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Original paper

Coniella granati and *Phytophthora palmivora*: the main pathogens involved in pomegranate dieback and mortality in north-eastern Italy

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Abstract

Severe branch dieback, root rot and sudden death symptoms have recently been observed on pomegranate in several orchards in Veneto (north-eastern Italy). Since there is little information about the aetiology of these diseases and given the growing economic interest in this crop, an in-depth study was conducted. From winter 2017 to autumn 2018, forty-five symptomatic plants in two orchards were monitored and sampled to isolate the associated pathogens. Based on morphology, colony appearance and DNA sequence data, eight species belonging to seven genera and five families were isolated and identified. These included *Botryosphaeria dothidea* and *Neofusicoccum parvum* (Botryosphaeriaceae), *Diaporthe eres* (Diaporthaceae), *Coniella granati* (Schizoparmaceae), *Cytospora punicae* (Valsaceae), *Phytophthrora palmivora*, *Phytophthora pseudocryptogea* and *Phytopythium vexans* (Peronosporaceae). Pathogenicity trials conducted on 3-year-old pomegranate seedlings showed that three species, *C. granati*, *N. parvum* and *P. palmivora* are aggressive pathogens on pomegranate. Results obtained have allowed us to establish that the number of pathogens associated with emerging diseases of pomegranate is greater than previously recognised. The species *B. dothidea*, *P. pseudocryptogea* and *P. vexans* are reported for the first time on pomegranate in Italy.

Keywords
emerging diseases; *Neofusicoccum*; *Botryosphaeria*; *Phytopythium*; *Cytospora*; pathogenicity

Introduction

Pomegranate (*Punica granatum* L.) is a small shrub native to the region extending from Iran to northern India (Mars, 2000). It has been cultivated for its edible fruits throughout the Mediterranean region since ancient times (Adiletta et al., 2018). Its adaptation to the Mediterranean climate has contributed to a wide diffusion in various countries, originating several local genotypes varying from sweet to sour in fruit taste and morphology (Holland et al., 2008; Ferrara et al., 2011). In Italy pomegranate cultivation is concentrated chiefly in the southern regions where several local genotypes are present (Calani et al., 2013; Ferrara et al., 2014).

The growing consumption of pomegranate fruits for their beneficial nutritional and medicinal properties in preventing cancer and cardiovascular disease, has favoured a rapid expansion of cultivation of this crop in several countries outside the traditional areas (Lansky and Newman, 2007; Seeram et al., 2008; Basu and Penugonda, 2009).

Together with the expansion of cultivation areas, there has been an increase of new disease reports for this crop worldwide, including foliar and fruit diseases caused by *Colletotrichum gloeosporioides*

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(Penz.) Penz. & Sacc. and *Dwiroopa punicae* K.V. Xavier, A.N. KC, J.Z. Groenew., Vallad & Crous (Uysal and Kurt, 2019; Xavier et al., 2019), branch cankers and dieback caused by *Cytospora punicae* Sacc., *Coniella granati* (Sacc.) Petr. & Syd. and *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (KC and Vallad, 2016; Thomidis and Exadaktylou, 2011; Triki et al., 2015) and root and collar rot caused by *Phytophthora* spp. (Kurbetli et al., 2020). In particular, in Italy severe attacks of *N. parvum* and *C. granati* have recently been reported in the main growing areas of central and southern regions (Pollastro et al., 2016; Riccioni et al., 2017). Since 2017, a serious decline of pomegranate plants with a notable impact on their vitality and productivity has also been observed in several orchards in Veneto (north-eastern Italy).
Since there is no information about the aetiology of this emerging disease, a survey was conducted to establish the causal agents.

Materials and Methods

Field surveys and sampling procedure

From winter 2017 to autumn 2018, the health status of pomegranate plants was monitored in two orchards located in Veneto (north-eastern Italy) (Table 1). The plants were checked for the presence of symptoms on branches and trunk (dieback, exudates, cankers and epicormic shoots), and in the collar and root system (exudates, necrosis and loss of fine roots).
A total of 40 symptomatic plants were randomly chosen for sampling. From each plant a cankered branch and a portion of soil (300 g) including fine roots around the collar was sampled. Additional necrotic root samples were collected from five plants showing sudden death symptoms in the site 2 (Table 1).

Table 1 - Information of sampling sites and number of symptomatic plants sampled

Study sites	Geographic coordinates	Cultivar	Number of plant monitored
1	45°36'58"N - 12°28'15"E	Mollar de Elche	10
2	45°56'20"N - 12°18'14"E	Dente di Cavallo	35*

*Including five plants with sudden death symptoms

Isolation of canker-causing agents

All branch samples were taken to the laboratory to be visually inspected and the outer bark surface was removed with a sterile scalpel. Longitudinal and transversal cuts were made to observe any internal symptom. Isolations were made from approx. 5 mm^2 chips of inner bark and xylem cut aseptically from the margin of necrotic lesions (Smahi et al., 2017). All chips were placed on 90 mm Petri dishes containing potato dextrose agar (PDA, Oxoid Ltd). After incubation at 25 ± 1 °C for 5–7 days in the dark, hyphal tips from the emerging fungal colonies were sub-cultured onto half-strength PDA and incubated at room temperature under natural daylight to enhance sporulation.

Isolation of root rot pathogens

In the laboratory rhizosphere soil samples were placed in a plastic box and flooded with 2 L of distilled water. After 24 h, young cork oak and elder leaves were placed on the water surface and used as baits. Boxes were kept at 18-20 °C under natural daylight and after 3-5 days, leaves showing dark spots were cut in pieces of 5 mm^2 and placed on 90 mm Petri dishes containing PDA supplemented with 100 ml/L of carrot juice, 0.015 g/L of pimaricin and 0.05 g L^{-1} of hymexazol (PDA+) (Linaldeddu et al., 2020).
Isolation of *Phytophthora* was also directly attempted from roots of five plants showing sudden death

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symptoms. Necrotic roots were cut in 3 cm long samples, rinsed in distilled water, blotted dry on filter paper and then small inner bark pieces were cut from the margin of necrotic lesions and placed onto PDA+.
Petri dishes were incubated in the dark at 20 °C and examined every 12 h. Hyphal tips from the emerging colonies were sub-cultured on carrot agar (CA) (Erwin and Ribeiro, 1996) and PDA and incubated at 20 °C in the dark. To enhance sporangia production, CA plugs (5 mm diameter) of each isolate were placed in Petri dishes containing unsterile pond water. Sporangial production was assessed every 12 h for 4 days by microscopic observation.

Identification of isolates
All isolates were initially grouped in morphotypes on the basis of colony growth characteristics including surface and reverse colony appearance observed after 7 days of incubation on PDA and CA at 25 °C in the dark and morpho-biometric data of conidia or sporangia. Measurements of conidia and sporangia were taken with the software Motic Images Plus 3.0 paired with a Moticam 10+ camera connected to a Motic BA410E microscope. In particular, *Phytophthora* isolates were grouped according to morphological descriptions provided by Erwin and Ribeiro (1996).
Molecular analysis was used to confirm the identity of all isolates at species level. InstaGene Matrix (BioRad Laboratories, Hercules) was used to extract genomic DNA from mycelium of 5-day-old cultures grown on PDA and incubated at 25 °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. Polymerase chain reaction (PCR) mixtures and amplification conditions were as described by Linaldeddu et al. (2016). The PCR products were purified using a EUROGOLD gel extraction kit (EuroClone S.p.A.) following the manufacturer's instructions.
The ITS regions were sequenced by the BMR Genomics DNA sequencing service (www.bmr-genomics.it), 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 (type material) retrieved from GenBank using the BLASTn algorithm. ITS sequences from representative isolates obtained in this study were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank).

Pathogenicity test
The pathogenicity of 7 isolates (4 fungi and 3 oomycetes), representative of the seven main species isolated in this study, was assessed on 3-year-old pomegranate seedlings in March-June 2019. Ten seedlings were inoculated with each isolate, and ten seedlings were used as controls.
The fungal isolates were inoculated in the main stem. The inoculation point was initially surface-disinfected with 90% ethanol and then a small U-shaped wound made with a flamed scalpel was inoculated with an agar-mycelium plug taken from the margin of an actively growing colony on PDA. The inoculation site was covered with cotton wool soaked in sterile water and wrapped in a piece of aluminium foil secured with masking tape. Controls were inoculated with a sterile PDA plug applied as described above.
The oomycetes were inoculated at the collar. After disinfecting the bark with 90% ethanol, the seedlings were inoculated under the bark after removing a piece of outer bark (5 mm Ø) with a sterile cork borer. A same-sized agar-mycelium plug cut from the margin of a 4-day-old CA colony was used as inoculum (Linaldeddu et al., 2020). The inoculation point was protected as described above. Ten control plants were inoculated with a sterile CA plug. All inoculated plants were kept in field conditions at 6.1 to 37.6 °C and watered regularly for 3 months.
Re-isolation of inoculated species was attempted by transferring 10 pieces of inner bark and wood tissues taken around the margin of each lesion onto PDA (fungi) and PDA+ (oomycetes). Cultures

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obtained were grown in daylight at room temperature and then identified by morphological and molecular analysis (ITS region).

Statistical analyses

Pathogenicity assay data were checked for normality, then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using Fisher's least significant differences (LSD) multiple range test ($P = 0.05$) using XLSTAT software (Addinsoft).

Results

Field surveys

Field inspections conducted over a two-year period showed the widespread presence of symptomatic pomegranate plants in both orchards. Disease incidence (estimated as number of symptomatic plants out of the total number of plants) ranged from 54% (site 2) to 71% (site 1) and the rate of mortality from 17% to 43%. Plants showed a complex symptomology including leaf chlorosis, shoot blight, branch canker and dieback, and epicormic shoots on trunk (Fig. 1a, b); in addition, several plants displayed sudden death symptoms (Fig. 1c).

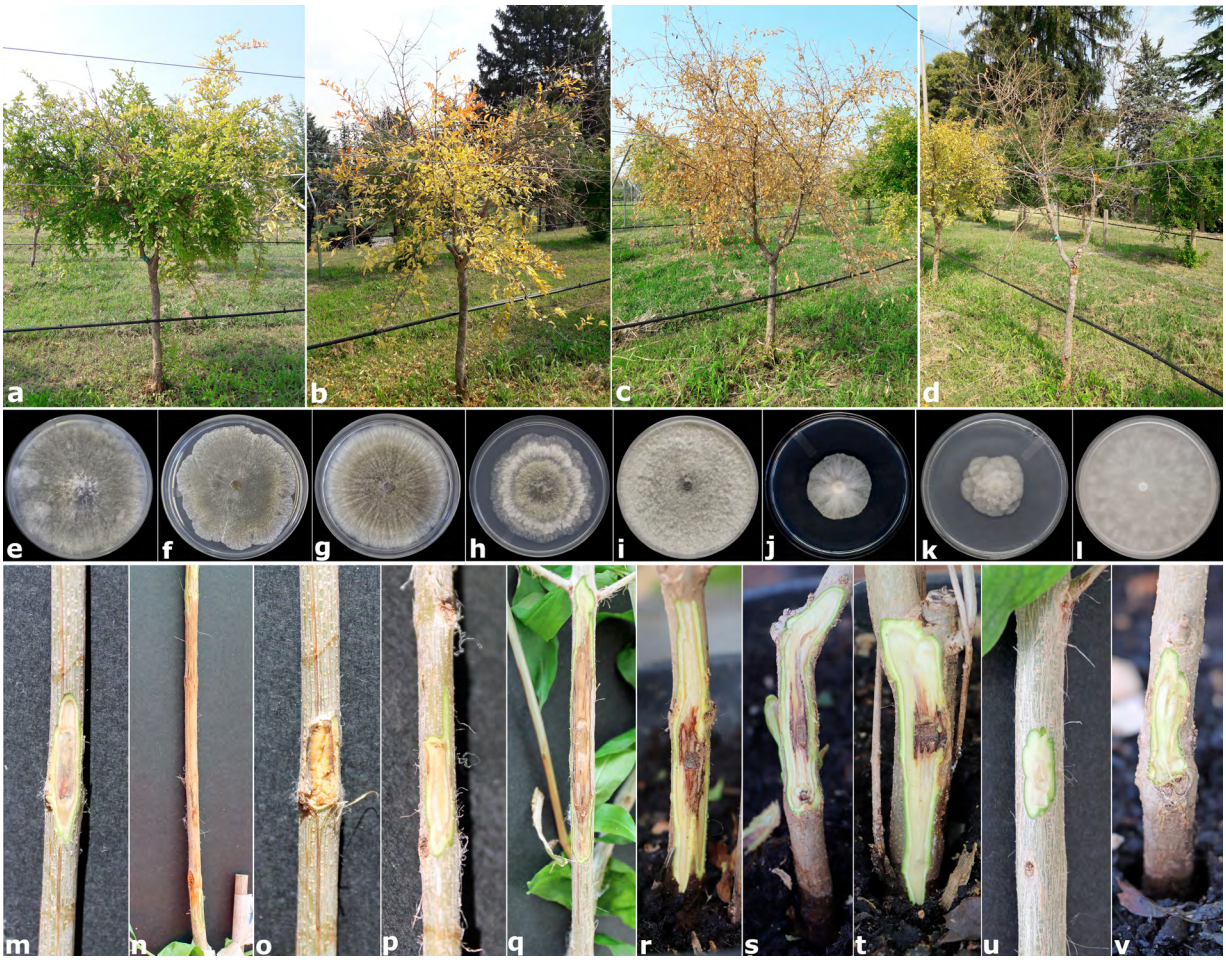

Fig. 1 - Main disease symptoms detected on pomegranate plants: progressive branch dieback and leaf chlorosis (a, b); sudden death (c); dead plant (d). Colony morphology of *Botryosphaeria dothidea* (e), *Coniella granati* (f), *Cytospora punicae* (g), *Diaporthe eres* (h), *Neofusicoccum parvum* (i), *Phytophthora palmivora* (j), *Phytophthora pseudocryptogea* (k) and *Phytopythium vexans* (l) after 7 days growth at 25 °C on PDA in the dark. Symptoms observed on pomegranate seedlings 90 days after inoculation with: *B. dothidea* (m), *C. granati* (n), *C. punicae* (o, p), *N. parvum* (q), *P. palmivora* (r), *P. pseudocryptogea* (s) and *P. vexans* (t). Asymptomatic control seedlings (u, v).

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Aetiology

Isolations performed from 40 cankered branch samples yielded a total of 51 fungal colonies. On the basis of morphological features and DNA sequence data (ITS region), five distinct species namely *C. granati* (26 isolates from both sites), *Botryosphaeria dothidea* (Moug.) Ces. & De Not. (9 isolates from both sites), *C. punicae* (8 isolates from both sites), *N. parvum* (6 isolates from the site 1) and *Diaporthe eres* Nitschke (2 isolates from the site 1) were identified (Fig. 1e-l).

Out of 40 plants investigated 36 were found to be infected by *Phytophthora* species. In total, 37 isolates belonging to two species, namely *Phytophthora palmivora* (E.J. Butler) E.J. Butler and *P. pseudocryptogea* Safaief., Mostowf., G.E. Hardy & T.I. Burgess were obtained from rhizosphere soil samples collected around the plant collar. All isolates conformed morphologically to the formal description of each *Phytophthora* species available in the literature (Erwin and Ribeiro, 1996; Safaiefarahani et al., 2015).

Phytophthora palmivora was the most common species, with an isolation frequency of 72.5%, whereas *P. pseudocryptogea* was isolated from the 20% of plants. Furthermore, *P. palmivora* was the only species obtained from the necrotic roots collected from the five plants with sudden death symptoms (Fig. 1c). From rhizosphere soil samples of 12 plants of the site 2, colonies of *Phytopythium vexans* (de Bary) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque were also obtained.

For each species BLAST searches against GenBank showed 100% identity to reference sequences of ex-type isolates and/or representative strains. The ITS sequence of a representative isolate of each species was deposited in GenBank: *B. dothidea* (MT611222), *C. granati* (MT611223), *C. punicae* (MT611224), *D. eres* (MT611225), *N. parvum* (MT611226), *P. palmivora* (MT611227), *P. pseudocryptogea* (MT611228) and *P. vexans* (MT611229).

Pathogenicity

At the end of the experimental period, all seedlings inoculated with *B. dothidea*, *C. granati*, *N. parvum*, *P. palmivora*, *P. pseudocryptogea* and *P. vexans* displayed necrotic lesions that spread up and down from the inoculation site (Fig. 1), whereas no disease symptoms were observed on seedlings inoculated with *C. punicae* and the stem wounds had begun to heal (Fig. 1o, p).

The average lesion length caused by the four fungal species differed significantly (Fig. 2a). Lesions caused by *C. granati* on the stem were significantly larger than those caused by other species. In addition, all plants inoculated with *C. granati* displayed wilting symptoms, congruent with field observations. The necrotic lesions caused by *N. parvum* appeared depressed due to the collapse of affected inner bark on xylem tissues.

No significant difference occurred among necrotic lesion sizes caused by *P. palmivora*, *P. pseudocryptogea* and *P. vexans* at the collar, although the longest lesions were detected on seedlings inoculated with *P. palmivora* (Fig. 2b). It is interesting to report that all the plants inoculated with *P. palmivora* showed leaf chlorosis.

Control seedlings inoculated with sterile PDA or CA plugs remained symptomless (Fig. 1u, v). All seven inoculated species were successfully re-isolated from the margin of symptomatic tissues of inoculated plants, thus fulfilling Koch's postulates.

Discussion

Results obtained have allowed us to clarify the aetiology of serious disease symptoms occurring on pomegranate in Veneto (north-eastern Italy). In recent years, several studies have been carried out throughout the world about postharvest fruit rots, shoot blights, branch cankers and root rots agents on pomegranate (Pollastro et al., 2016; Markakis et al., 2017; Jabnoun-Khiareddine et al., 2018; Lennox et al., 2018; Kurbetli et al., 2020). However, relatively few of these studies have reported the occurrence of two or more symptoms or pathogens in the same plant or orchard. In this study a

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complex symptomatology and aetiology was observed in both orchards. Pomegranate plants were characterized by different canopy and root disease symptoms.

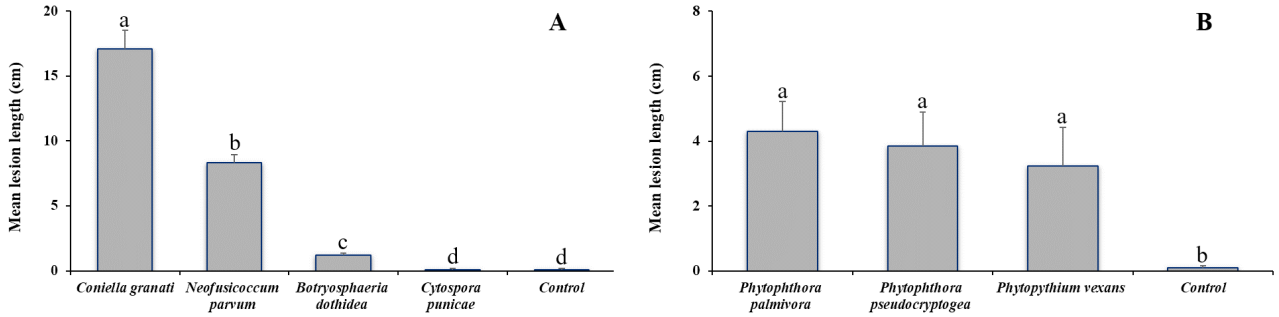

Fig. 2 - Mean lesion length (cm) caused by *Botryosphaeria dothidea* (strain MEL6), *Coniella granati* (MEL2), *Cytospora punicae* (MP4a) and *Neofusicoccum parvum* (MEL4) on the stem (A) and *Phytophthora palmivora* (MP24), *Phytophthora pseudocryptogea* (MP1) and *Phytopythium vexans* (MP43) at the collar (B) after 90 days on pomegranate seedlings. Error bars represent the standard deviations from the mean. Values with the same letter above the bar do not differ significantly at P = 0.05 according to LSD multiple range test.

Using comparisons of ITS sequence data combined with morphological and cultural features, eight species belonging to seven genera and five families were identified from a collection of symptomatic pomegranate samples. These included *B. dothidea* and *N. parvum* (Botryosphaeriaceae), *D. eres* (Diaporthaceae), *C. granati* (Schizoparmaceae), *C. punicae* (Valsaceae), *P. palmivora*, *P. pseudocryptogea* and *P. vexans* (Peronosporaceae). These findings show that the diversity of pathogenic species associated with canopy and root symptoms of pomegranate is greater than previously recognised. To our knowledge, *B. dothidea*, *P. pseudocryptogea* and *P. vexans* are reported for the first time on pomegranate in Italy.

The pathogenicity of all main species obtained in this study was confirmed through an inoculation experiment. All species tested, except *C. punicae*, were able to cause necrotic lesions and other canopy symptoms. In particular, the isolate of *C. granati*, *N. parvum* and *P. palmivora* proved to be highly aggressive. All these three species have recently been reported on pomegranate in different countries worldwide. In particular, *C. granati* has been reported in California (Michailides et al., 2010), Spain (Palou et al., 2010), Iran (Mirabolfathy et al., 2012), Turkey (Celiker et al., 2012), China (Chen et al., 2014), Greece (Thomidis, 2015) and Italy (Pollastro et al., 2016) as an agent of postharvest fruit rots, shoot blights, branch and stem cankers.

In this study *C. granati* was the main species associated with branch cankers and canopy dieback. Interestingly, the plants positive to *C. granati* were also positive to *P. palmivora*, a plurivorous pathogen recently reported on pomegranate in Greece and Turkey (Markakis et al., 2017; Kurbetli et al., 2020). *Phytophthora palmivora* is one of the most important *Phytophthora* species in tropical and sub-tropical regions. It is responsible for severe diseases on economically important tropical crops such as cocoa, citrus, durian, jackfruit, oil palm, papaya and rubber tree (Erwin and Ribeiro, 1996; Guest, 2007; Borines et al., 2014; Peter and Chandramohan 2014). Annual losses due to disease outbreaks in these crops impact for over US$ 2 billion (Derevnina et al., 2016; Torres et al., 2016). The widespread presence of this pantropical pathogen in northern Italy highlights its broad adaptability to different climatic conditions. Given the co-occurrence of these two aggressive pathogens in most of the plants examined a synergistic interaction is highly plausible.

At the same time, several of the examined plants were affected by two or three species of canopy and root pathogens suggesting a multitrophic interaction. In particular, in the site 1 *N. parvun* was isolated together with *B. dothidea* and *P. pseudocryptogea*. The co-occurrence of Botryosphaeriaceae and *Phytophthora* species on the same plants has also been detected in other pathosystems (Maloney

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et al., 2004; Linaldeddu et al., 2014, 2019). Recently, the presence of multiple *Phytophthora* species associated with severe decline and mortality of pomegranate trees has been reported in Turkey (Kurbetli et al., 2020).

Another interesting aspect of this research concerns *C. punicae*, which did not cause any disease symptom on inoculated seedlings in the pathogenicity assay, although it was successfully re-isolated from all plants. *Cytospora punicae* was detected from the same branch cankers affected by *C. granati*, which in the pathogenicity test has reproduced the symptoms observed in nature. This suggests an endophytic behaviour of *C. punicae* on pomegranate. This result is in contrast with the recent report of *C. punicae* as a canker agent on pomegranate in the United States (Peduto et al., 2014) and Tunisia (Triki et al., 2015).

This study represents the most comprehensive investigation of pomegranate-causing agents to date in Italy. The complex etiological scenario which emerged in this study indicates that to develop adequate management strategies to control emerging pomegranate diseases an accurate diagnosis is necessary on all plant organs. Still, it is not clear the origin of this high diversity of pathogens present in only two orchards.

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