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Update of pistachio leaf spot caused by *Septoria pistaciarum* in light of new taxonomic advances in Italy

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ABSTRACT

Septoria leaf spot is one the most widespread disease affecting pistachio (*Pistacia vera*) in countries of the Mediterranean region. Traditionally, three species have been associated with pistachio, including *Septoria pistaciae*, *Septoria pistaciarum* and *S. pistacina.* However, recent taxonomic studies have reordered and clarified the status of *Septoria* and septoria-like pathogens affecting pistachio. In our study, field surveys conducted in the traditional Sicilian pistachio production area of Bronte revealed the presence of trees showing characteristic septoria-like leaf spot. Collected isolates were morphologically and molecularly characterized. Morphological characterization was based on conidia measurements and evaluation of mycelial growth on different artificial media. Tested media included CMA, MEA, OA, PDA, and SNA. Phylogenetic analysis was conducted on a multi-locus approach (ITS + *tef1* + *tub2*) based on Maximum Parsimony and Maximum Likelihood. Results showed that our isolates clustered with *S. pistaciarum*. Pathogenicity test was conducted in the field using conidia suspensions in order to fulfill Koch's postulates. Presence of characteristic rounded spots and pycnidia was evaluated on the inoculated leaves 9 and 23 days after inoculation. This study represents the first update on *S. pistaciarum* in Italy since its first identification in 1934.

1. Introduction

Pistachio (*Pistacia vera* L.) is cultivated in the southern regions of Italy, of which Sicily is the main production area. The provinces of Catania, Agrigento and Caltanissetta are the largest pistachio-producing areas in Italy. Currently, the commune of Bronte in Catania province is considered the most important area of Sicily for pistachio production, representing an important economic resource for the territory (Barone and Marra, 2004). In this area, pistachio is grafted on spontaneous terebinth pistachio (*Pistacia terebinthus*) plants which are grown on volcanic soils, hence the name of "natural pistachio plantings" (Barone et al., 1985). However, mainly in Agrigento and Caltanissetta Provinces, there are "new" orchards, characterized by a rational design, irrigation, fertilization, and mechanical harvest (Marino and Marra, 2019). Few and outdated studies have been conducted to investigate pistachio diseases occurring in Italy until the last two years. The most recent studies on pistachio diseases in Italy focused on canker pathogens, including *Cytospora pistaciae*, *Eutypa lata* and *Liberomyces pistaciae*, recently taxonomically reordered as *Leptosillia pistaciae* (Aiello et al., 2019; Vitale et al., 2018; Voglmayr et al., 2019). Other recent reports in Italy described fruit pathogens, such as *Arthrinium xenocordella* and *Tuberculina persicina* (Aiello et al., 2018; Mirabile and Torta, 2020).

Septoria leaf and fruit spot is one among the most important worldwide diseases on many cultivated and wild plants. *Septoria s.l.* represents a polyphyletic group of genera including many plant pathogens (Bakhshi et al., 2019; Quaedvlieg et al., 2013; Verkley et al., 2013). Although the host-association has not to be considered a strict demarcating species factor, three different *Septoria* spp. have traditionally been associated with pistachio, including *Septoria pistaciae*, *Septoria pistaciarum* and *S. pistacina.* The oldest report of this disease date back to 1842 in France when Desmaziéres (1842) described *S. pistaciae* causing leaf spot on *P. vera*. Allescher (1901) introduced *S. pistacina,* and some years later, Caracciolo (1934) reported a third species named *S. pistaciarum* in Sicily. In 1956, two sexual morphs, namely *Mycosphaerella pistacina* (for *S. pistacina*) and *Mycosphaerella pistaciarum* (for *S. pistaciarum*) have been reported by Chitzanidis (1956) in Greece. Several

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countries reported the presence of these species associated with pistachio, in particular, *S. pistaciarum* in Arizona, New Mexico (US), Greece, India, Italy, Spain, and in East-Mediterranean and Southeast Anatolian regions (Ahmad et al., 2011; Caracciolo, 1934; Eskalen et al., 2001; French et al., 2009; López-Moral et al., 2021; Sarejanni, 1935; Young and Michailides, 1989), *S. pistaciae* in the United States (California), Italy, Greece, Ukraine and Egypt (Dudka et al., 2004; Haggag et al., 2006; Michailides, 1991; Montemartini, 1931; Pantidou 1973; Pupillo and Di Caro, 1952); and *S. pistacina* in Greece, Syria and Iran (Aghajani et al., 2009; Chitzanidis, 1956; Spaulding, 1961). Recently, Crous et al. (2013) elucidated the taxonomy of *Septoria*-like pathogens associated with pistachio, revealing three genera associated with this host, specifically *Cylindroseptoria*, *Pseudocercospora* and *Septoria s. str.* Until now, no molecular data from Italy confirmed the identity of *Septoria* spp. associated with *P. vera.* Due to the lack of a conspicuous number of gene sequences in published literature, and the outdated reports based on morphological characters only, the aim of this study was (a) to molecularly characterize representative isolates of *Septoria* spp. associated with pistachio leaf spot in Sicily, using a multi-locus approach in order to confirm the identity of the causal agent, and (b) to test the pathogenicity and provide updated information for further investigations.

2. Materials and methods

2.1. Field surveys and morphological characterization

During June of 2019, pistachio trees "Bianca" from three farms of Bronte area (Sicily, Italy), showing leaf spot symptoms were surveyed. Leaves showing red-brown spots with black margins were brought to the laboratory for isolation and further investigations. Field observations, and sampling of leaf material, were conducted in springtime (May), summertime (June) and also in autumn (November) in order to observe possible reproductive structures on leaf litter material. Observations were made using an Olympus SZX-ILLB2-200 stereoscope (Olympus, Tokyo, Japan). From the symptomatic samples, small pieces of 0.2–0.3 cm^2 were surface sterilized for 1 min in 1.5% sodium hypochlorite solution, rinsed with sterile water, air dried in a laminar hood and placed on Potato Dextrose Agar (PDA, Lickson, Vicari, Italy) amended with 100 mg/L of streptomycin sulfate (Sigma–Aldrich, St. Louis, MO, USA). All the Petri plates were incubated at 25 °C for 7–10 days. The slowly-growing, septoria-like colonies were transferred to PDA plates and single-hyphae isolates were obtained from pure cultures. Due to the very slow growth of the isolates, in order to obtain larger colonies in plates for further analysis, mycelial plugs were collected into 2.0 mL Microcentrifuge tubes with sterile deionized water and vortexed at high speed for 3–5 min. Few drops of the turbid suspension were streaked on PDA. Representative single-hyphae isolates from three different surveyed farms were stored in the fungal collection of the Dipartimento di Agricoltura, Alimentazione e Ambiente, Sezione di Patologia Vegetale, University of Catania. Six representative isolates namely SD6, SE, S1, S13, S14, and S15 were selected and used for further investigations. Isolates S13, S1 and S15 were also registered in the Westerdijk Fungal Biodiversity Institute (CBS culture collection), Utrecht, The Netherlands as follows: CBS 146141, CBS 146142, and CBS 146143, respectively.

A total of five artificial media were evaluated for the optimal mycelial growth, including: corn meal agar (CMA, Oxoid, UK), malt extract agar (MEA, Lickson, Vicari, Italy), oatmeal agar (OA, Difco, Detroit, MI, USA), PDA, and synthetic nutrient agar (per Liter of SNA, 1g of KH_2PO_4; 1g of KNO_3; 0.5g $MgSO_4$ * $7H_2O$; 0.5g of KCl; 0.2g of Glucose; 0.2g of Saccharose; 20g of Bacto Agar) following recipe label instructions. A 5-mm plug of a 7-days old colony of isolate CBS 146142 (S1) was removed and transferred to the center of a 90-mm Petri plate, and the plates were incubated at 25 °C for 14 days. Four Petri plates were used for each tested media. The experiment was repeated once. Two perpendicular diameters were recorded after 14 days of incubation, and data were transformed to radial growth rate. Data were analyzed with ANOVA and mean differences were compared with Fisher's least significance difference test (LSD) at $P = 0.05$ using Statistix 10 (Analytical Software, 2013). Additionally, some isolates were cultivated on water agar (WA) containing sterile stinging nettle (*Urtica dioica*) stems but were not considered as a treatment in this assay.

In order to study conidial morphology, a total of 50 conidia were collected from 7-days-old colonies of representative isolates grown on PDA. Length and width were measured using an Olympus-BX61 fluorescence microscope (Olympus, Tokyo, Japan) coupled to an Olympus DP70 digital camera; images and measurements were captured using the software analySIS 3.2 (Soft Imaging System GmbH, Münster, Germany) and DP Controller 1.1.1.89 (Olympus Optical Co., LTD).

2.2. DNA extraction and amplification

Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation, WI, USA) and Gentra Puregene Yeast/Bact. Kit (Qiagen). The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA operon was amplified with primers ITS5 and ITS4 (White et al., 1990), the primers EF1-728F (Carbone and Kohn, 1999) and EF-2 (O'Donnell et al., 1998) were used to amplify part of the translation elongation factor 1-alpha gene (*tef1*), and primers set T1 (O'Donnell and Cigelnik, 1997) and β-Sandy-R (Stukenbrock et al., 2012) were used for the partial beta-tubulin (*tub2*). PCR amplification conditions for ITS were set as follows: initial denaturation temperature of 94 °C for 5 min, followed by 35 cycles at the denaturation temperature of 94 °C for 30 s, annealing temperature of 48 °C for 50 s, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min. PCR conditions for *tef1* and *tub2*, were: 96 °C for 2 min, 40 cycles at 96 °C for 45 s (denaturation), 52 °C for 30 s (annealing), 72 °C for 90 s (extension) and final extension at 72 °C for 2 min. The PCR products were purified and sequenced in both directions by Macrogen Inc. (Seoul, South Korea).

2.3. Phylogenetic analysis

The DNA sequences generated were analyzed and computed using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar et al., 2018). Sequences were submitted to GenBank. The sequences obtained in this study were preliminarily blasted against the NCBIs GenBank nucleotide database to determine the closest relatives. Multiple sequences alignment was conducted based on the available recent literature (Crous et al., 2013; Quaedvlieg et al., 2013; Verkley et al., 2013) using MEGA X. A total of 34 taxa were considered in the analysis, and *Dothistroma pini* isolates CBS 121005 and CBS 116485 served as the outgroup (Table 1). Phylogenetic analyses were based on Maximum parsimony (MP) and Maximum Likelihood (ML). Sequences of ITS, *tef1* and *tub2* were concatenated using MEGA X, and manual adjustments of alignments were made when necessary. A partition homogeneity test with heuristic search and 1000 homogeneity replicates was performed using PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0a (Swofford, 2003) to test the discrepancy among the three genes dataset. The analysis of the combined dataset was obtained with the heuristic search function and tree bisection and reconstruction (TBR) as branch swapping algorithms with the branch swapping option set on "best trees" only. Gaps

Table 1
Information and GenBank accession numbers of the isolates used in the phylogenetic analysis. Isolates in bold are of the present study.

Species	Isolate no.	Host	Location	Collector	GenBank accession no.		
					ITS	*tef1*	*tub2*
Dothistroma pini	CBS 116485	*Pinus nigra*	USA	G. Adams	JX901739	JX901625	JX902196
D. pini	CBS 121005	*Pinus pallasiana*	Russia	T. S. Bulgakov	KF251155	KF253115	KF252653
Septoria astragali	CBS 109117	*Astralagus glycyphyllos*	Austria	G.J.M. Verkley	KF251349	KF253296	KF252821
S. astragali	CBS 123878	*Astralagus glycyphyllos*	Czech Rep.	G.J.M. Verkley	KF251350	KF253297	KF252822
S. hippocastani	CPC 23103	*Aesculus* sp.	Netherlands	S.I.R. Videira	KF251563	KF253510	KF253031
S. hippocastani	CBS 411.61	*Aesculus hippocastanum*	Germany	W. Gerlach	KF251435	KF253383	KF252907
S. justiciae	CBS 128610	*Justicia procumbens*	South Korea	H.D. Shin	KF251436	KF253384	KF252908
S. justiciae	CBS 128625	*Justicia procumbens*	South Korea	H.D. Shin	KF251437	KF253385	KF252909
S. lamiicola	CBS 109112	*Lamium album*	Austria	G.J.M. Verkley	KF251445	KF253393	KF252917
S. lamiicola	CBS 109113	*Lamium album*	Austria	G.J.M. Verkley	KF251446	KF253394	KF252918
S. pistaciae	CBS 420.51	*Pistacia vera*	Italy	G. Goidánich	KF251520	KF253469	KF252989
S. pistaciarum	CPC 23116; 5DMR032	*Pistacia vera*	Turkey	K. Sarpkaya	KF442651	KF442635	KF442737
S. pistaciarum	CPC 23114; 003c	*Pistacia vera*	Turkey	K. Sarpkaya	KF442652	KF442641	KF442738
S. pistaciarum	CPC 23115; 002B	*Pistacia terebinthus*	Turkey	K. Sarpkaya	KF442653	KF442642	KF442739
S. pistaciarum	CBS 135838; 45sln034	*Pistacia vera*	Turkey	K. Sarpkaya	KF442654	KF442643	KF442740
S. pistaciarum	CBS 135839; 001A	*Pistacia vera*	Turkey	K. Sarpkaya	KF442655	KF442644	KF442741
S. pistaciarum	**CBS 146141; S13**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268214**	**MZ285907**	**MZ285913**
S. pistaciarum	**CBS 146142; S1**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268215**	**MZ285908**	**MZ285914**
S. pistaciarum	**CBS 146143; S15**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268216**	**MZ285909**	**MZ285915**
S. pistaciarum	**SD6**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268217**	**MZ285910**	**MZ285916**
S. pistaciarum	**SE**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268218**	**MZ285911**	**MZ285917**
S. pistaciarum	**S14**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268219**	**MZ285912**	**MZ285918**
S. protearum	CPC 19691	*Zanthedeschia aethiopica*	South Africa	P.W. Crous	KF251525	KF253474	KF252994
S. protearum	CBS 113114	*Geum* sp.	New Zealand	G.J.M. Verkley	KF251510	KF253459	KF252980
S. protearum	CBS 119942	*Asplenium ruta-muraria*	Germany	G.J.M. Verkley	KF251512	KF253461	KF252982
S. protearum	CBS 135477; CPC 19675	*Zanthedeschia aethiopica*	South Africa	P.W. Crous	KF251524	KF253473	KF252993
S. protearum	CBS 164.78	*Nephrolepis* sp.	New Zealand	H.J. Boesewinkel	KF251513	KF253462	KF252983
S. protearum	CBS 179.77	*Myosotis* sp.	New Zealand	H.J. Boesewinkel	KF251515	KF253464	KF252985
S. protearum	CBS 364.97	*Skimmia* sp.	Netherlands	J. de Gruyter	KF251517	KF253466	KF252986
S. protearum	CBS 390.59	*Ligustrum vulgare*	Italy	M. Ribaldi	KF251518	KF253467	KF252987
S. rumicum	CBS 503.76	*Rumex acetosa*	France	H.*A. van* der Aa	KF251529	KF253478	KF252998
S. stellariae	CBS 102376	*Stellaria media*	Netherlands	G.J.M. Verkley	KF251574	KF253521	KF253042
S. stellariae	CBS 102378	*Stellaria media*	Netherlands	G.J.M. Verkley	KF251575	KF253522	KF253043
S. stellariae	CBS 102410	*Stellaria media*	Netherlands	G.J.M. Verkley	KF251576	KF253523	KF253044

were treated as "missing", the characters were unordered and of equal weight and Maxtrees were limited to 100. Tree length (TL), consistency index (CI), retention index (RI), rescaled consistence index (RC), and Homoplasy index (HI) were calculated. A total of 1000 bootstrap replicates were performed to test the robustness of the tree topology. For the ML analysis, MrModeltest v. 2.4 (Nylander, 2004) was used to identify the best-fit model of nucleotide evolution for each gene according to the Akaike Information Criterion (AIC). The ML analysis of the combined genes was performed in GARLI v.0.951 (Zwickl, 2006), and clade support was assessed by 1000 bootstrap replicates.

2.4. Pathogenicity test

Pathogenicity test was conducted in an experimental field of the University of Catania (unsprayed orchard). A total of six trees "Bianca" grafted on *P. terebinthus* were randomly selected and one shoot on each selected tree having 25–35 leaves was inoculated by spraying an aqueous conidial suspension (8×10^5 spores/ml) on both sides of the leaves. Three shoots on three different trees (35–68 leaves) were sprayed with sterile deionized water and served as negative controls. Inoculated shoots were covered with transparent plastic bags for 48 h. Inoculum was prepared culturing the representative isolate CBS 146142 (S1) on PDA and OA for 5–7 days at 26 ± 1 °C. Disease incidence was evaluated as the percentage (%) of leaves showing characteristic spots 9 and 23 days after the inoculation. In the second evaluation, 30 random lesions were measured (length and width) and observed with the stereoscope to detect the presence of any pycnidia.

3. Results

3.1. Field surveys and morphological characterization

Symptomatic samples collected in the fields showed irregular red lesions with black margins on both sides of the leaves, usually confined by leaf veins, and increasing slightly in size with time. Sometimes, symptomatic material showed the presence of cirri exuding from the lesions (Fig. 1a–e). Stereoscopic observations conducted on leaves collected during springtime and summertime, revealed the presence only of solitary (~200 μm) or aggregated pycnidia bearing pycnidiospores (conidia). Examination of leaves collected in November showed the presence of fruiting bodies, similar to pycnidia or spermogonia. In both cases no conidia and/or spermatia have been found inside the reproductive structures, a reason why it was not possible to ascertain with confidence the identity of each of the reproductive structures. Results of isolations from symptomatic leaves showed the constant presence of a septoria-like fungus, characterized by slow growing, gray/black, immersed colonies (Fig. 1f). This fungus was able to grow on each medium, showing different pigmentation, specifically red to salmon on OA (Fig. 2a), faint white on SNA and CMA (Fig. 2b and c), white on WA containing sterile stinging nettle (Fig. 2d), and gray/black on MEA and PDA, sometimes with red pigmentation, depending on the isolate (Fig. 2e–i). Presence of conidia was observed on each tested medium, except on CMA and SNA where pycnidia formation was not observed. Differences between each medium are shown in Fig. 3. Yellow mucilaginous matrix exuded from black conidiomata, often covered by white mycelium, was

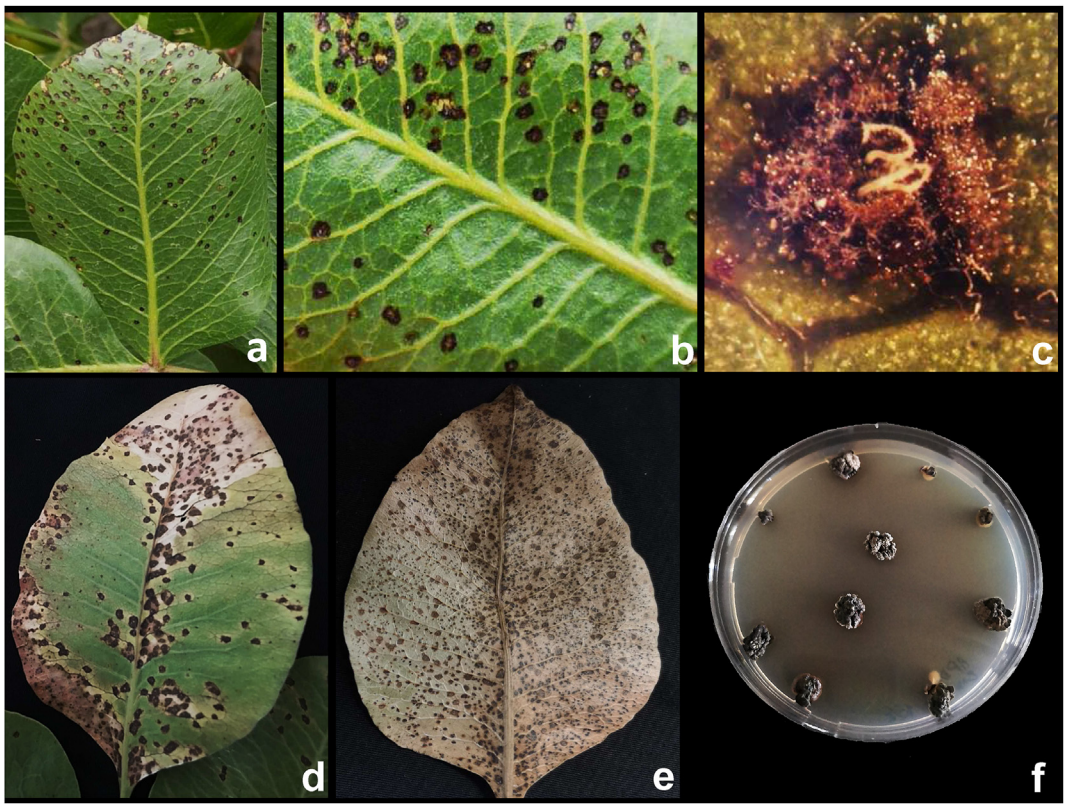

Fig. 1. Septoria leaf spot of pistachio caused by *Septoria pistaciarum*. **a**. Symptoms observed in the field (June); **b**. Details of leaf spot; **c**. Crystalline cirrus exuded from a lesion; **d.** Symptoms observed at the end of July (coalesced lesions leading to necrotic patches); **e.** Symptomatic senescent fallen leaf (November); **f.** Results of isolations showing constant presence of the same *Septoria*-like colony.

observed after 7 days from cultures grown on PDA (Fig. 4a). Mucilaginous material was also observed from colonies grown on OA and MEA after 14 days of incubation at 25 °C (Fig. 4b and c). Conidiophores (= conidiogenous cells) ~ 10 × 3 μm. Conidia were hyaline, curved to falcate, showing 1 to 5 septa (Fig. 4d and e). Measurements results, summarized in Table 2, were compared to the taxonomic key provided by Crous et al. (2013).

3.2. DNA amplification and phylogenetic analysis

For all representative isolates, PCR edited amplicons resulted in 533 bases for partial ITS region, approximately in 522 for *tef1*, and approximately in 446 for *tub2* gene. The preliminary BLASTn search resulted in high identity values (99–100%) with the species confirmed in the phylogenetic tree. The ITS, *tef1*, and *tub2* sequences generated in this study were deposited in GenBank (Table 1). Result of the partition-homogeneity test ($P = 0.441000$) indicates no significant differences in the three genes dataset. The MP analysis of the combined dataset showed that of 1599 total characters (34 taxa), 393 were parsimony-informative, 74 parsimony-uninformative, 1132 were constant. A total of 40 trees were retained. Tree length was = 783, CI = 0.775, RI = 0.923, RC = 0.715, and HI = 0.225. For ML analyses, the best-fit model of nucleotide evolution resulted SYM for ITS, GTR + G for *tef1* and GTR + I for *tub2*. The ML analysis showed that of 1599 total characters, 1132 were constant, 418 parsimony-informative, and 49 autapomorphic. Our isolates strongly clustered with *S. pistaciarum* strains (100/100, MP and ML bootstrap support %, respectively). According to our results, our isolates were identified as *S. pistaciarum* Caracc. 1934 (Fig. 5).

3.3. Pathogenicity test

The pathogenicity test revealed that *S. pistaciarum* is responsible for causing leaf spot in pistachio. At the first symptoms evaluation, occurring nine days after the inoculation, 28% of the inoculated leaves developed tiny (~1.00 mm^2) characteristic, light brown, rounded spots, still not confined by black margins. In the second evaluation, performed 23 days after inoculation, lesions enlarged and showed characteristic black margins surrounding red to dark brown necrotic spots (Fig. 4f). Lesions ranged from 1.00 to 2.00 mm^2 and 51% of inoculated leaves were infected. In the second evaluation pycnidia were observed in the lesions, isolated and aggregated together as well. Very small lesions (<1.00 mm^2) did not show pycnidia. Controls did not develop lesions. Re-isolations of *S. pistaciarum* from the leaf spots fulfilled the Koch's postulates.

4. Discussion

Results of our study confirm, in light of new taxonomic reclassification, that our isolates belong to *S. pistaciarum*, the species described by Caracciolo in 1934 in Sicily (Caracciolo, 1934). Although other authors reported *S. pistaciarum* worldwide (Ahmad et al., 2011; Eskalen et al., 2001; Mass et al., 1971; Young and Michailides, 1989) no molecular data were provided, but identification was done based on only morphological observations and conidia size measurements. For the first time, a study conducted by Crous et al. (2013) elucidated the taxonomic status of septoria-like pathogens associated with pistachio, revealing new taxa classification on the basis of a multi-locus phylogenetic analysis. Results from the study of Crous et al. (2013) led to distinguish *Cylindroseptoria pistaciae*, *Pseudocercospora pistacina* (ex *S. pistacina*),

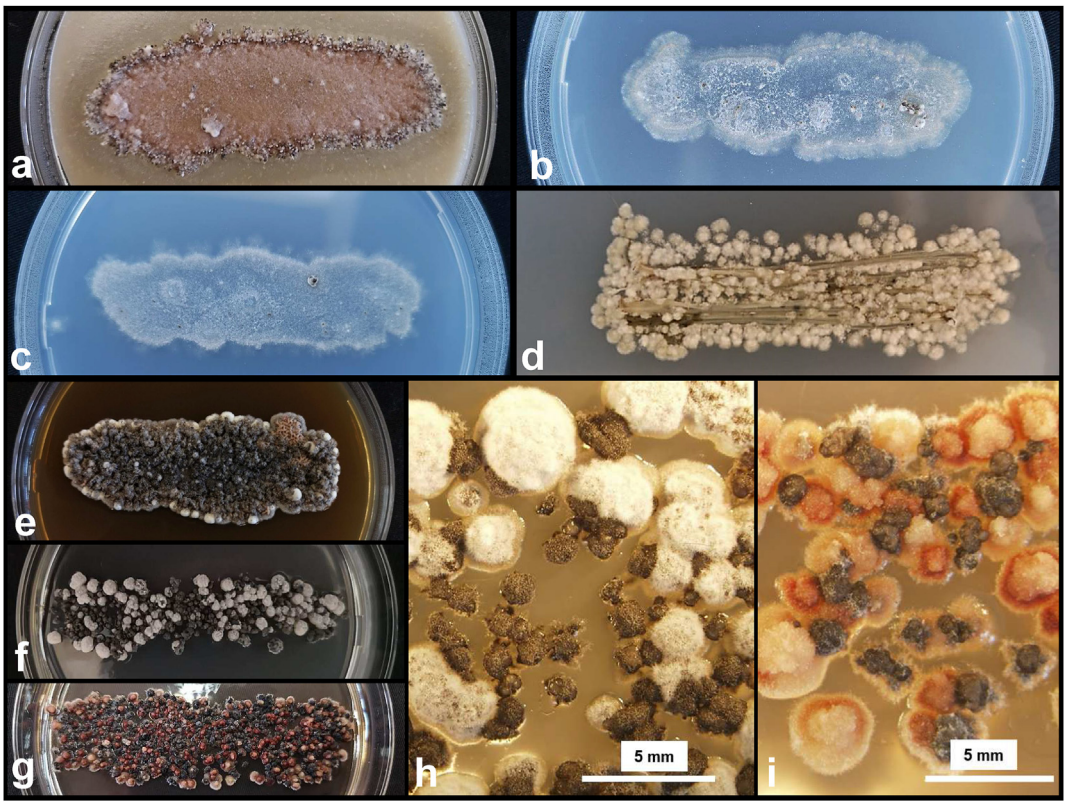

Fig. 2. Cultural characteristic of *Septoria pistaciarum*. **a-g**. 14 days old colony grown on OA (**a**); SNA (**b**); CMA (**c**); WA with stinging nettle (**d**); MEA (**e**); PDA gray/black colony (**f**), red/black colony (**g**); **h, i**. Details of two different pigmented isolates grown on PDA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

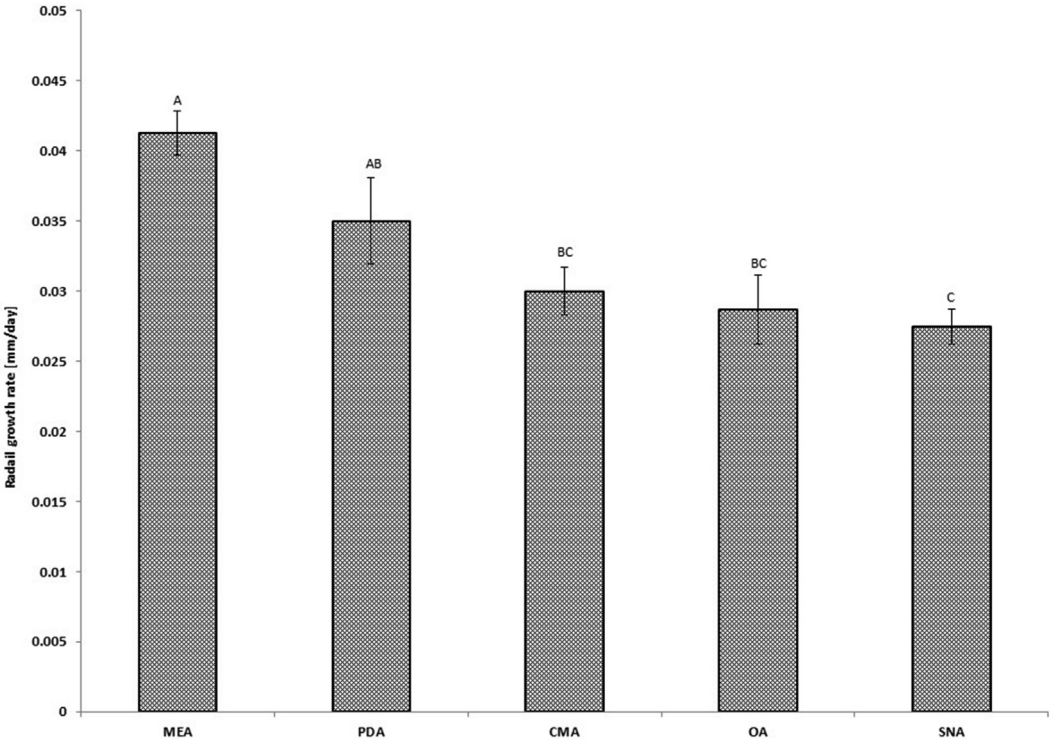

Fig. 3. Radial growth rate [mm/day] on different artificial media after 14 days of incubation at 25 °C. Letters above the columns indicate treatments significantly different ($P < 0.05$) according to the LSD test. Vertical bars are the standard errors.

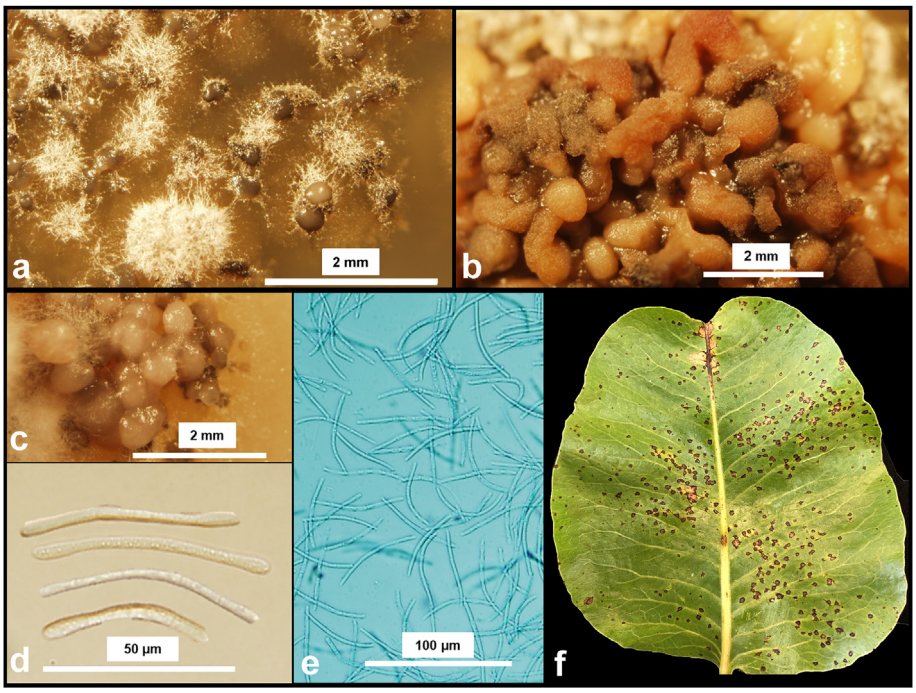

Fig. 4. Cultural feature details of *Septoria pistaciarum*. **a**. Mucilagineous matrix exuded from conidiomata on PDA; **b**, **c**. Mucilagineous material from colonies grown on MEA and OA; **d**, **e**. Conidia of *S. pistaciarum*; **f**. Pistachio leaf showing symptoms 23 days after inoculation.

Table 2
Measurements of conidia of three *Septoria pistaciarum* isolates from pistachio in Italy.

Isolate	Conidia
	Length × Width (µm)[a]
S13 CBS 146141	(23.9–) 36.9 ± 4.5 (–50.2) × (2.0–) 2.7 ± 0.5 (–3.8)
S1 CBS 146142	(21.8–) 45.2 ± 8.5 (–64.4) × (1.7–) 2.9 ± 0.5 (–4.2)
S15 CBS 146143	(32.5–) 42.4 ± 5.2 (–59.9) × (1.8–) 2.8 ± 0.6 (–4.2)

[a] L × W = length × width, (minimum –) average ± standard deviation (– maximum).

Septoria pistaciae and *S. pistaciarum*. *Septoria pistaciae* belongs to the *S. protearum* species complex. Traditionally, the classification of this extensive group of fungi was based on the host association, leading, however, to unreliable identifications. Many *Septoria* species have broader host range and many species can be found on the same host; reason why the coevolution hypothesis for *Septoria* is rejected, and the "trans-family host jumping" seems to be the major evolution force-driving for *Septoria* (Quaedvlieg et al., 2013; Verkley et al., 2013). After the first description in 1934 (Caracciolo, 1934), *S. pistaciarum* was denied by Pupillo and Di Caro (1952) who considered it a synonym of *S. pistacina*. Later, Chitzanidis (1956) confirmed the validity of *S. pistaciarum* and described the sexual stage of this species in Greece as *M. pistaciarum*, although, nowadays, the name *Mycosphaerella* referred for the mycosphaerella-like sexual morphs linked to *Septoria* should be avoided (Crous et al., 2013; Quaedvlieg et al., 2013). Results of phylogenetic analysis in this study confirmed that the isolates collected in Sicily strongly grouped into the *S. pistaciarum* clade.

Old studies reported also the presence of *S. pistaciae* in Sicily, but only morphological observations were available at that time (Montemartini, 1931; Pupillo and Di Caro, 1952). Crous et al. (2013), provided a taxonomic key (morphology) and our measurements of conidia agree with their description, although *Septoria* specimen collected in the same location, but under different environmental conditions, may result in considerable differences in conidial sizes (Jørstad, 1965). This explains why previous identification, before the molecular era, was particularly difficult and questionable. *S. pistaciarum* was able to grow on each tested media, although best results, in terms of growth and sporulation, was obtained on PDA, MEA and OA. Interestingly, conidia were observed on CMA and SNA, where pycnidia were not observed. Pupillo and Di Caro (1952) erroneously denied *S. pistaciarum*, affirming its synonymy with *S. pistacina*. Describing its morphological characters they noticed hyphal conidia, formed from fertile hypha, and this could explain our observations. Previous studies on many *Septoria* spp. show that shape of conidia on OA generally agree best with those found in the natural substrate (Verkley et al., 2013). Symptoms observed in the field, and replicated in our pathogenicity test, are in accordance with those described by other authors (Caracciolo, 1934; Chitzanidis, 1956). Spots caused by *S. pistaciarum* are easily distinguishable in the field, being more angular and confined by leaf veins (Crous et al., 2013). Our field surveys revealed the presence of leaf spot on *P. vera* as well as on *P. terebinthus*, the latter grown wild and usually used as a rootstock in Sicily, compared to what observed by Young and Michailides (1989), who did not note the disease on *P. atlantica* and *P. terebinthus* leaves. Although it does not represent the most harmful disease for pistachio production in Sicily, it seems to be the most common and widespread among different production areas of the Island. Observations conducted in Arizona (US) revealed that the onset and severity of the disease were affected by summer rainfall temperatures from 15 to 25 °C (Matheron and Call, 1998). Recent investigations in Aegina Island, Greece, showed that the optimum temperature for conidium germination is 23 °C with the appearance of first symptoms in the field around mid-May (Thomidis et al., 2021). These results agree with our observations, probably due to the very similar Mediterranean environmental conditions, the island Aegina, Greece and Sicily, Italy. Very few, sporadic and outdated data is available about the epidemiology of this fungus. In this study, conidium was the only reproductive spore observed in the field. Based on our field

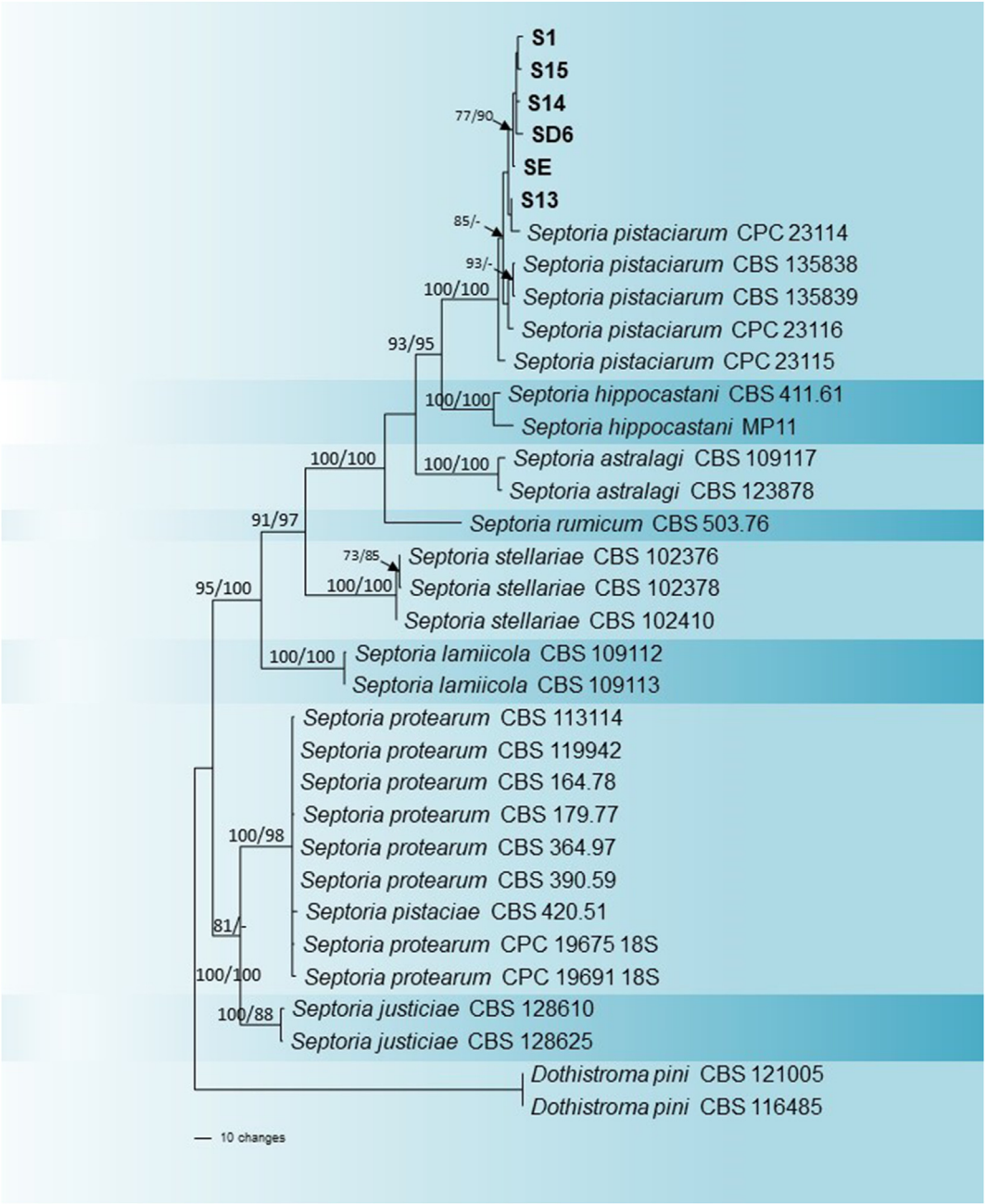

Fig. 5. One of 40 equally most parsimonious trees resulting from the analysis of the three-gene combined dataset (ITS + *tef1* + *tub2*) of known *Septoria* species and isolates of this study. Numbers in front and after the slash represent Maximum Parsimony and Maximum Likelihood bootstrap values, respectively. Values represented by "—" were less than 70%. Scale bar represents the number of nucleotide changes.

surveys, and literature available information, we hypothesize *S. pistaciarum* lifecycle characterized by three "states". As observed in this study, and confirmed by other authors (Thomidis et al., 2021), symptoms occur from springtime to the end of summertime with the imperfect form named *S. pistaciarum*, producing conidia from pycnidial conidiomata on the leaf surface. Pycnidiospores germinate on the leaf surface and penetrate through the stomata (Tzavella-Klonari, 1989). Conidia are then dispersed during the season triggering new infections. The second lifecycle state seems to be characterized by the spermatial morph named *Asteromella pistaciarum*, described for the first time in 1947 (Bremer and Petrak, 1947). *Asteromella* was traditionally assigned to those leaf-inhabiting, pycnidia-forming fungi producing tiny rod-shaped, one-celled, hyaline conidia (spermatia) (Ruszkiewicz-Michalska, 2016). However, *Asteromella* classification significantly changed over the years, being defined as a taxonomic entity (von Thümen, 1880), an anamorph genus (Sutton and Hennebert, 1994) and a supposed fertilizing agent in sexual reproduction (andromorph) (Crous, 2009; Parbery, 1996). Chitzanidis (1956) observed spermogonia and spermatia of *A. pistaciarum* in Greece from the middle of September throughout the autumn, whereas Zachos and Tzavella-Klonari (1971) in December, but nowadays no other recent findings are available to confirm this. In this study, examinations conducted in November on leaves debris showed the presence of empty fruiting bodies similar to pycnidia and/or spermogonia. Since immature pycnidia can be very small and very

similar to spermogonia, propagules formation is needed to ascertain the identity of the structure. Moreover, more than one species of leaf-inhabiting fungi produces spermatia within one leaf, and this, certainly, complicates the correct identification (Gerard Verkleij *personal communication*). In Sicily, the species *L. pistaciae* (ex *L. pistaciae*), recently described, was initially misidentified as *A. pistaciarum* (Vitale et al., 2018). The perfect form (third state) was described by Chitzanidis (1956) as *Mycosphaerella pistaciarum* in Greece. The perfect form is supposedly formed with the andromorphic contribution of *A. pistaciarum*. About the latter, Zachos and Tzavella-Klonari (1971) described in Greece the formation and development of spermogonia and ascocarps of *S. pistaciarum*. The authors observed the presence of spermatia by the tips of trichogynes, this reinforces the assumption of their role as male gametes, although they did not obtain the evidence of fusing and passing of the nucleus into the trichogyne (Zachos and Tzavella-Klonari, 1971). As ascertained for *Sphaerulina musiva* (ex *Septoria musiva*), causal agent of poplar leaf spot and canker, the spermogonial stage was identified in senescent leaves (Luley et al., 1987; Thompson, 1941; Quaedvlieg et al., 2013) and pseudothecia were obtained *in vitro* culturing spermatia with *S. musiva* cultures (Luley et al., 1987). The teleomorph state of *S. pistaciarum* produces ascospores within perithecia from the beginning of February to the beginning of March (Chitzanidis, 1956). Tzavella-Klonari and Zachos (1976) described six distinct phases of the development of perithecia of *M. pistaciarum*, showing how temperatures play a key role in its sexual reproduction. Although the possibility of ascocarps formation exists since early fall, nonoptimal temperatures lead to the ascocarps degeneration, and only low temperatures (winter) favor their full maturation (Tzavella-Klonari and Zachos, 1976). Ascospores are ready for discharge (wind and rain dispersion) in springtime (April–May) from leaf debris, being the starting point of the primary infection (Fig. 6). The possibility of additional sources of inoculum represented by overwintering mycelia in the tree (i.e., within buds, bark, etc.) should be further investigated. However, due to the lack of molecular data for the spermatial and teleomorphic states in previous literature, the lifecycle of *S. pistaciarum* needs to be entirely investigated and confirmed. Further investigations must be conducted to clarify the epidemiology of this important pathogen of pistachio. The present study represents the first update of Septoria leaf spot on pistachio in Italy since its first description in 1934, and new information is reported here to proceed with additional investigations on an important and widespread disease affecting pistachio in Sicily as well as in many other countries around the world.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

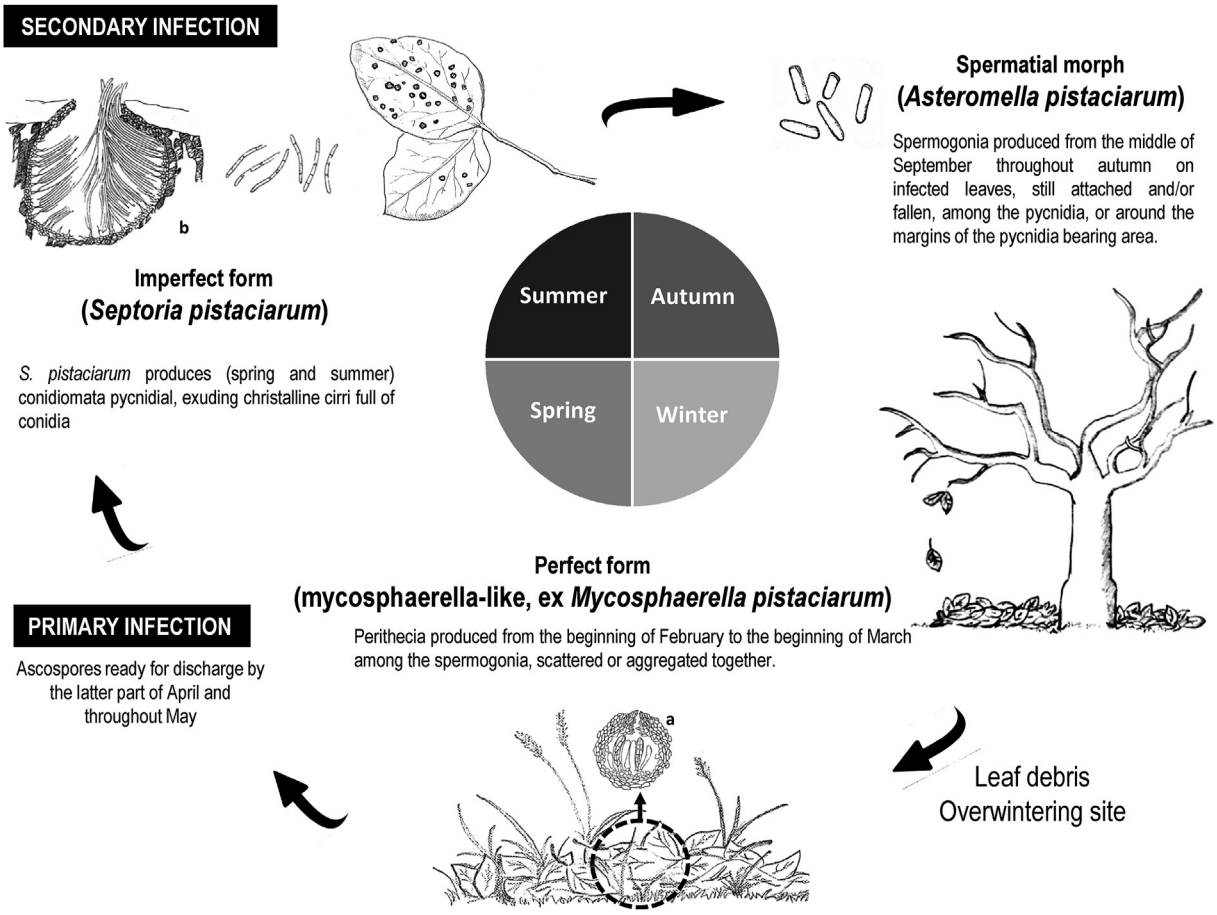

Fig. 6. Possible cycle of *Septoria pistaciarum*. Seasons details, perithecium with asci (**a**) and pycnidium with pycnidiospores (**b**) from Chitzanidis (1956). Illustrations by G. Gusella.

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