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Short note

First report of *Neofusicoccum parvum* and *Phytophthora palmivora* causing fruit rot of pomegranate in Italy

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Abstract
Severe pomegranate yield losses due to fruit diseases have recently been observed in several orchards in Veneto (north-eastern Italy). Given the economic relevance of these emerging diseases, an in-depth study was conducted in ten orchards distributed in the main producing areas in order to investigate the aetiology. From autumn 2020 to autumn 2023, eighty-two symptomatic fruits were sampled to isolate the causal agents. Based on morphology, colony appearance and DNA sequence data, seventy-seven isolates were obtained and identified. These included *Coniella granati* (Fam. Schizoparmaceae, 39 isolates), *Neofusicoccum parvum* (Fam. Botryosphaeriaceae, 29) and *Phytophthora palmivora* (Fam. Peronosporaceae, 9). Pathogenicity trials conducted on ripe pomegranate fruits confirmed the aggressiveness of the three species. Results obtained have allowed us to expand knowledge on emerging pomegranate pathogens. *Neofusicoccum parvum* and *P. palmivora* are reported here for the first time as fruit rot agents on pomegranate in Italy.

Keywords
Emerging diseases, pathogenicity, fruit rot, pomegranate

Introduction

Pomegranate (*Punica granatum* L., family: Punicaceae, order: Myrtales) is one of the most ancient cultivated fruit crops in the Mediterranean region (Adiletta et al., 2018). In the last decades pomegranate cultivation has been expanding rapidly in different geographic areas thanks to the adaptability of this crop to different environmental conditions. The growing interest is also due to an increased market demand linked to the beneficial nutritional properties of its fruit (Lansky and Newman, 2007).

In Italy, the production is concentrated especially in southern regions (Apulia and Sicily) but is also growing rapidly in central and northern regions (ISTAT, 2022). With the expansion of cultivated areas, reports of new or unusual diseases have progressively increased in all Italian regions (Pollastro et al., 2016; Linaldeddu et al., 2020; Mincuzzi et al., 2020). Among the main pomegranate diseases, the most common are related to fruit rot, known as heart or black rot disease (Tziros et al., 2008; Day and Wilkins, 2011; Munhuweyi et al., 2016). Recent studies showed that in aetiology of pomegranate fruit rot a plethora of pathogens are involved, including *Alternaria* spp. (Tziros et al., 2008; Faedda et al., 2015; Luo et al., 2017; Aloi et al., 2021), *Aspergillus tubingensis* (Guo et al., 2021), *Botryosphaeria dothidea* (Gu et al., 2021), *Botrytis* spp. (Alam et al., 2019; Testempasis et al., 2020), *Colletotrichum* spp. (Mincuzzi et al., 2017; Xavier et al., 2019a), *Coniella granati* (Levy et al., 2011; Chen et al., 2014; Mincuzzi et al., 2016; Cintora-Martínez et al., 2017; Jabnoun-Khiareddine et al., 2018; Lennox et al., 2018; Mahadevakumar et al., 2019; Novak et al., 2023), *Corynespora cassiicola* (Gajbhiye et al., 2019), *Cytospora punicae* (Venter et al., 2017; Mincuzzi et al., 2022), *Dwiroopa punicae* (Xavier et al., 2019b), *Penicillium* spp. (Labuda et al., 2004; Mincuzzi et al., 2020) and *Phoma aliena* (Palavouzis et al., 2015). In addition to fungal species, some oomycetes have also been reported as fruit rot agents, such as *P. palmivora* and *P. nicotianae* (Erwin and Ribeiro, 1996; Khosla and Bhardwaj, 2013; Markakis et al., 2017). This complex aetiology associated with fruit rot makes it difficult to develop adequate disease control measures.

Given the rapid diffusion of fruit rot diseases of pomegranate in the main producer area in north-eastern Italy and the still limited information on the pathogens involved, a study was conducted to isolate, identify and characterize the main causal agents and clarify the symptomatology.

Materials and Methods

Sampling procedure, isolation and identification of pathogens

From September 2020 to October 2023, field surveys were conducted in ten pomegranate orchards located in Veneto (north-eastern Italy) (Table 1). The plants were monitored for the presence of fruit rot symptoms during the growing season. In five sites (1-4-6-9-10), disease incidence was estimated along rows, as the number of plants with symptomatic fruits with respect to the total number of plants. In order to ascertain the causal agents of the fruit rot symptoms observed, a total of 82 symptomatic fruits were randomly chosen for sampling. Symptomatic samples were stored at 4 °C and processed in the laboratory within 24 hours.

In the laboratory, all fruit samples were visually checked, and the outer peel surface removed with a sterile scalpel after disinfection with 70% ethanol for 30 s. The fruits were also transversely and longitudinally sectioned to observe the internal symptoms. Isolations were performed from approx. 5 mm^2 fragments of fruit tissues cut aseptically from the margin of necrotic lesions. All fragments were placed in 90 mm Petri dishes containing potato-dextrose-agar (PDA, Oxoid Ltd.) and a selective media for *Phytophthora* (PDA+) as reported in Linaldeddu et al. (2023). After incubation at 20 °C for 1-5 days in the dark, hyphal tips from the emerging colonies were sub-cultured onto PDA and carrot agar (CA). The isolates obtained were initially grouped in morphotypes based on micromorphological features and colony growth patterns, including surface and reverse colony appearance observed after 7 days of incubation on PDA and CA at 20 °C in the dark.

Table 1 – Study sites information.

Study sites	Locality	Geographic Coordinates		Cultivar	Number of samples
1	Mareno di Piave	45°50'50.1"N	12°19'58.2"E	Acco	12
2	Codognè	45°52'48.7"N	12°26'41.1"E	Acco, Parfianka	6
3	Fossalta di Portogruaro	45°46'34.8"N	12°53'01.8"E	Wonderful	4
4	San Giorgio al Tagliamento	45°47'26.9"N	12°57'39.7"E	Mollar	8
5	Marcon	45°33'13.7"N	12°19'59.7"E	Acco	2
6	Urbana	45°11'35.8"N	11°26'39.5"E	Mollar, Acco	9
7	Legnaro	45°20'43.7"N	11°57'20.0"E	n/d	13
8	Sariano	45°00'09.6"N	11°24'35.2"E	Dente di cavallo	1
9	Valeggio sul Mincio	45°19'40.8"N	10°47'34.0"E	Big fruit	17
10	Valeggio sul Mincio	45°20'57.4"N	10°45'01.9"E	Big fruit	10

Molecular analysis was used to identify all isolates at species level. InstaGene Matrix (BioRad Laboratories, Hercules) was used to extract genomic DNA from the mycelium of 5-day-old cultures grown on PDA and incubated at 20 °C in the dark. The primers ITS1 and ITS4 (White et al., 1990) were used to amplify and sequence the internal transcribed spacer (ITS) regions, including the complete 5.8S gene. In addition, the primer-pairs TUBUF2/TUBUR1 (Kroon et al., 2004) were used to amplify and sequence a portion of the β-tubulin (Btub) region of *Phytophthora* isolates, whereas for Botryosphaeriaceae isolates a portion of the translation elongation factor 1 alpha (tef1-α) gene was amplified and sequenced with primers EF446f and EF1035r (Inderbitzin et al., 2010). Polymerase chain reaction (PCR) mixtures and amplification conditions were as described by Linaldeddu et al. (2023). The PCR products were purified using an ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermo Fisher Scientific Inc.) following the manufacturer's instructions. The PCR products were sequenced by the BMR Genomics DNA sequencing service (www.bmr-genomics.it) (Padova, Italy), in both directions, with the primers used for amplification. The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc. http://www.geospiza.com/finchtv) and then compared with reference sequences (ex-type culture) retrieved from GenBank using the BLASTn algorithm. ITS, Btub and *tef*1-α sequences from representative isolates obtained in this study were deposited in GenBank (Table 2).

Table 2 – Accession numbers of the sequences deposited in GenBank and number of isolates of each species obtained from fruit rot symptoms.

Fungal Species	Accession Number			Number of isolates	Number of Sites
	ITS	Btub	*tef*1-*α*		
Coniella granati	PP266025	-	-	39	8
Neofusicoccum parvum	PP266026	-	PP498840	29	7
Phytophthora palmivora	PP266027	PP498841	-	9	2

Pathogenicity test

The pathogenicity of a representative isolate of the three species obtained was assessed on mature pomegranate fruits of bittersweet cultivar. Fifteen fruits per isolate were inoculated on the fruit albedo after peel surface-disinfection with 90% ethanol for 30 s. A 5 mm disc of peel was removed with a flamed cork-borer and replaced with an agar-mycelium plug of the same size, taken from the margin of an actively growing colony on PDA. The inoculation site was covered with cotton wool soaked in sterile water. Fifteen control fruits were inoculated with a sterile PDA plug applied as described above. All inoculated fruits were kept in humid chambers at 25 °C for three days.

Re-isolation of the inoculated species was attempted by transferring 10 pieces of albedo taken around the margin of each lesion onto PDA and PDA+. Cultures obtained were grown in daylight at room temperature and then identified by morphological and molecular analysis (ITS region).

Statistical analysis

Data from the pathogenicity assay were first checked for normality, and then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using LSD Multiple Range test (P = 0.05) following one-way ANOVA. Statistical analyses were performed using XLSTAT software (Addinsoft, France).

Results and Discussion

In all investigated sites, reddish to brown soft rot or sunken necrotic lesions starting from the stamen cluster were observed on fruits in different stages of maturation (Fig. 1). Soft rot symptoms progressively enlarged superficially and internally, causing total fruit rot. Carpoptosis was associated especially with soft rot symptoms in both early and advanced stages of infection (Fig. 1a,c,d). Whereas fruits with epicarp dark brown sunken necrosis progressively blackened and mummified remaining attached to the plant (Fig. 1b,e). On mummified fruits, fungal pycnidia were often visible on the epicarp (peel) surface. Early disease symptoms appeared mainly in the calyx area after rainfalls in autumn (September). Disease incidence ranged from 10 (site 10) to 70% (site 1) with an average intensity of 20%.

Isolation performed on 82 symptomatic fruit samples yielded a total of 77 isolates among fungi and oomycota. Colonies were grouped into three morphotypes on the basis of colony appearance and morphological features (shape and size of conidia and sporangia). BLAST analysis of ITS sequences confirmed the identity of isolates as *Coniella granati* (39 isolates), *Neofusicoccum parvum* (29) and *Phytophthora palmivora* (9) (Table 2). *Neofusicoccum parvum* and *Phytophthora palmivora* identification was also confirmed by *tef*1-α and Btub sequences, respectively. In particular, *C. granati* was the main isolated species from soft rot symptoms. It was recorded in 8 sites (Table 2). *Neofusicoccum parvum* was the second most frequent species. This pathogen was associated with sunken necrotic lesions, whereas *P. palmivora* was isolated from two sites.

In the pathogenicity test, all three species proved to be pathogenic on pomegranate fruits, although with different aggressiveness (Fig. 1). Statistically significant differences emerged among the three pathogens in terms of lesion size (area) and ability to colonize fruit tissues (Table 3). In particular, *C. granati* caused extensive soft rot symptoms that quickly expanded towards internal tissues and also arils. *Phytophthora palmivora* caused extensive but superficial soft rot lesions, easily recognizable by touch. Finally, the lesions caused by *N. parvum* were less extensive but characterized

by collapse of the peel towards the albedo, confirming field observations. Neither other fungi nor oomycetes were isolated from inoculated fruits. Control fruits inoculated with sterile PDA plugs remained asymptomatic. All three species were successfully re-isolated from the margin of necrotic lesions on albedo, thus fulfilling Koch's postulates.

Table 3 – Mean lesion length (± standard deviation) caused by *Coniella granati*, *Neofusicoccum parvum* and *Phytophthora palmivora* on pomegranate fruits after 72 h post inoculation and percentage of positive re-isolations.

Species	Isolate code	Lesion surface (mm^2)*	Lesion depth (mm)*	Re-isolation frequency (%)
Coniella granati	ME34	168 ± 51a	16 ± 3a	100
Neofusicoccum parvum	ME66	30 ± 8c	12 ± 5a	100
Phytophthora palmivora	ME75	112 ± 57b	8 ± 3b	100
Control	-	9 ± 2d	2 ± 1c	-

* Values with the same letter do not differ significantly at P = 0.05, according to LSD multiple range test.

This study allowed us to clarify the symptomatology and aetiology of emerging fruit rot disease symptoms occurring on pomegranate in north-eastern Italy. During the last decade several researches have highlighted the role played by different fungi and oomycetes in pomegranate diseases, including black fruit rots, shoot blights, cankers and root rots in the main producing areas (Pollastro et al., 2016; Markakis et al., 2017; Jabnoun-Khiareddine et al., 2018; Lennox et al., 2018; Kurbetli et al., 2020). Among the three pathogens detected in this study, *C. granati* confirmed its key role in pomegranate diseases. It has been reported in North America (Michailides et al., 2010; KC and Vallad, 2016), Asia (Mirabolfathy et al., 2012; Chen et al., 2014), Africa (Lennox et al., 2018; Jabnoun-Khiareddine et al., 2008) and Europe (Tziros et al., 2008; Palou et al., 2010, Novak et al., 2023) as an agent of postharvest fruit rots, shoot blights, open longitudinal cankers on stem and branches. In Italy it was first reported in Apulia in 2016 on stem cankers and fruit rot (Pollastro et al., 2016; Mincuzzi et al., 2016). A recent study carried out in northern Italy has ascertained the primary role of *C. granati* in pomegranate canker aetiology (Linaldeddu et al., 2020).

Results obtained in this study, besides confirming the role of *C. granati* in pomegranate fruit rot in Italy, showed that other two species, *N. parvum* and *P. palmivora* are involved in the aetiology of fruit diseases. Both species are reported here for the first time as fruit rots on pomegranate in Italy. In particular, the symptoms caused by *N. parvum* on fruits were different from those of *C. granati* and *P. palmivora*. Although, *N. parvum* has long been known on pomegranate as a canker and shoot blight agent in many areas of the world, it has never been associated with fruit necrosis (Palavouzis et al., 2015; KC and Vallad, 2016; Golmohammadi et al., 2020). In Italy, this botryosphaeriaceous fungus is widespread across 14 regions, causing disease symptoms in a huge number of agricultural crops (Aiello et al., 2023). The necrotic lesions caused by *N. parvum* are less rapid than those of the other two species, but easily recognisable as they are characterized by a sunken necrotic lesion of the outer peel. From an epidemiological point of view, it is important to underline how both *N. parvum* and *C. granati* are able to produce pycnidia on the surface of mummified fruits. Both pathogens overwinter as pycnidia in mummies; this allows the two species to increase the inoculum potential.

Fig. 1 – Pomegranate fruit disease symptoms detected during field surveys: light-brown soft rot caused by *Coniella granati* (a), sunken necrotic lesions caused by *Neofusicoccum parvum* (b), dark-brown soft rot caused by *Phytophthora palmivora* (c), section of a fruit with pulp rot (d), mummified fruit with pycnidia of *N. parvum* and *C. granati* (e). Colony morphology of *Coniella granati* (f), *Neofusicoccum parvum* (g), and *Phytophthora palmivora* (h) after 7 days growth at 25 °C on PDA in the dark. Symptoms observed on ripe fruits 72h after inoculation with *Coniella granati* (i,m), *N. parvum* (j,n) and *P. palmivora* (k,o). Control (l,p).

Phytophthora palmivora is one of the most dangerous *Phytophthora* species in sub-tropical areas but it has recently been emerging as an invasive pathogen in temperate zones (Erwin and Ribeiro, 1996; Guest, 2007; Borines et al., 2014; Linaldeddu et al., 2023). The abundant production of zoospores and caducous sporangia of *P. palmivora* allows this species to have both a soilborne and airborne lifestyle. In late autumn soft rot lesions on ripe fruits were covered by a whitish mycelium with papillate sporangia. On pomegranate *P. palmivora* has been reported as a root rot agent in Turkey and Italy and on fruit in Greece (Markakis et al., 2017; Türkölmez et al., 2015; Linaldeddu et al., 2020). The most recent studies have shown that some pomegranate isolates actually belong to *P. heterospora*, a cryptic species closely related to *P. palmivora* (Scanu et al., 2021). Further studies

have ascertained the widespread and simultaneous presence of both *P. palmivora* and *P. heterospora* as root rot agents of pomegranate in Veneto (Linaldeddu, data not shown).

In conclusion, this study allowed us to expand knowledge on symptomatology and aetiology of pomegranate fruit rots in Italy, confirming that both *Phytophthora* and *Neofusicoccum* species represent a growing threat to forest ecosystems and agriculture crops (Bregant et al., 2023; Aiello et al., 2023). Given the ever-increasing number of reports of combined attacks by *Phytophthora* and Botryosphaeriaceae species it is crucial to extend the research into the pathways of these pathogens in Italian nurseries and orchards, aiming to develop early detection methods and improve disease management strategies. In particular, it appears essential to protect fruits from infection during the blooming stage.

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