the isolate Cg10 was the highest as compared to the two other isolates (Figure 7b).

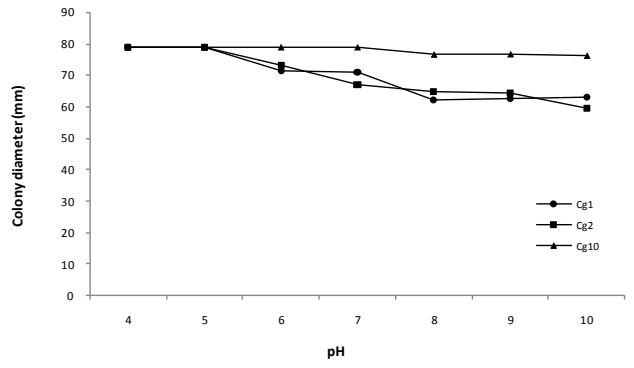

Figure 5: Effect of pH on the mycelial growth (mm) of three *Coniella granati* isolates recorded after 6 days of incubation on PDA at 25°C.
LSD (Temperatures× *Coniella granati* isolates) = 1.17 mm at $p \leq 0.05$.

Tested media[a]	Colony diameter (mm)			Mean colony diameter per medium[b]	% colony area covered with pycnidia			Mean % colony area covered with pycnidia per medium[d]
	Cg1	Cg2	Cg10		Cg1	Cg2	Cg10	
PDA	75.8 ab	75.4 a	79 a	76.7 a	0.00 e	0.00 d	0.00 c	0.00 d
OA	74.6 b	73.2 a	74.7 b	74.2 a	77.21 b	78.46 a	51.61 b	69.09 a
CA	79.0 a	69.3 ab	78.6 a	75.6 a	89.11 a	40.42 c	82.69 a	70.74 a
PJA	60.5 c	63.8 b	72 b	65.4 b	28.58 d	9.87 d	60.63 b	33.02 c
MEA	59.6 c	53.7 c	72.1 b	61.8 c	59.90 c	57.37 b	55.67 b	57.64 b
Sabouraud	28.1 e	49.2 c	66.2 c	47.8 d	0.00 e	0.00 d	0.00 c	0.00 d
AJA	36.0 d	32.5 d	45 d	37.8 e	0.00 e	0.00 d	0.00 c	0.00 d
Mathur	26.0 e	35.4 d	34.9 e	32.1 f	0.00 e	0.00 d	0.00 c	0.00 d
CzA	0.0 f	0.0 e	0.0 f	0.00 g	0.00 e	0.00 d	0.00 c	0.00 d
Mean colony diameter/% colony area covered with pycnidia per isolate[c,e]	48.8 b	50.3 b	58.1 a		28.31 a	20.68 b	27.84 a	

[a] PDA (Potato Dextrose Agar), OA (Oatmeal Agar), CzA (Czapeck Agar), PJA (Pomegranate Juice Agar), Malt Extract Agar (MEA), Sabouraud, AJA (Apple Juice Agar), Mathur, CA (Carrot Agar)
[b] Mean colony diameter per medium for the three isolates combined
[c] Mean colony diameter per isolate for all media combined
[d] Mean % colony area covered with pycnidia per medium for the three isolates combined
[e] Mean % colony area covered with pycnidia per isolate for all media combined
LSD (Tested culture media × C. granati isolates)=4.84 mm at $p \leq 0.05$ (mycelial growth)
LSD (Tested culture media × C. granati isolates)=9.81% at $p \leq 0.05$ (pycnidial production)

Table 1: Mycelial growth and pycnidial production of three *Coniella granati* isolates (Cg1, Cg2 and Cg10) recorded after 6 days of incubation at 25°C on nine culture media.

It should be noted that, after 10 days of incubation and under all the tested light regimes, pycnidia were observed in all pathogen colonies.

Pathogenicity on detached leaves

After 2-3 days of incubation at 25°C, small necrotic lesions appeared on all inoculated cv. Gabsi leaves and spread from the inoculation sites toward their both extremities whereas control leaves remained symptomless (Figure 8). These lesions increased progressively in size resulting in complete leaf necrosis and degeneration after 5 days of inoculation with the formation of abundant black pycnidia on the upper sides of all inoculated leaves (Figure 8). On the lower sides, these pycnidia become visible within 10 days following inoculation. Fungus isolates were 100%-re-isolated from all inoculated leaves but not from the control ones thus confirming Koch's postulates.

Pathogenicity on detached fruits

Pathogenicity tests showed that all pomegranate cv. Gabsi fruits artificially inoculated with *C. granati* developed typical symptoms

of fruit rot that were visible by 3 days of incubation at 25°C, while control fruits remained symptomless. In fact, brown circular lesions appeared firstly around the inoculation site and expanded rapidly to the fruit rind and the arils as well as the membranes separating the aril compartments. The infected tissues turned brown, soft and juicy (Figure 9). After 9 days of incubation, the lesion diameter and mean rot penetration reached 98-99.5 and 56.9-58 mm, respectively, but no significant differences ($p \leq 0.05$) were noted among the three tested *C. granati* isolates. The infected fruits were completely rotted within 11 to 15 days and red-violet juice leaked from the rotten tissues. Abundant dark brown to black spherical pycnidia of the pathogen developed on the surface of inoculated fruits within 20 days after inoculation (Figure 9). In general, all *C. granati* isolates produced symptoms similar to those observed in naturally infected fruits and were re-isolated from infected tissues, thus confirming Koch's postulates.

Pathogenicity on detached branches

Results from pathogenicity tests showed that all tested *C. granati* isolates were pathogenic to pomegranate cv. Gabsi detached branches. They produced dark to light brown necrotic lesions that developed both upward and downward from the inoculation points after 30 days of incubation. The length of necrosis ranged from 20 to 35 mm and there were no significant differences among the three isolates (Figures 10a and 10b). These lesions became more pronounced as the outer bark layer was scraped away (Figure 10c). Few pycnidia were visible around the inoculation points. No necrosis were observed on control pomegranate branches. Furthermore, when the detached branches were devoid of bark layers, brown lesions were induced by *C. granati* which length (32-50 mm) was longer than that noted on the intact inoculated branches and abundant pycnidia were produced and covered the entire necrosis colonized surface within 12 days after inoculation (Figures 10d and 10e).

Pathogenicity on attached pomegranate shoots

In the potted plant trials, *C. granati* isolates were shown able to induce symptoms at 30 days after inoculation. These symptoms consisted on brown lesions which lead to shoot dieback after 60 days of incubation under greenhouse conditions. Necrosis length noted on inoculated pomegranate shoots was statistically similar for the three pathogen isolates and reached 110 to 130 mm, whereas control plants remained symptomless. In addition, inoculated shoots showed necrotic lesions on leaves similar to those observed on naturally occurring *Coniella* infections. Koch's postulates were satisfied after re-isolating the fungus from inoculated shoots.

Discussion

The disease caused by *Coniella granati* (syn. *Pilidiella granati*) is an expanding threat to the pomegranate cultivation and industry and is rapidly emerging in almost all pomegranate producing regions of the world. Chronologically, this pathogen has been previously reported in Greece [27], Spain [24], Israel [28], USA [21], Iran [23], Turkey [18], China [19], Cyprus [16], Italy [22], and Mexico [20]. Although *C. granati* has been reported as causal agent of pomegranate shoot blight,

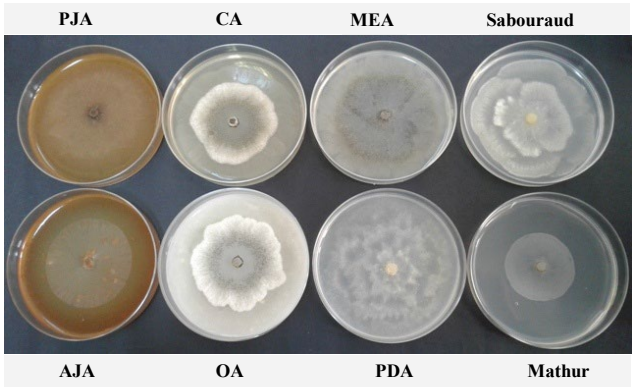

Figure 6: Variation in *Coniella granati* Cg10 (MG256184) colony morphology when grown on different culture media noted after 6 days of incubation at 25°C. PDA (Potato Dextrose Agar), OA (Oatmeal Agar), CA (Czapeck Agar), PJA (Pomegranate Juice Agar), Malt Extract Agar (MEA), Sabouraud, AJA (Apple Juice Agar), Mathur, CA (Carrot Agar). Cg10: *C. granati* isolate recovered from pomegranate rotten fruits.

Figure 8: Necrosis of pomegranate cv. Gabsi leaves noted after 2 (a), 3 (b), 4 (c) and 5 (d) days post-inoculation with *Coniella granati* Cg1 accompanied with the formation of abundant black pycnidia on upper (e,f) and lower leaf sides (g), compared to control.

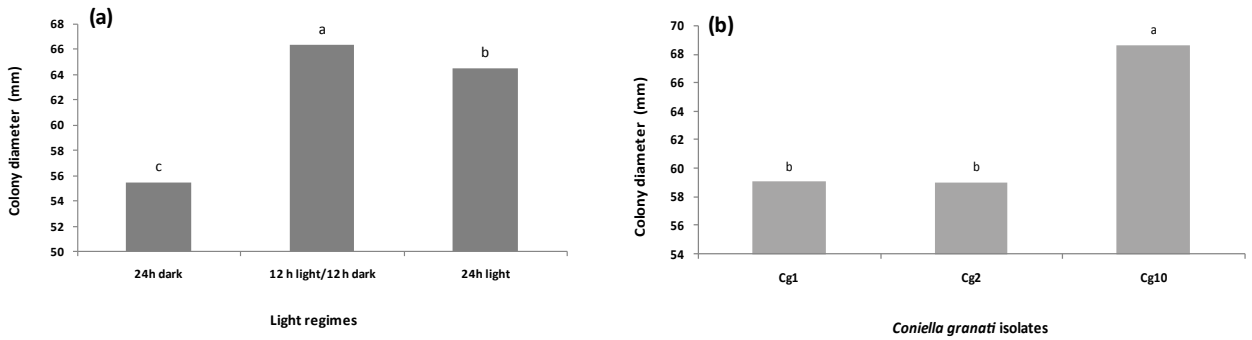

Figure 7: Effect of different light regimes (a) and tested isolates (b) on *Coniella granati* mycelial growth (mm) recorded after 6 days of incubation at 25°C on PDA medium. Bars affected with the same letter are not significantly different according to Student-Newman-Keul's test at $p \leq 0.05$.

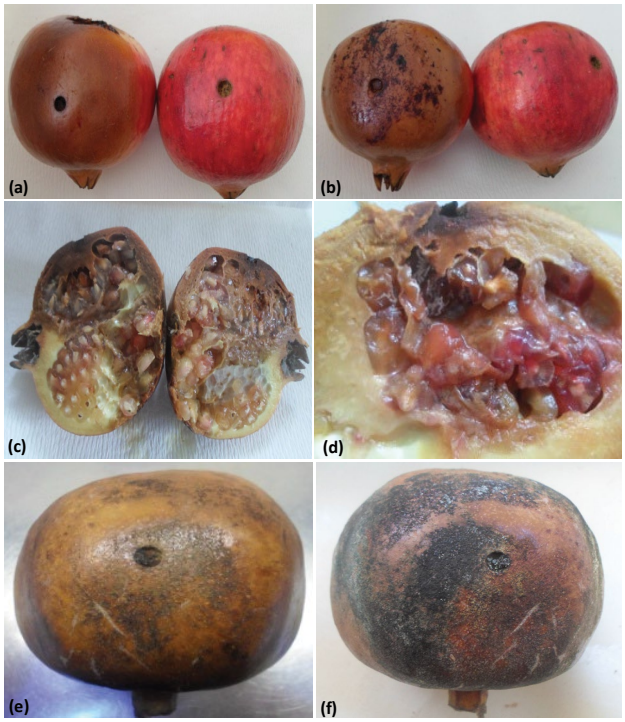

Figure 9: Typical symptoms of *Coniella granati* infection observed on detached pomegranate cv. Gabsi fruits inoculated with PDA plugs colonized by the pathogen. (a) Brown circular lesion formed around the inoculation site (on the left) compared to symptomless control (on the right); (b) Completely rotten fruit leaking red-violet juice after 13 days after inoculation; (c, d): fruit rind, arils as well as the membranes separating the aril compartments displaying rot; (e, f): Abundant dark brown to black spherical pycnidia of the pathogen developed on the surface of the fruit within 14 (e) and 20 days (f) after inoculation.

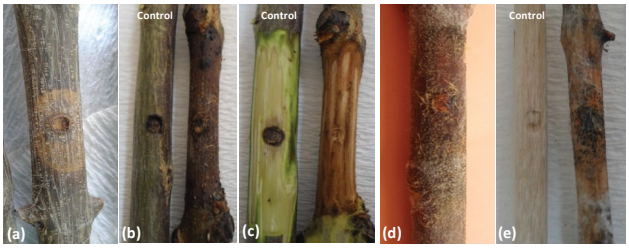

Figure 10: Severity of *Coniella granati*-induced lesions observed on pomegranate cv. Gabsi detached branches inoculated with PDA plugs colonized by the pathogen noted after 15 (a) and 30 days (b, on the right) of incubation at 25°C. Internal brown lesions induced by *C. granati* after 30 days of incubation at 25°C (c). Abundant pycnidia covering the entire necrosis colonized surface within 12 days after inoculation noted on detached branch devoid of outer bark layer (d) and those without bark layers (e).

dieback, crown rot and/or fruit rot in all these countries, this study is a recent record of this fungus on pomegranate in Tunisia, associated with dieback, shoot blight and fruit rot [29]. In the current investigation, *C. granati* isolates were identified based on cultural and morphological characteristics and molecular data which are consistent with previous descriptions of this species [19,23,26]. A high homology (99%) was obtained for the representative isolate in relation to reference isolates available in the GenBank. Mirabolfathy et al. [23] also identified the causal pathogen of dieback and fruit rot of pomegranate as *C. granati* on the basis of cultural, morphological, genetic (ITS) and pathogenicity analyses. Although *C. granati* is a threat for pomegranate production, only limited information about the effects of abiotic factors (temperature, culture media, pH and light) on its growth has been published. The current study presents the first attempt to characterize *C. granati* associated with pomegranate dieback and fruit rot in Tunisia. In fact, *C. granati* isolates tested grew between 10 and 30°C and showed an optimal mycelial growth at 20-25°C but they did not grow at 35°C. These results are in agreement with Sharma and Tegta [25] study reporting that optimum temperature for vegetative growth of *C. granati* was 25°C and those of Michailides et al. [21] and Thomidis [10] who found that 25-30°C was the optimum temperature for *C. granati* mycelial growth. Furthermore, although *C. granati* was not able to grow at 35°C, this pathogen resumed growth after the cultures were moved to 25°C. This indicates its ability to survive under these relatively high temperatures. This could explain the incidence of the disease in almost all the pomegranate-producing countries and the adaptation of the pathogen to different climatic conditions. Furthermore, in Tunisia, average temperatures in the spring and summer are disease conducive. In the same sense, Kumari [30] pointed out that *Coniella* disease appear in the last week of June-first week of July when mean temperature of 24°C is prevalent. In fact, the knowledge of the most favorable temperatures for a pathogen development is essential to determine the optimal period of its activity and the best method for its management [10]. While temperature can affect *C. granati* growth, it can be concluded from the results reported here, that this species has the ability to be active over a wide pH range. All isolates were able to grow between pH 4-10 with an optimum growth at pH 4 and 5 for Cg1 and Cg2 isolates. This corresponds with a previous study [25] reporting that a range of 4-5 pH favored better growth of this fungus. Also Carlile et al. [31] mentioned that when nutrient requirements are satisfied, most fungi grow well over the pH range 4-7. Thus, it appeared that acid and alkaline conditions favor mycelial growth of this fungus and explain its virulence towards pomegranate sweet and sour varieties with different pH levels (data not shown). As well as for pH, the effect of culture media on *C. granati* mycelial growth has not been previously investigated. In this study, we evaluated the suitability of nine culture media for mycelial growth of the fungus at 25°C. We found that Potato Dextrose Agar (PDA), Carrot Agar (CA) and Oatmeal Agar (OA) followed by pomegranate juice agar (PJA) and Malt Extract Agar (MEA) favored its growth. The last four media were also favorable for pycnidial production. Using a natural medium prepared from parts of susceptible host plants may enhance growth and sporulation of fungal pathogens [32]. However, pomegranate juice agar (PJA) tested in this study did not support fungal growth well as compared to Carrot Agar (CA) and Oatmeal Agar (OA) media. Apple Juice Agar, Sabouraud and Mathur appeared to be not suitable for rapid mycelial growth nor for pycnidial production of this fungus while Czapeck Agar did not support fungal growth. Nutrients and their availability on these media might be related to the differences in fungus mycelial growth and pycnidial production. *C. granati* growth was reduced under continuous light in comparison with the 12 h light/12 h dark regime and was significantly slowest under 12 h dark regime. This could be in relation with activity of the fungus in the spring-autumn when light exposure is lengthened. In fact, when environmental conditions are favorable, this pathogen can cause important damages to pomegranate [33]. In the current study, it has been shown that *C. granati* isolates were responsible for soft rot of pomegranate cv. Gabsi after 9 days of incubation at 25°C. This fungus has been reported to cause pomegranate fruit rots in many other countries [21,23]. For instance, Kumari [30] proved the pathogenicity of *C. granati* to pomegranate fruits in India that

developed typical symptoms of dry rot within 7-8 days after inoculation, whereas other researchers reported that pomegranate fruits from Greece, Spain and Mexico developed *C. granati* rot symptoms within 10, 7 and 6 to 9 days after inoculation, respectively [20,24,27]. Furthermore, the typical symptoms of the disease on fruits were found identical as described by different workers from different regions of the world [21,23,25,27,34,35]. Indeed, as reported by in another study [34,35], *C. granati*-incited rot differs from Alternaria, Aspergillus and Botrytis fruit rots, in that it causes breaking of the peel, unevenly distributed black dots on fruit peel, and a black circle around the black dots. It should be noted that according to our results, the infected fruits were completely rotted within 11 to 15 days after inoculation confirming previous reports showing that pomegranate is highly susceptible to *C. granati* infection that resulted in substantial losses of this culture in many countries [10]. Indeed, *P. granati* (Sacc.) has been identified as a pathogen causing pre-harvest fruit rots on pomegranate [24]. Thomidis [14] showed that *P. granati* was isolated from about 60% of the pre-harvest rotted fruits without symptoms of cracking, while Cintora-Martínez et al. [20] mentioned that fruit rot disease was observed on 60 to 85% of pomegranate fruits in an orchard located in Oaxaca, Mexico. *P. granati* was also identified as a pathogen causing postharvest pomegranate fruit rot but in much lower percentage than that induced by *B. cinerea* [14,24]. Also, amongst fungal diseases, fruit rot caused by *C. granati* results in rotting of pomegranate fruits within a week of infection, causes drastic reduction in yield as well as marketability of the produce [30]. Fruit losses up to 80% due to this disease have been reported in pomegranate orchards in Mandi district of Himachal Pradesh in India [30]. From the foregoing results, it is also concluded that pomegranate leaves are highly susceptible to *C. granati* infection as complete degeneration was observed within 5 days post inoculation as indicated in a previous study [30]. Disease symptoms on leaves were also described as small necrotic angular lesions which started from the leaf tip region towards the proximal end leading to desiccation and premature shedding of leaves [36]. These leaves showed abundant gritty black, minute pycnidial bodies, which spread throughout on the leaf surface. In the present study, *C. granati* isolates were found to be pathogenic on pomegranate cv. Gabsi attached shoots and detached branches, giving rise to brown lesions. Our results matched those of Mirabolfathy et al. [23] and Chen et al. [19] who reported that *P. granati* isolates from Iran and China, respectively, were also pathogenic on pomegranate twigs, resulting in brown lesions that were 2 to 5 cm long after 2 months of incubation. In inoculation tests, *P. granati* was pathogenic to annual shoots of pomegranate cv. Wonderful and to experimental genotypes tested where lesion's length was 6.3 to 12.1 cm long 30 days after inoculation [10]. Furthermore, when the detached branches were devoid of bark layers, abundant pycnidia were produced and covered the entire necrosis colonized surface within 12 days after inoculation. These results highlighted the importance of the pruning material left in the orchard in the inoculum multiplication and spread of the pathogen. In fact, in 2006, all the pomegranate grafting material that was imported from India was destroyed after the diagnosis of *C. granati*. This study also showed that all three *C. granati* isolates were equally virulent when tested on pomegranate parts. These results are in agreement with a previous work [10] indicating no significant differences in virulence among the isolates of *P. granati* used for pomegranate shoots inoculation.

Conclusion

In conclusion, this study showed that *C. granati* is responsible for fruit rot, leaf necrosis, shoot blight and branch dieback of pomegranate cv. Gabsi, the most known cultivar in Tunisia. The results obtained here improve understanding of the biology of this important emerging pomegranate pathogen. However, these results do not exclude the possibility that other species of phytopathogenic fungi (e.g., *Botryosphaeria*, *Pestalotia*, and *Phomopsis* spp.) may eventually be involved in the symptoms observed in the orchards. As there is very little information about pomegranate fungal pathogens in Tunisia, more studies are needed for their identification and characterization. Keeping in view the importance of the pomegranate crop and the destructive nature of *Coniella*-incited diseases and the extent of losses it caused, further studies should be undertaken in order to define the pathogen host range, its aggressiveness towards pomegranate Tunisian varieties and to search for suitable control methods.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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