Journal of Horticulture
ISSN: 2376-0354

Jabnoun-Khiareddine et al., J Hortic 2018, 5:4
DOI: 10.4172/2376-0354.1000245

Research Article Open Access

Response of Tunisian Pomegranate (*Punica granatum* L.) Cultivars and Several Plant Hosts to *Coniella granati* (Saccardo)

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Abstract

Pomegranate (*Punica granatum* L.) disease incited by *Coniella granati* (Saccardo) has been recently reported in Tunisia causing branch dieback, shoot blight and fruit rot. The present study was undertaken in order to evaluate the response of nine known and widely grown pomegranate cultivars throughout Tunisia toward this emergent pathogen, to study the host range of *C. granati* and to identify its extracellular cell wall degrading enzymes. Plant response was determined based on fruit lesion diameter and length of external, internal and pycnidia-bearing lesions recorded on wounded and detached branches. All cultivars tested were susceptible to *C. granati* fruit rot where the lowest lesion diameters (87-88 mm), recorded 9 days post-inoculation (DPI), were noted on cultivars Gabsi and Zaghouani. At 30 DPI, intact detached branches were shown to be moderately susceptible to *C. granati* infection with only cv. Chetwi being moderately tolerant. On altered detached branches (branches devoid of bark layers) Jbali, Zehri and Zaghouani were the most susceptible to *C. granati* giving rise at 12 DPI to longer lesions bearing pycnidia than the other cultivars. *C. granati* isolates tested were able to cause dry rot on nine out of ten different fruits, with apple, tomato and fakous (*Cucurbita melo* var. *flexuosus*) fruits being the most susceptible ones while they were nonpathogenic on zucchini. On detached and intact branches, *C. granati* was weakly pathogenic on orange and pear compared to apple and loquat and was non pathogenic to olive, fig, peach, prune, and apricot branches. Using specific media, *C. granati* isolates were shown able to produce laccase, protease, pectinase, pectate transeliminase, and cellulase but no amylolytic and lipolytic activities were detected. Thus, as *C. granati* represents a threat to all Tunisian pomegranate cultivars as well as for other tree fruit species, the search for effective management methods towards this disease is of urgent need.

Keywords: *Coniella granati;* Cultivars; Fruit rot; Hosts; Necrosis; Enzymes; *Punica granatum* L.

Introduction

In Tunisia, pomegranate (*Punica granatum* L.) has been cultivated traditionally since ancient times [1,2] and has an important ecological and socio-economic role as it can valorize marginal soils and saline waters and is well adapted to arid and semi-arid environment [1]. Pomegranate cultivation is spread throughout the country with the main producing areas are the southern oases of Gabes, Kairouan, the North-east coasts and Testour in the northwest [1,3].

In Tunisia, pomegranate has been long considered as secondary fruit tree [1]. However, in the last decades, there has been an impressive increase of interest in this ancient fruit species due to its numerous medicinal as well as nutritional properties. In fact, its production, beginning from September to December, has increased from 64797 tons in 2010 to 74500 tons in 2017 [4] which is destinated mainly to local market and small quantities were exported. This production is issued from few cultivars with interesting market characteristics with cv. Gabsi being the predominant one [1].

Tunisia is considered as a micro-gene center for pomegranate [1]. Numerous pomegranate cultivars are grown in Tunisia, alone or very often associated with other fruit species such as olive, apricot, peach, apple or palm trees and sometimes with vegetables such as solanaceous and cucurbit species. Cultivars are often classified as sweet, sweet-sour and sour, early, mid season and late, juicy and table fruit, soft-seeded and hard-seeded or major and minor. The names originate frequently either from growing site or attributed to fruit colour [5].

Nevertheless, pomegranate cultivation is affected by various problems such as fruit cracking, fruit rots and pest attacks. In fact, pomegranate fruits are often infected by numerous fungal pathogens causing pre and/or post-harvest spots or rots such as *Alternaria* spp., *Penicillium* spp., *Aspergillus* spp., *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and *Pestalotia brevista* [6-11]. Furthermore, several fungi have been reported to cause wilt, shoot blight, branch dieback and root and crown rot [12-15]. *Coniella granati* Saccardo (an obligate synonym of *Pilidiella granati* according to MycoBank database) has been also reported in most pomegranate-producing countries causing dieback and twig blight of pomegranate trees and also fruit dry rot and/or crown rot and was responsible of substantial economic losses to pomegranate industry [15-23].

In Tunisia, *C. granati* was recently reported in several orchards located in Sousse governorate, on most-known cultivars such as cvs. Gabsi and Kalai [24]. Under optimum conditions, this fungus caused on cv. Gabsi a complete fruit rotting within 11-15 days showing that this pomegranate cultivar is highly susceptible to *C. granati* infection. Furthermore, this pathogen infects the aerial parts of the host giving

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Received August 31, 2018; **Accepted** September 21, 2018; **Published** September 28, 2018

Citation: Jabnoun-Khiareddine H, Ibrahim N, Aydi Ben Abdallah R, Mars M, Daami-Remadi M (2018) Response of Tunisian Pomegranate (*Punica granatum* L.) Cultivars and Several Plant Hosts to *Coniella granati* (Saccardo). J Hortic 5: 245. doi: 10.4172/2376-0354.1000245

rise to a variety of symptoms which include brown necrotic lesions in woody tissues and leaf necrosis [24].

As *C. granati* represents a threat for the expansion of pomegranate cultivation in Tunisia, the search for efficient control methods is urgently needed to prevent disease propagation. Among the most important methods to control a disease is the use of resistant cultivars, which can also be a useful complementary control measure [15]. However, there is no available data on the level of susceptibility of Tunisian pomegranate cultivars to *C. granati*-induced disease. Furthermore, little information is also available on the response of pomegranate-associated vegetables and fruit trees toward this pathogen and on the extracellular enzymes secreted by *C. granati* probably included in its pathogenesis. In fact, phytopathogenic fungi are known by their ability to produce an array of extracellular hydrolytic enzymes such as cutinases, cellulases, pectinases, xylanases and proteases that can degrade the plant cell wall components and favor penetration and spreading of fungal pathogens within plant tissues thus acting as virulence factors [25].

Therefore, the aim of the current investigation was to (i) assess the response of pomegranate cultivars to *C. granati*, (ii) determine the host range of this emergent pathogen, and (iii) identify its extracellular cell wall-degrading enzymes. This study will provide important and useful information for a better understanding on the development and the potential spreading of this emerging disease.

Materials and Methods

Pathogen isolation and culture

Pomegranate twigs showing necrosis and dieback symptoms and decayed fruits were collected in 2016 from naturally infected orchards located in Sousse region, East coast Tunisia. Symptomatic twigs and fruits were cut into 0.5 cm-pieces, surface-disinfected in 10% NaOCl for 3 min, rinsed thrice 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). After incubation for 10-15 days at 25°C, developing fungal cultures were purified by hyphal tip transfer on freshly poured PDA medium. Three single-spore *C. granati* isolates (namely Cg1, Cg2 and Cg10) were used in the present investigation. Cg1 and Cg2 were isolated from symptomatic pomegranate twigs whereas Cg10 was recovered from decayed fruits.

These isolates were maintained on PDA medium at 4°C and are held in the laboratory of Phytopathology at the Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia.

Before use, isolates were grown at 25°C on PDA medium for 10-15 days.

Plant material

Nine Tunisian pomegranate cultivars (namely cvs. Gabsi, Zaghouani, Garroussi, Chetwi, Zehri, Khedhri, Tounsi, Kalai and Jebali) were used in the current study to assess their response to *C. granati* infection on detached fruits and branches.

To determine the host range of *C. granati*, six rosaceous fruit tree species that are commonly grown in Tunisia including apricot (*Prunus armeniaca.* L.), apple (*Malus domestica*), Pear (*Pyrus communis* L.), peach (*Prunus persica*), prune (*Prunus domestica*), and loquat (*Eriobotrya japonica*) together with three non-rosaceous fruit tree species, orange (*Citrus Sinensis*), fig (*Ficus carica*) and olive (*Olea europaea*), were tested for their relative susceptibility to pathogen on detached branches.

Host range investigations were also carried out using detached fruits of peach, loquat, apricot, apple, and lemon (*Citrus limon* L.), and also on other fruits species including strawberry (*Fragaria vesca* L.), tomato (*Solanum lycopersicum* L.), pepper (*Capsicum annuum* L.), fakous (*Cucurbita melo* var. *flexuosus* (L.) Naudin)) and zucchini (*Cucurbita pepo* L.).

All detached pomegranate branches and fruits were collected from visibly symptomless trees grown in the experimental station of the Regional Research Centre on Horticulture and Organic Agriculture and the Technical Center of Organic Agriculture, in Chott-Mariem region, whereas the other fruits were purchased from local market.

Detached fruit inoculation

Ripe middle size fruits were surface-sterilized in **10%** NaOCl for 5 min, washed thrice with SDW and allowed to dry at ambient temperature on sterilized filter papers. Fruit inoculation was performed using mycelial plugs, obtained from 7-day-old culture of *C. granati* isolate grown on PDA medium, which was inserted into each wound (3 mm in depth and 6 mm in diameter) performed on fruits using a sterile cork borer (one plug per fruit). Similarly wounded fruits inoculated with non-colonized agar plugs served as controls.

Detached branch inoculation

Apparently healthy and intact segments of tree branches (15 cm long and 1 to 1.6 cm in diameter) were taken away, directly brought to laboratory to ovoid desiccation and disinfected with 10% NaOCl (3 min) and 70% ethanol (1 min). Three alternate wounds (3 mm in diameter and in depth) were made in these disinfected segments using a sterile cork borer and mycelium plugs (3 mm diameter), cut from 7-days-old culture, were inserted into each wound and the inoculated area was wrapped with parafilm. Control branches were similarly wounded and inoculated with non-colonized PDA plugs.

Assessment of cultivar response to *Coniella granati* infection

For the assessment of pomegranate cultivar response, one isolate of *C. granati*, Cg10, was chosen for fruit and branch inoculation tests since this isolate was shown, in previous work (Jabnoun-Khiareddine et al., unpublished data) to be the most aggressive one on pomegranate fruits, leaves and branches.

Detached fruit test

All inoculated and pathogen-free pomegranate fruits, belonging to the nine tested cultivars, were placed in plastic boxes and maintained at 70% relative humidity at 25°C for 9 days. After this incubation period, fruits were examined and the mean diameter of the developed external lesion was measured. Ten fruits were used for each individual treatment.

Detached branch test

Inoculated and control pomegranate segments belonging to the nine tested cultivars were incubated in humid chambers and maintained at 25°C for 30 days. Twelve replicate branch segments were used for each individual treatment.

Altered segments, with the outer and inner bark removed, were also included in the inoculation tests and treated as described above in order to prove pathogen growth on old detached branches left in

orchards. They were incubated in humid chambers as described above and maintained at 25°C for only 12 days.

For each intact branch segment, the length of the induced external and internal necrosis or lesion was measured after 30 days of incubation and the presence of pycnidia was visually examined. For the altered segments, the length of the developed external lesion together with that of the lesion bearing pycnidia were measured after 12 days of incubation.

Coniella granati host range analysis

For host range analysis, three *C. granati* isolates (Cg1, Cg2 and Cg10) were used in order to assess the fruit response to pathogen isolates derived from pomegranate twigs (Cg1 and Cg2) and from pomegranate decayed fruits (Cg10). However, for branch inoculation tests, the most aggressive isolate, Cg10 isolate was chosen.

Detached fruits trial

Inoculated and pathogen-free fruits belonging to tree (peach, loquat, apricot, apple, and lemon) and herbaceous species (tomato, pepper, strawberry, fakous, and zucchini) were incubated for 7 days at 25°C under high relative humidity. After this period, the external mean lesion diameter of the occasioned rot, developed from inoculation sites, was measured. Five fruits were used per species and per treatment (inoculated or non with colonized PDA plugs).

Detached branches trial

All tested branches (inoculated or non with colonized PDA plugs), belonging to nine fruit tree species (Apricot, apple, pear prune, peach, loquat, olive, orange, and fig), were incubated for 30 days at 25°C under high humidity. Ten replicate branch segments were used for each individual treatment.

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.

Detection of Extracellular Enzymes Production

The three *C. granati* isolates tested were screened for their ability to produce cell wall degrading enzymes (amylase, laccase, protease, pectinase, pectate transeliminase, cellulase and lipase) using a qualitative agar-plate method as follow:

Amylase activity

Amylolytic activity was assessed by growing fungal isolates on Glucose Yeast Peptone (GYP) (1 g glucose + 0.1 g yeast extract + 0.5 g peptone, 16 g agar per liter of distilled water) medium adjusted at pH 6 and amended with 0.2% soluble starch. After incubation at 25°C for 3-5 days, plates were flooded with iodine solution and the appearance of yellow areas around fungal colony in an otherwise purple medium indicated positive amylase activity [26].

Laccase activity

For laccase activity, fungal isolates were grown on GYP medium amended with 0.05 g/l 1-naphthol at pH 6. Change in color of the medium from colorless to blue, within 3 days of incubation at 25°C, indicated positive laccase activity (laccase oxidation of 1-naphthol) [26].

Protease activity

Proteolytic activity was determined by growing fungal isolates on GYP medium containing 0.4% gelatin at pH 6. After incubation at 3-5 days, the plates were flooded with aqueous solution of ammonium sulphate and the appearance of clear zone around the fungal colony in an otherwise opaque medium indicated positive protease activity [26].

Pectinase activity

To detect pectinolytic activity, *C. granati* isolates were grown on Pectin Agar medium (1 g yeast extract, 5 g pectin, 15 g agar per liter of distilled water in pH 5). After 3-5 days of incubation, the plates were flooded with 1% aqueous solution of hexadecyltrimethylammonium bromide (CTAB). The formation of a clear zone around the fungal colony indicated positive pectinase activity [26].

Pectate transeliminase activity

Pectate transeliminase activity was detected by growing fungal isolates on Pectin Agar medium adjusted at pH 7. After 3-5 days of incubation, the plates were flooded with 1% aqueous solution of hexadecyltrimethylammonium bromide (CTAB). A clear zone formed around the fungal colony indicated pectinase activity [26].

Cellulase activity

For cellulase activity, fungal isolates were grown on Yeast Extract Petone (YEP) medium (0.1 g yeast extract + 0.5 g peptone, 16 g agar per liter of distilled water) amended with 0.5% Na-carboxy-methylcellulose. After 3-5 days of incubation, the plates were flooded with 2% aqueous Congo red solution and destained with 1 M NaCl (15 min each). Appearance of yellow areas around the fungal colony in an otherwise red medium indicated positive cellulase activity [26].

Lipase activity

For lipase activity, *C. granati* isolates were grown on agar medium (Peptone 10 g, NaCl 5 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, Agar 20 g per liter of distilled water at pH 6) and supplemented with 1% of separately sterilized Tween 20. The formation of a clear zone or a precipitation around the fungal colony after inoculation and incubation at 25°C for 3-5 days, indicates positive lipase activity [26].

Statistical Analyses

Cultivar response trials were conducted according to a completely randomized design where pomegranate cultivars were the only fixed factor. Ten and twelve replicates per individual treatment were respectively used for fruit and branch trials.

For host range analysis using fruit tests, statistical analysis were performed following a completely randomized factorial design where fungal treatments (*C. granati* isolates) and plant species (tree or vegetable fruits) were the two fixed factors. Five replicates were used per individual treatment. For host range tests using detached branches, trials were conducted according to a completely randomized design where tree species were the only fixed factor. Ten replicates were used per individual treatment.

It is to note that, for all trials performed in the present study, non-inoculated controls were not included in the statistical analyses since they were symptomless and no lesions were developed on fruits or branches of pomegranate as well as of the other plant species tested.

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. Means were separated using Fisher's protected LSD or Students Neuman Keuls test (at $P \leq 0.05$).

Results

Cultivar response to *Coniella granati* infection

Comparative infection of detached fruits: All *C. granati*-inoculated pomegranate fruits developed typical symptoms of fruit rot while control fruits remained symptomless. However, the fruit lesion diameter, noted after 9 days of incubation at 25°C, varied significantly ($P \leq 0.05$) depending on cultivars tested. As shown in Figure 1, the lowest lesion diameters (of about 87-88 mm) were recorded on cvs. Gabsi and Zaghouani fruits, followed by cvs. Garroussi, Chetwi and Zehri. Conversely, disease development was highest on cv. Khedhri, followed by cvs. Tounsi and Kalai where lesion diameters reached or exceeded 120 mm.

It should be noted that all the inoculated pomegranate fruits belonging to all cultivars completely rotted within 11-20 days after inoculation.

Comparative infection of detached branches: On 1-year-old detached pomegranate branches, *C. granati* isolate Cg10 inoculated to intact and altered detached branches of nine cultivars induced typical symptoms. All infected branches showed external and internal lesions which were brownish in color, necrotic and spreading in both directions from the point of inoculation.

Intact branches: *C. granati* Cg10 isolate induced external necrotic lesions on all inoculated intact-detached branches compared to control branches which remained symptomless. In fact, after 30 days of incubation at 25°C, lesion length varied significantly ($P \leq 0.05$) depending on pomegranate cutlivars tested. Data presented in Table 1 showed that *C. granati* induced the longest external lesions on cv. Jebali reaching 29.33 mm, followed by cvs. Gabsi, Zehri and Kalai. However, the lowest lesion length was recorded on cv. Chetwi (10.45 mm).

Similarly, the internal lesion induced by *C. granati* on intact-detached pomegranate branches was significantly ($P \leq 0.05$) different depending on cultivars. As shown in Table 1, the longest internal lesion (22-27 mm) was recorded on cvs. Zehri, Jebali and Gabsi, followed by cvs. Tounsi and Kalai. In contrast, cvs. Chetwi and Garroussi developed the lowest internal lesion (8-9 mm) 30 days post-inoculation with *C. granati*.

It should be highlighted that on all inoculated branches, few pycnidia were visible around the inoculation points.

Altered detached branches: *C. granati* induced brown necrotic lesions on all altered detached branches which length differed significantly ($P \leq 0.05$) depending on tested cultivars. At 12 days post-inoculation, the necroses noted on cvs. Zaghouani, Zehri and Jebali, of about 33 mm, were longer than that noted on detached branches of the other tested cultivars, ranging between 23 and 28 mm (Table 1).

On *C. granati*-inoculated and altered detached branches, the length of lesions bearing pycnidia varied also significantly ($P \leq 0.05$) depending on tested pomegranate cultivars with cvs. Zaghouani, Zehri, Jebali and Gabsi being the most susceptible by showing the longest lesions estimated at 27.33-29.89 mm after 12 days of incubation (Table 1).

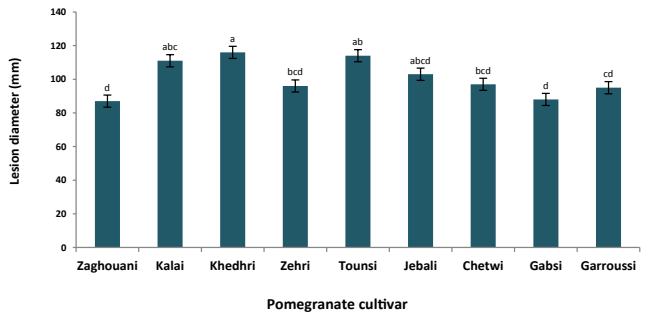

Figure 1: Diameter of *Coniella granati*-induced lesion on detached fruits of nine pomegranate cultivars noted after 9 days of incubation at 25°C. Bars sharing the same letter are not significantly different according to Student-Newman-Keul's test at $P \leq 0.05$.

Determination of *Coniella granati* Host Range

Comparative pathogenicity on detached fruits

Three isolates were chosen to determine *C. granati* host range including rosaceous and non rosaceous fruit species and also some vegetables, commonly grown in Tunisia.

The results showed that all three isolates were able to induce brown lesions on all tested hosts, except for zucchini, that started since 2 days after inoculation (Tables 2 and 3).

On tested fruits, lesion length varied significantly depending on tested hosts and isolates used (Table 2). In fact, 7 days post-inoculation, these isolates produced significantly longer lesions on apple with an average of 79 mm in diameter, compared to 45.3, 39.8,

Pomegranate cultivar	Intact detached branches		Altered detached branches	
	External lesion length (mm) ± SE	Internal lesion length (mm) ± SE	External lesion length (mm) ± SE	Lesion bearing pycnidia length (mm) ± SE
Zaghouani	18.875 ± 0.6 b[y]	16.96 ± 1.1 abc	33.22 ± 0.2 a	29.89 ± 0.3 a
Kalai	22.58 ± 0.5 ab	19.67 ± 1.1 ab	25.11 ± 0.5 b	20.22 ± 0.3 b
Khedhri	19.29 ± 0.7 b	17.33 ± 0.8 abc	24.50 ± 0.5 b	24.50 ± 0.5 ab
Zehri	25.71 ± 0.6 ab	24.38 ± 0.9 a	33.61 ± 0.1 a	28.72 ± 0.1 a
Tounsi	20.58 ± 0.8 b	20.50 ± 0.8 ab	23.39 ± 0.1 b	18.28 ± 0.5 b
Jebali	29.33 ± 0.7 a	26.71 ± 0.6 a	33.67 ± 0.04 a	27.33 ± 0.7 a
Chetwi	10.46 ± 0.1 c	7.88 ± 1.2 c	23.72 ± 0.5 b	20.78 ± 0.5 b
Gabsi	22.50 ± 0.5 ab	22.29 ± 0.7 a	26.67 ± 0.3 b	27.56 ± 0.2 a
Garroussi	20.04 ± 0.8 b	9.42 ± 0.9 bc	28.44 ± 0.2 b	21.22 ± 0.2 b

[x] Lesion lengths were noted after 30 and 12 days of incubation at 25°C for intact and altered branches, respectively.

[y] In each column, values (± standard error) sharing the same letter are not significantly different according to Student-Newman-Keuls test at $P \leq 0.05$.

Table 1: Length of external, internal and pycnidia-bearing lesions induced by *Coniella granati* on intact and altered detached branches of nine pomegranate cultivars[x].

Fruit species	*C. granati* isolates			Mean lesion diameter per fruit species[a]
	Cg1	Cg2	Cg10	
Apple	80.85 ± 1.1 a*	71.17 ± 0.3 a	85.75 ± 1.1 a	79.25 a
Apricot	44.92 ± 0.3 a	30.57 ± 0.2 b	43.98 ± 0.1 a	39.82 b
Peach	50.17 ± 0.2 a	32.96 ± 0.4 b	52.85 ± 0.1 a	45.33 b
Loquat	36.05 ± 0.1 a	33.48 ± 0.4 b	33.83 ± 0.3 a	34.46 c
Lemon	37.40 ± 0.6 a	33.90 ± 0.7 b	42.10 ± 0.1 a	37.80 bc
Mean lesion diameter per isolate[b]	43.83 a	35.96 b	44.68 a	-
[a] Mean lesion diameter per fruit species for the three isolates combined.				
[b] Mean lesion diameter per isolate for all fruit species combined.				
*For the lesion diameter noted on each fruit species, and for its mean per isolate, values followed by the same letter are not significantly different according to Student-Newman-Keul's test at $P \leq 0.05$.				

Table 2: Diameter of lesions induced by three *Coniella granati* isolates on different tree and vegetable fruits noted after 7 days of incubation at 25°C.

Fruit species	*C. granati* isolates			Mean lesion diameter per fruit species[a]
	Cg1	Cg2	Cg10	
Strawberry	24.90 ± 0.05 b*	18.60 ± 0.2 c	30.30 ± 0.2 a	24.60 c
Tomato	50.3 ± 1.1 a	51.7 ± 0.6 a	52.2 ± 0.4 a	51.40 a
Pepper	18.8 ± 0.1 b	35.3 ± 0.6 b	48 ± 1.4 a	34.03 b
Fakous	54.3 ± 0.2 a	51.7 ± 0.6 a	52.2 ± 0.4 a	52.73 a
Zucchini	12:00 AM	12:00 AM	12:00 AM	0.00 d
Mean lesion diameter per isolate[b]	29.66 b	31.46 b	36.54 a	-
[a] Mean lesion diameter per fruit species for the three isolates combined.				
[b] Mean lesion diameter per isolate for all fruit species combined.				
*For the lesion diameter noted on each fruit species, and for its mean per isolate, values followed by the same letter are not significantly different according to Student-Newman-Keul's test at $P \leq 0.05$.				
LSD (Fruit species × *Coniella granati* isolates) = 0.94 mm at $P \leq 0.05$.				

Table 3: Diameter of lesions induced by three *Coniella granati* isolates on five vegetable fruits noted after 7 days of incubation at 25°C.

37.8 and 34.5 mm noted respectively on peach, apricot, lemon, and loquat. Furthermore, for all fruits species combined isolates Cg1 and Cg10 caused significantly comparable lesion diameters on apple, apricot, peach and loquat which were higher than that caused by Cg2 isolate.

A variable response was also noted on herbaceous fruits after inoculation with *C. granati* (Table 3). In fact, the lesion diameter varied significantly ($P \leq 0.05$) depending on pathogen isolates used and fruits tested; a significant interaction was also noted between these two factors at ($P \leq 0.05$). In fact, at 7 days post-inoculation, tomato and fakous fruits showed the highest lesion diameters, with an average of 51.40 and 52.73 mm compared to 31.37 and 24.60 mm noted on pepper and strawberry, respectively. However, none of *C. granati* isolates was able to induce brown lesion on zucchini.

Significant differences were noted among pathogen isolates. In fact, Cg10 isolate occasioned the highest rot diameter compared to the other isolates.

Comparative pathogenicity on detached branches

Pathogenicity analyses conducted with mycelial inoculation of wounded detached branches showed that the different host species tested have responded differently to *C. granati* Cg10 infection (Table 4). In fact, at 30 days post-inoculation, Cg10 isolate produced lesions only on 4 (orange, apple, peach and loquat) out of 11 hosts tested. In fact the greatest lesion lengths, of about 11 mm, were noted on orange and pear branches compared to 7.5 and 8.5 mm, recorded respectively on apple and loquat branches.

Enzymatic activity of tested *Coniella granati* isolates

When grown on specific agar media, all three *C. granati* isolates tested in the present study were shown able to produce 5 out of the 7 enzymes studied (Table 5). In fact, these isolates expressed laccase, protease, cellulase, pectate-transeliminase and pectinase activities. However, none of the tested isolates were able to secrete two enzymes, lipase and amylase (Table 5).

Discussions

The disease caused by *C. granati*, associated with dieback, shoot blight and fruit rot, has been recently reported in Tunisia [24]. However, this disease might have been present for many years on pomegranate or other host plants which are naturally present within or around pomegranate orchards. Furthermore, in Tunisia, average temperatures in the spring and summer are disease conducive and the pathogen can be adapted to different climatic conditions. Given that, the knowledge of the susceptibility of Tunisian pomegranate cultivars toward this pathogen and also its host range could be useful for integrated disease management. Added to this, many efforts have been done and continue for the preservation of pomegranate genetic resources in Tunisia which is represented in the numerous cultivated pomegranate cultivars.

Therefore, the present investigation was undertaken to evaluate the behavior of known pomegranate cultivars commonly grown throughout Tunisia toward *Coniella* disease. It has been shown that all nine pomegranate cultivars tested were susceptible to *C. granati* fruit rot, with the lowest lesion diameters (87-88 mm) recorded on cvs. Gabsi and Zaghouani fruits, 9 days post-inoculation with Cg10 isolate.

Plant species	Lesion length (mm) ± SE
Orange (*Citrus Sinensis*)	11.1 ± 0.21 a*
Apple (*Malus domestica*)	7.5 ± 0.18 b
Pear (*Pyrus communis* L.)	11.1 ± 0.25 a
Loquat (*Eriobotrya japonica*)	8.5 ± 0.13 b
Peach (*Prunus persica*)	0 c
Fig (*Ficus carica*)	0 c
Prune (*Prunus domestica*)	0 c
Apricot (*Prunus armeniaca*. L.)	0 c
Olive (*Olea europaea*)	0 c
*In each column, Values (± standard error) sharing the same letters are not significantly different according to Student-Newman-Keuls test at $P \leq 0.05$.	

Table 4: Length of *Coniella granati*-induced lesion on detached intact branches of nine fruit tree species noted after 30 days of incubation at 25°C.

Enzymes tested	*C. granati* isolates		
	Cg1	Cg2	Cg10
Pectate-transeliminase	+ (4)*	+ (4)	+ (4.2)
Pectinase	+ (4.6)	+ (4.8)	+ (5)
Protease	+ (3.27)	+ (3.16)	+ (3.47)
Cellulase	+ (3)	+ (4.6)	+ (3.8)
Lipase	-	-	-
Amylase	-	-	-
Laccase	+	+	+
+: Presence of enzymatic activity; -: Absence of enzymatic activity.			
* Values in parentheses represent the mean diameter of clearing zone (mm).			

Table 5: Enzymatic activity displayed by three *Coniella granati* isolates on specific media.

From the abovementioned results, pomegranate fruits were shown to be highly susceptible to *C. granati* infection as they totally rotted within 11-20 DPI. Our results are in accordance with Thomidis [15] studies confirming the susceptibility of pomegranate to *C. granati* disease and the resulting substantial losses recorded in many countries [15]. In the same sense, Kumari and Ram [27] found that among twenty three different cultivars and forty exotic cultivars screened under natural field conditions for resistance against dry fruit rot caused by *C. granati*, only six were shown to be moderately resistant. Likewise, when studying the varietal behavior of five pomegranate cultivars, Tegta [28] found that none was resistant to *C. granati* dry fruit rot. He also showed that the fruit qualitative characters i.e. pH, titratable acidity, total sugars and reducing sugars had significant positive correlation with dry fruit rot whereas fruit ascorbic acid, total phenols and OD phenols were negatively correlated with disease development.

In the present study, the severity of dry rot induced by *C. granati* Cg10 isolate seems to be not correlated to the characteristics of pomegranate cultivars tested such as pH and sugar content. Indeed, statistically similar rot lesion diameters were noted on sweet cultivars like cvs. Gabsi and Zaghouani as well as on sweet-sour one like cv. Garroussi. In fact, in previous work, we demonstrated that acid as well as alkaline conditions favor fungus mycelial growth and could thus explain its virulence towards pomegranate sweet and sour cultivars with different pH levels. Furthermore, according to our results, skin thickness appears to be not a preference for *C. granati* as it did not influence its development. For instance, cv. Tounsi with thick skin showed comparable rot severity than that noted on cv. Kalai with less skin thickness.

The response of Tunisian pomegranate cultivars to *C. granati* infection was also estimated based on the length of the external and internal lesions and those bearing pycnidia on detached intact branches. It should be noted that according to our results, all pomegranate cultivars tested were moderately susceptible to *Coniella* infection with cv. Chetwi being moderately tolerant. However, on altered detached branches (branches devoid of bark layers), cvs. Jbali, Zehri and Zaghouani were the most susceptible to *C. granati* giving rise to longer lesions bearing pycnidia than the other cultivars. In fact, abundant pycnidia were produced and covered the entire necrotic surface developed within 12 days after inoculation. These results highlighted the importance of prunings left in orchards for inoculum multiplication and spread and the potential risk to infect wounded plants. Our results are in accordance with those of Thomidis [15] who reported that in inoculation tests, *C. granati* was pathogenic to all experimental genotypes tested.

It is also to note that the response of pomegranate cultivars to Coniella disease differed depending on tested organs i.e. fruit, intact or altered branches.

Although *C. granati* is a threat for pomegranate production, only limited information about its host range has been published. The current study presents an attempt to characterize *C. granati* virulence on pomegranate associated fruits in Tunisia. In fact, *C. granati* isolates tested were able to cause dry rot on all tested fruits i.e. apple, peach, apricot, loquat and lemon, with apple fruits being the most susceptible. Moreover, on herbaceous fruits, tomato and fakous fruits showed the highest lesion diameters compared to pepper and strawberry, while *C. granati* isolates were nonpathogenic on zucchini. In this sense, Tegeta [28] found that out of thirteen fruit hosts tested, *C. granati* could infect the fruit of seven test hosts including apple and china pear among pome fruits; apricot and plum among stone fruits and the other hosts were litchi, tomato and papaya.

Furthermore, given that the pomegranate trees were grown in mixed plantings or at least in close proximity with other fruit tree species in many regions, 9 plant species were tested using detached intact branch tests. In fact, *C. granati* was weakly pathogenic on orange and pear branches compared to apple and loquat branches. However, this fungus posed little or no virulence risk in the tested plant branches belonging to olive, fig, peach, prune and apricot trees according to our assay conditions.

This host range evaluation suggested that *C. granati* can attack most tree fruits as well as some vegetables. These diseased fruits together with diseased apple and loquat branches could act as an infection source in the field.

Although it is expected that *C. granati*, due to its ability to induce fruit rot and branch necrosis, produces a high number of degrading enzymes, the number of reports describing extracellular enzymes produced by this pathogen is rather small. In the current investigation, we assessed the ability of three *C. granati* isolates to produce extracellular enzymes using various solid media. We showed that the tested isolates are able to secrete laccase, protease, pectinase, pectate transeliminase and cellulase, however, amylolytic and lipolytic activities were not detected. In this sense, Sunitha et al. [29] reported that cellulases, amylases and pectinases are major enzymes involved in plant polysaccharide degradation along with protease. Sieber-Canavesi et al. [30] reported that the production of extracellular cellulase and pectinase by pathogens shows that the fungus is well equipped for penetration, through the decomposition of host cells. In the same sense, Gajbhyie et al. [31] reported that *Chaetomella raphigera*, the causal agent of pomegranate fruit rot, produced pectinase, cellulase, xylanase,

and protease. They concluded that the high amount of these enzymes in diseased tissues, in comparison to control, imply their probable role in pathogenesis and acts as main virulence factors in the development of fruit rot in pomegranates. Thus, the production of these cell wall degrading enzymes by *C. granati* isolates may explain their virulence towards pomegranates and the other fruits tested. As reported by Velho et al. [32], the secretion of extracellular enzymes may lead to a greater fungal adaptability on distinct host plant tissues.

Conclusion

The results of this study showed that no pomegranate cultivar was resistant to *C. granati* fruit rot and branch dieback in artificial infections and expand the host ranges of this pathogen to numerous fruit species which could act as infection sources for pomegranates. In addition, this pathogen was shown able to produce major cell wall degrading enzymes. Further researches are needed to study the production of these hydrolytic enzymes in infected tissues and their correlation with disease severity. In fact, as these enzymes have a key role in the virulence of various fungal pathogens, the development of formulations with enzyme inhibitors is needed in order to reduce fruits damage.

Our findings highlighted that *C. granati* is a potential high-threat fungal pathogen for all Tunisian pomegranate cultivars as well as for other tree fruit species. Accordingly, further investigations are urgently needed to manage this economically important disease.

Acknowledgment

This work was funded by the Ministry of Higher Education and Scientific Research in Tunisia through the budget assigned to UR13AGR09-Integrated Horticultural Production in the Tunisian Centre-East, The Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem, University of Sousse, Tunisia, and by IRESA through the funding attributed to the multidisciplinary and the multi-institutional project CONEVAL Fruits. Authors would like to thank Prof. Walid Hamada for assistance with molecular identification of pathogen isolates.

References

1. Mars M (2001) Genetic resources of grenadier (*Punica granatum* L.) in Tunisia: prospecting, conservation and diversity analysis (Doctoral dissertation). Faculty of Sciences of Tunis, El Manar University, Tunis, Tunisia.
2. Hasnaoui N, Jbir R, Mars M, Trifi M, Kamal-Eldin A, et al. (2011) Organic acids, sugars and anthocyanins contents in juices of Tunisian pomegranate fruits. Int J Food Prop 14: 741-757.
3. Mansour E, Ben Khaled A, Haddad M, Abid M, Ferchichi A, et al. (2011) Selection of pomegranate *(Punica granatum L.)* in south-eastern Tunisia. Afr J Biotechnol 10: 9352-9361.
4. Anonymous (2017) Groupement Interprofessionnel des fruits (Tunisie).
5. Mars M, Marrakchi M (1998) Conservation and enhancement of the genetic resources of grenadier (*Punica granatum* L.) in Tunisia. Plant Genet Resour Newslett 118: 35-39.
6. Ezra D, Kirshner B, Hershcovich M, Shtienberg D, Kosto I, et al. (2015) Heart rot of pomegranate: disease etiology and the events leading to development of symptoms. Plant Dis 99: 496-501.
7. Bardas GA, Tzelepis GD, Lotos L, Karaoglanidis GS (2009) First report of *Penicillium glabrum* on pomegranate *(Punica granatum)* in Greece. Plant Dis 93: 1347.
8. Khokhar I, Bajwa R, Nasim G (2013) New report of *Penicillium implicatum* causing a postharvest rot of pomegranate fruit in Pakistan. Australas Plant Dis Notes 8: 39-41.
9. Thomidis T (2014) Fruit rots of pomegranate (cv. wonderful) in Greece. Australas Plant Pathol 43: 583-588.
10. Palou L, Taberner V, Guardado A, Del Río MA, Montesinos-Herrero C, et al. (2013) Incidence and etiology of postharvest fungal diseases of pomegranate (*Punica granatum* cv. Mollar de Elche) in Spain. Phytopathol Medit 52: 478-489.
11. Kanetis L, Testempasis S, Goulas V, Samuel S, Myresiotis C, et al. (2015) Identification and mycotoxigenic capacity of fungi associated with pre- and postharvest fruit rots of pomegranates in Greece and Cyprus. Int J Food Microbiol 208: 84-92.
12. Jadhav VT, Sharma KK (2011) Integrated management of diseases in pomegranate. Acta Hortic 890: 467-474.
13. Peduto Hand F, Choudhury RA, Gubler WD (2014) First report of *Cytospora punicae* causing wood canker and branch dieback of pomegranate *(Punica granatum)* in the United States. Plant Dis 98: 853.
14. Türkölmez Ş, Çiftçi O, Serçe ÇU, Derviş S (2016) First report of *Phytophthora palmivora* causing Crown and Root Rot on pomegranate *(Punica granatum)* in Turkey. Plant Dis 100: 227.
15. Thomidis T (2015) Pathogenicity and characterization of *Pilidiella granati* causing pomegranate diseases in Greece. Eur J Plant Pathol 141: 45-50.
16. Çeliker NM, Uysal A, Çetinel B, Poyraz D (2012) Crown rot on pomegranate caused by *Coniella granati* in Turkey. Australas Plant Dis Notes 7: 161-162.
17. Chen Y, Shao DD, Zhang AF, Yang X, Zhou M, et al. (2014) First report of a fruit rot and twig blight on pomegranate *(Punica granatum)* caused by *Pilidiella granati* in Anhui Province of China. Plant Dis 98: 695.
18. Cintora-Martínez EA, Leyva-Mir SG, Ayala-Escobar V, Ávila-Quezada GD, Camacho-Tapia M, et al. (2017) Pomegranate fruit rot caused by *Pilidiella granati* in Mexico. Australas Plant Dis Notes 12: 4.
19. Michailides TJ, Puckett R, Reyes H, Morgan DP (2011) Fruit decay caused by *Pilidiella granati* in California. Phytopathology 100: S83.
20. Mincuzzi A, Garganese F, Ippolito A, Sanzani SM (2016) First report of *Pilidiella granati* causing postharvest fruit rot on pomegranate in southern Italy. J Plant Pathol 98: 377.
21. Mirabolfathy M, Groenewald JZ, Crous PW (2012) First report of Pilidiella granati causing dieback and fruit rot of pomegranate *(Punica granatum)* in Iran. Plant Dis 96: 461.
22. Palou L, Guardado A, Montesinos-Herrero C (2010) First report of *Penicillium spp*. and *Pilidiella granati* causing postharvest fruit rot of pomegranate in Spain. New Disease Reports 22: 21.
23. Sharma RL, Tegta RK (2011) Incidence of dry rot of pomegranate in Himachal Pradesh and it's management. Acta Hortic 890: 491-499.
24. Jabnoun-Khiareddine H, Ibrahim N, Aydi Ben Abdallah R, Mars M, et al. (2018) First report of *Coniella granati* causing dieback and fruit rot of pomegranate in Tunisia. New Disease Reports 37: 17.
25. Bellincampi D, Cervone F, Lionetti V (2014) Plant cell wall dynamics and wall-related susceptibility in plant-pathogen interactions. Front Plant Sci 5: 228.
26. Sun X, Guo L-D, Hyde KD (2011) Community composition of endophytic fungi in *Acer truncatum* and their role in decomposition. Fungal Divers 47: 85-95.
27. Kumari N, Ram V (2015) Evaluation of pomegranate germplasm for resistance against leaf spot and dry fruit rot (Coniella granati). Inter J Farm Sci 5: 97-104.
28. Tegta RK (2008) Studies on fruit rot of pomegranate caused by *Coniella granati* (Sacc.) Petrak and Sydow. PhD Thesis, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan (HP), India, p: 113.
29. Sunitha V, Nirmala D, Srinivas C (2013). Extracellular enzymatic activity of endophytic fungal strains isolated from medicinal plants. World J Agr Sci 9: 1-9.
30. Sieber-Canavesi F, Petrini O, Sieber TN (1991) Endophytic Leptostroma species on Picea abies, Abies alba and Abies balsamea: a cultural, biochemical and numerical study. Mycologia 83: 89-96.
31. Gajbhiye M, Sathe S, Shinde V, Kapadnis B (2016) Morphological and molecular characterization of pomegranate fruit rot pathogen, *Chaetomella raphigera*, and its virulence factors. Indian J Microbiol 56: 99-102.
32. Velho AC, Mondino P, Stadnik MJ (2018) Extracellular enzymes of *Colletotrichum fructicola* isolates associated to Apple bitter rot and Glomerella leaf spot. Mycology 9: 145-154.