Rotting fungi of pomegranate fruit from southern Italy
Symptoms, fungal characterization, and pathogenicity

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Abstract. Pomegranate (*Punica granatum* L.) is an increasingly important crop in Apulia region (southern Italy), where most of the Italian production occurs. The main yield problem is related to postharvest losses caused by fungi. The present research was conducted using fruit of various cultivars (Acco, Mollar de Elche, Wonderful, and Wonderful One), from local markets, orchards, and packinghouses. Four new postharvest fungal pathogens of pomegranate fruit were identified: *Pilidiella granati*, *Cytospora punicae*, *Talaromyces albobiverticillius,* and *Colletotrichum acutatum s.s.*. The last fungus caused antrachnose, whereas the three others rots with characteristic symptoms. The macro- and micro-morphological traits and the molecular characterization of the pathogens allowed the species identification. Koch's postulates supported the pathogenicity of these fungi. They might represent a serious threat for this promising crop.

Keywords. Pomegranate - *Pilidiella* - *Cytospora* - *Talaromyces* - *Colletotrichum*.

Champignons pourris de la grenade de Sud de l'Italie. Symptômes, caractérisation fongique et pouvoir pathogène

Résumé. *La grenade (*Punica granatum *L.) est une culture de plus en plus importante dans les Pouilles, où l'essentiel de la production italienne est produite. Le principal problème de rendement est lié aux pertes post-récolte causées par les champignons. La présente recherche a été menée à partir de fruits de divers cultivars (Akko, Mollar de Elche, Wonderful et Wonderful One), de marchés locaux, de vergers et d'ateliers de conditionnement situés dans le sud de l'Italie. Quatre nouveaux agents pathogènes fongiques post-récolte des fruits de la grenade ont été identifiés:* Pilidiella granati, Cytospora punicae, Talaromyces albobiverticillius *et* Colletotrichum acutatum ss*. Les traits macro- et micro-morphologiques et la caractérisation moléculaire des agents pathogènes ont permis l'identification de l'espèce. Les postulats de Koch soutenaient la pathogénicité de ces champignons. Ils pourraient représenter une menace sérieuse pour cette culture prometteuse..*

Mots-clés. *Grenade - Pilidiella - Cytospora - Talaromyces - Colletotrichum.*

I - Introduction

Pomegranate (*Punica granatum* L.) is an increasingly important crop in Apulia, where most of the Italian production occurs. The main yield problem is related to postharvest losses caused by fungi; indeed, in optimal conditions, pomegranates are stored for up to 6 months (Arendse, 2014). Fruit are placed in micro-perforated plastic bags, one-layer arranged in cartoon or plastic boxes and stored at 7±1°C, 90-95% RH. In local markets, orchards, and packinghouses of southern Italy, fruit of various cultivars (Wonderful, Wonderful One, Mollar de Elche, and Acco) exhibiting peculiar symptoms have been noticed: three different rots and an anthracnose. Twenty-six percent of stored pomegranate fruit showed circular brownish-yellow lesions, beginning in the crown area and quickly expanding to the entire fruit, with softening of the tissues including arils. In another 5% of fruit, circular creamy-brownish lesions, centrally darker, with tissue softening, were observed. Finally, on 18% of fruit 'Wonderful one', circular brownish lesions on the rind were detected, eventually covered by a greenish sporification present also on stamens. This last soft rotting, originating from wounds, without nesting or connection with stamen colonization, was characteristic of *Penicillium sensu lato* species. Anthracnose symptoms, typical of *Colletotrichum* species, were observed on 11% of cold-stored

pomegranate fruit cv. Wonderful coming from an Apulian packinghouse. They consisted of circular, concentric, and brown lesions with darker spots. Increasing in diameter, those soft sunken injuries merged and white mycelium and black acervuli grew on them. The aim of this study was to identify the causal agents of these postharvest diseases.

II - Materials and methods

Symptomatic pomegranate fruit were surface-sterilized by 2-min dipping in a 2% sodium hypochlorite solution and 70% ethanol spraying, rinsed in sterile water for 1 min, and air-dried. Marginal rotted tissue fragments were aseptically plated on semi-selective PDA amended with 250 mg L^{-1} of streptomycin and 250 mg L^{-1} of ampicillin, and incubated at 24±1°C in the dark. Mono-conidial colonies were sub-cultured on PDA or MEA. Initially, fungal isolates were morphologically identified according to Barnett and Hunter (1999). Species identification was confirmed by sequencing DNA barcoding regions. DNA was extracted from mycelium by Plant/Fungi DNA Isolation Kit according to the manufacturer's recommendations (Norgen, Thorold, ON, Canada). PCR reactions were performed in a 50 µL volume containing 50 ng of DNA, 10 µM of each primer, and 1× Master Mix (EmeraldAmp PCR Master Mix, Takara, Clontech, USA), using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers (ITS), an intron of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and partial sequences of actin (ACT) and glutamine synthetase (GS) genes were amplified using the primer pairs ITS5/4 (White *et al.*, 1990), GDF1/GDR1 (Guerber *et al.*, 2003), ACT-512F/ACT-783R (Carbone & Kohn, 1999) and GSF1/GSR1 (Stephenson *et al.*, 1997), respectively. Amplification of the β-tubulin gene (TUB2) was performed with primers Bt2a and Bt2b (Glass & Donaldson, 1995). Amplification products were sequenced by Macrogen Europe (Amsterdam, The Netherlands) and the sequences were deposited in GenBank. For pathogenicity tests, surface-sterilized fruit of two cultivars were wounded with a cork-borer (5 mm Ø) at two opposite sides along the equatorial axis and inoculated with mycelial plugs. Sterile plugs were used as controls. Fruit were incubated in the dark at 24-28°C and high relative humidity for 5-14 days.

III - Results and discussion

Colonies obtained from the first type of dry rot were white to creamy, leathery, and covered by abundant dark-greenish-brown to black spherical pycnidia (80-140 µm Ø) with thin membranous walls. Hyphae were septate, and conidia hyaline, one-celled, 10-17.5×2-5 µm, ellipsoid to fusiform, straight or slightly curved. These characteristics corresponded to *Pilidiella granati* (Saccardo) (Berlese & Voglino, 1886). The other soft-rotted tissues originated whitish colonies, turning olive green and dark brown at maturity, were covered by globose dark brown pycnidia (200-250 µm Ø). Conidia were 4-6×1-2 µm in size, allantoid, hyaline, and aseptate. These morphological features identified the fungus as *Cytospora punicae* (Saccardo, 1884). Finally, the last colonies were velvety and 25-28 mm in size after 7 days at 24°C. The 3-8 acerose phialides (8-13×2-3 µm) were slender aculeate or lanceolate, highlighting the symmetrical, biverticillate conidiophores; interlaced hyphae constituted the layers of the typical soft ascomatal wall; mature asci were chain-like. Pinkish-white conidia, from globose to sub-globose, measured 2-4×1.5-2.5 µm. The divergent metulae (8-13×1.5-4.5 µm, each) were 3-8. Stipes were 200-400×2.5-4 µm with a smooth surface. These traits, together with the production of red soluble pigments, identified the pathogen as *Talaromyces albobiverticillius* Samson *et al.* (Yilmaz *et al.*, 2014). *Colletotrichum* sp. colonies were fluffy, initially whitish with a salmon-greyish reverse, then peachy-pink and covered by salmon conidial masses. Single elliptical-fusiform conidia measured 8.47-14.05×3.07-5.37 µm. These characteristics corresponded to *C. acutatum sensu stricto* (*s.s.*) Simmonds (Damm *et al.*, 2012). The identification of *T. albobiverticillius* (M49-1 strain) was confirmed by sequencing of a portion of β-tubulin gene. The amplicon (accession No. KY563698) showed 100% identity with other sequences deposited in the GenBank. *P. granati* (strain M0_C2) and *C. punicae* (M76-Bn) identities were confirmed by

sequencing ITS region. The 506 (accession No. KU821701) and 606 (accession No. KY496629) bp amplicons proved 100% identical to reference strains. Finally, a multilocus approach (using primer pairs ITS5/4, ACT512F/783R, Bt2a/2b, GDF1/R1, and GSF1/R1) confirmed the identification of M146-2 as *C. acutatum sensu stricto*. The related amplicons (accession Nos.: MF581923, MF581920, MF581919, MF581921, and MF581922) were 100% identical to reference sequences. For pathogenicity assays *T. albobiverticillius* and *C. punicae* were tested on cvs Acco and Wonderful One, *P. granati* on cvs Wonderful and Mollar de Elche, and *C. acutatum* on cvs Acco and Wonderful fruit. The typical symptoms of each disease developed only on the inoculated fruits and each re-isolated fungus corresponded to the tested causal agent, fulfilling Koch's postulates. *P. granati* has been already reported as postharvest rot agent of pomegranate in Spain (Palou *et al.*, 2010). *T. albobiverticillius* has never been reported on pomegranate fruit; however, being mistaken with a *Penicillium sensu stricto*, its actual incidence might have been underestimated (Samson *et al.*, 2011). *C. punicae* has been reported as a rot agent of pomegranate fruit in South Africa (Venter *et al.*, 2017). Finally, although *Colletotrichum* has already been reported on pomegranate fruit (Thomidis and Exadaktylou, 2011), its identification at species level (as *C. gloeosporioides*) was probably inaccurate since not relying on a multilocus approach. As such our study identify the antrachnose causal agent as *C. acutatum s.s.* within *C. acutatum* "complex" that includes some phylogenetically closely related species.

IV - Conclusions

In this study, *P. granati*, *T. albobiverticillius*, *C. punicae,* and *C. acutatum s.s.* were identified for the first time as causal agents of postharvest rots of *P. granatum* fruit in southern Italy. *P. granati* and *C. punicae* might infect both the pomegranate shrub and fruit, so they might represent a serious threat both in the field and in postharvest. These pathogens together with *T. albobiverticillius* and *C. acutatum s.s.* deserve attention since may cause an important economic loss on a product with high added value. Overall, fruit of cv. Acco proved to be the most susceptible; on these fruits the lesions were doubled compared to cv. Wonderful One.

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