NEWSLETTER

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The ENI CBC Programme "Italy-Tunisia" 2014-2020 is a bilateral cross-border cooperation programme, co-financed by the European Union under the European Neighbourhood Instrument (ENI). With a budget of EUR 33.3 million, the programme, which joint management has been assigned to the Sicilian Region's Programming Office, aims to promote fair, equitable and sustainable economic, social and territorial development in order to foster cross-border integration and enhance the territories and resources of the two participating countries. https://www.italietunisie.eu/

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SPECIAL EDITION: SCIENTIFIC PLANT PATHOLOGY PROTOCOLS OF THE PROMETEO PROJECT

This special issue of the Prometeo periodic Newsletter has a purely scientific character and is entirely dedicated to the publication of the first part of scientific protocols on Plant Pathology, which are the result of research activities and discussions between scientific experts and stakeholders of the Prometeo project, with the objective of creating a cross-border network of collaboration between Italian and Tunisian researchers, companies and other actors to foster the development of innovative and sustainable technical solutions for the protection of Mediterranean tree crops against quarantine pathogens or emerging pests that threaten their profitability and survival.

These results will be useful to guide agricultural policies, strengthen phytosanitary ser-

vices, increase production efficiency, competitiveness and sustainability of these sectors, and improve quality standards in food safety.

Enjoy your reading!

Almond anthrachnose - Anthracnose de l'amande - Antracnosi del mandorlo *Colletotrichum* spp.

Almond anthracnose (AA) caused by *Colletotrichum* spp. has been described as one of the most important diseases of this nut crop in the main almond-growing regions worldwide, including California, Australia, Spain and Italy. Currently, AA is considered a re-emerging disease in the countries across the Mediterranean Basin due to the shift of plantations from the original crop areas to others with climatic, edaphic and orographic conditions favoring crop growing and yield. The pathogen mainly affects fruit at the youngest maturity stages, causing depressed, round and orange or brown lesions with abundant gum. The affected fruits can fall prematurely and lead to the drying of branches, causing significant economic losses in years of epidemics (López-Moral et al., 2020).

Diagnosis

***In field* - symptoms and signs**

The pathogen affects mainly almond fruit, but flowers, leaves and woody tissues can also be affected. Infected flowers become blighted, often with orange droplets of conidia on the floral cup (Palacio-Bielsa et al., 2017). However, the most characteristic symptoms of the disease are observed in green fruits. The infected fruit show depressed, round and orange or brown lesions from 5 to 12 mm in diameter that develop on the fruit surface in spring–summer and produce abundant gum **(Figure 1a).**

The diagnosis of the disease is difficult in the incipient lesions since the color of affected areas does not clearly differ from the asymptomatic epidermis. Whenever symptoms progress, abundant whitish mycelium and orange masses of conidia are produced on the surface of infected fruit. Subsequently, fruit mummify **(Figure 1b)** and fall prematurely to the soil (Förster et al., 1999; López-Moral et al., 2017; Palacio-Bielsa et al., 2017; Peres et al., 2005).

The mummies that remain in the tree canopy during autumn and winter will be the main inoculum source for infection in the following year **(Figure 1c).** Although the pathogen causes mainly fruit rot, a secondary syndrome consisting of leaf necrosis, defoliation, shoot blight and branch dieback has also been observed in seriously affected trees **(Figure 1d)** (Adaskaveg et al., 2017; Förster et al., 1999; López-Moral et al., 2017).

Leaves show necrosis starting from the tips and margins and extending to the entire leaf blade **(Figure 1e)** (Palazón et al., 1979). This secondary syndrome seems to be caused by the translocation of the toxins produced by the pathogen in the affected fruit remaining in the tree canopy. The pathogen can be isolated consistently from the affected tissues of almond fruit.

However, it cannot be isolated from necrotic leaves as well as from the wood of shoots and branches showing blight and dieback, probably because this syndrome is caused directly by the phytotoxins, but the pathogen is no longer present in the tissues (Adaskaveg et al., 2017; Förster et al., 1999; López-Moral et al., 2017).

Figure 1. Characteristic symptoms of almond anthracnose caused by *Colletotrichum* spp. **(a)** depressed, sunken, round and orange lesions on green almonds; **(b)** branch with mummified fruits and necrotic leaves; **(c)** mummified fruits from infections caused the previous year and remaining in the tree canopy; **(d)** defoliation and dieback of shoots and branches as a consequence of the toxins produced by the pathogen; **(e)** necrotic irregular lesions in the tips and margins of the leaves (López-Moral et al., 2020).

In laboratory – isolation and characteristics of cultures

Isolation from symptomatic fruits. Affected fruit are surface disinfected with commercial bleach (Cl at 50 g liter–1) at 10% (vol/vol) in sterile water for 1 min, and air dried on sterile filter paper for 30 min. Affected tissues are cut with a sterile scalpel and plated on potato dextrose agar (PDA) (Difco Laboratories) containing streptomycin (PDA+S). When the affected fruit tissues show abundant pathogen sporulation, masses of conidia are removed using a sterile needle and cultured in petri dishes on PDA+S. Petri dishes are incubated at 25 ± 2˚C under a 12-h diurnal photoperiod of cool fluorescent light (350 µmol m–2 s–1) for 5 days, and hyphal tips from the colonies are transferred to PDA and incubated as described above.

In general, fungal colonies of *Colletotrichum* spp. associated with AA show radial growth with concentric circles and abundant aerial mycelium on PDA when they are incubated at 23 ± 2 ˚C with a 12-h photoperiod. Colony color varies from light to dark gray for *C. godetiae* isolates (gray subpopulation); and from salmon to pink–orange for *C. acutatum* s.s. **(Figure 2)** or *C. fioriniae* isolates (pink–orange subpopulations) (Förster et al., 1999; Freeman et al., 1998; López-Moral et al., 2017). The mycelial growth rate ranges from 3.0 to 6.0 mm/day on PDA at 25 ˚C (Förster et al., 1999; López-Moral et al., 2017).

In spite of conidium morphology may vary with the *Colletotrichum* species, the morphology of conidia of *Colletotrichum* isolates from almond is not useful to distinguish them between species. In general, all *Colletotrichum* species associated with AA show unicellular, hyaline conidia with two sharp ends. Interestingly, conidia of *C. godetiae* from almond show similar morphology to those of *C. acutatum* from almond (two sharp ends in both cases), whereas conidia of *C. godetiae* from other hosts such as olive usually show a single sharp end (López-Moral et

al., 2017) **(Figure 2).** However, this morphologic differences on conidium morphology between isolates of *C. godetiae* from different hosts do not result in genetic differences when using six genomic regions (López-Moral et al., 2017).

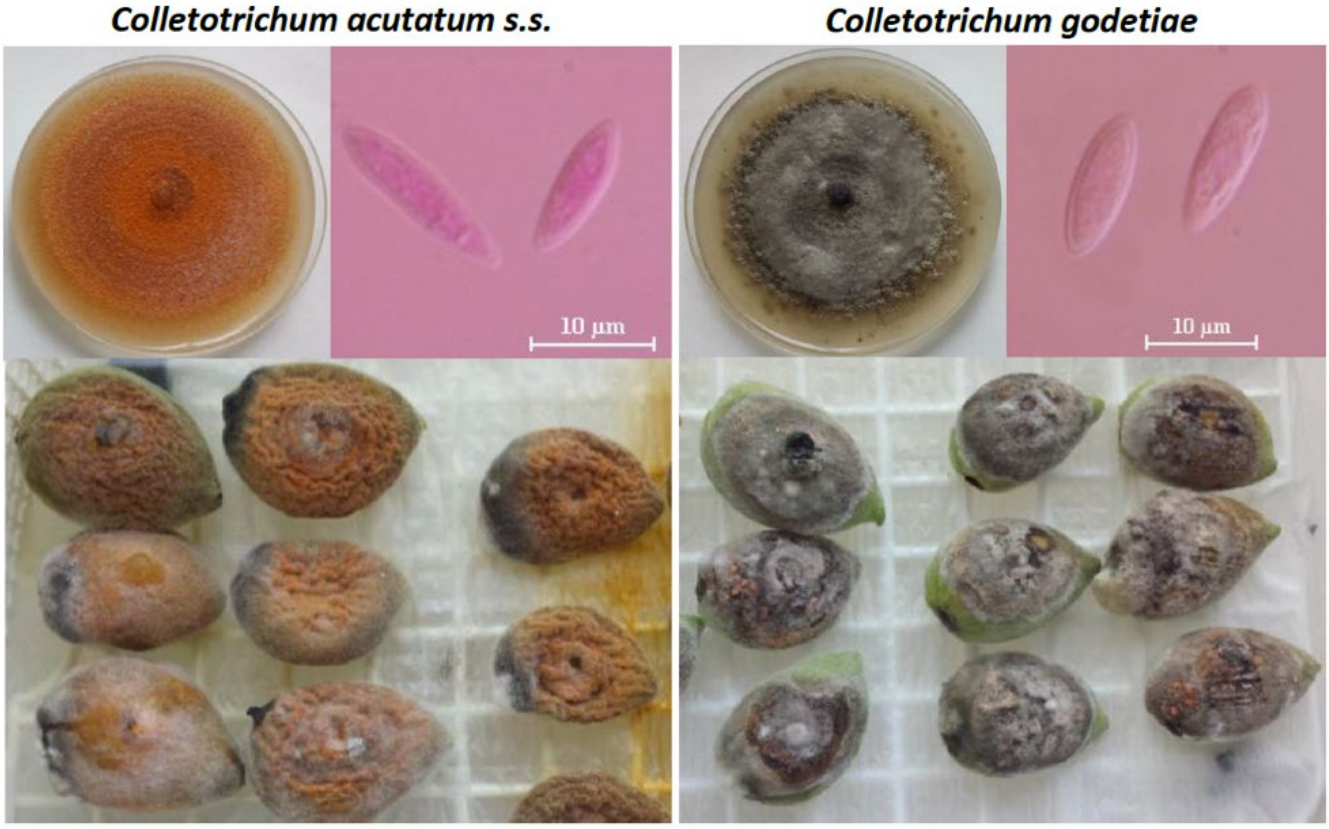

Figure 2. Colonies and conidia of *Colletotrichum acutatum* s.s. (isolate Col-506; pink–orange subpopulation) and *C. godetiae* (isolate Col-522; gray subpopulation) identified as causal agents of almond anthracnose. Colonies grown on PDA and on inoculated almond fruits at 23 ± 2 °C with a 12-h photoperiod for 7 and 14 days, respectively. Scale bars: (conidia) 10 µm (López-Moral et al., 2020).

In laboratory – molecular detection

DNA Extraction

For genomic DNA, fungal mycelium and conidia from single-spore cultures grown on PDA at 23 ± 2°C with a 12-h photoperiod for 7 days are used. Total DNA is extracted using commercial kit following the manufacturer's instructions. The concentration and purity of the extracted DNA are determined with a MaestroNano spectrophotometer (MaestroGen) (López-Moral et al., 2017).

Conventional PCR

Six genomic areas are amplified and sequenced, including the 5.8S nuclear ribosomal gene with two flanking internal transcribed spacers (ITS), a 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and partial sequences of the chitin synthase 1 (CHS-1), histone 3 (HIS3), actin (ACT), and β-tubulin (TUB2) genes. The primer pairs used for each genomic area, as well as their sequences, are shown in **Table 2.**

Table 2. Primers used to amplify the six genomic areas studied by DNA analysis

Primer[y]	Primer pairs	Genomic sequence (5′–3′)	Size (bp)[z]	Reference
ITS	ITS4	TCCTCCGCTTATTGATATGC	540	White et al. 1990
	ITS5	GGAAGTAAAAGTCGTAACAAGG	…	…
TUB2	BT2A	GGTAACCAAATCGGTGCTGCTTTC	429	Glass and Donaldson 1995
	BT2B	ACCCTCAGTGTAGTGACCCTTGGC	…	…
ACT	ACT-512F	ATGTGCAAGGCCGGTTTCGC	246	Carbone and Kohn 1999
	ACT783R	TACGAGTCCTTCTGGCCCAT	…	…
CHS-1	CHS-79F	TGGGGCAAGGATGCTTGGAAGAAG	282	Carbone and Kohn 1999
	CHS-354R	TGGAAGAACCATCTGTGAGAGTTC	…	…
HIS3	CYLH3F	AGGTCCACTGGTGGCAAG	382	Crous et al. 2004
	CYLH3R	AGCTGGATGTCCTTGGACTG	…	…
GAPDH	GDF1	GCCGTCAACGACCCCTTCATTGA	250	Guerber et al. 2003
	GDF1	GGGTGGAGTCGTACTTGAGCATGT	…	…

[y] ITS = internal transcribed spacer, TUB2 = β-tubulin, ACT = actin, CHS-1 = chitin synthase 1, HIS3 = histone 3, and GAPDH = glyceraldehyde-3-phosphate dehydrogenase.
[z] Expected polymerase chain reaction product.

Real-time PCR (TaqMan®) assays for the diagnosis of *Colletotrichum* spp., *C. acutatum* and *C. gloeosporioides* from culture and plant material.
Diagnosis, based on real-time PCR, gives a rapid, sensitive and accurate result in 1–2 days, allowing high-throughput and inexpensive screening of samples. These methods also permit the detection of the pathogens from artificially infected symptomless plant material. The methods described could prove useful for studying the epidemiological routes of these pathogens in fields and nurseries. In addition, the methods could be used in future surveys monitoring fungal populations, or in studies concerning the biology and infection strategies of *Colletotrichum* spp. (Garrido et al., 2009)

Three specific assays (primers and TaqMan® probes), for *C. acutatum*, *C. gloeosporioides* and *Colletotrichum* spp. detection and discrimination, are designed using the primer express™ software (PE Biosystems).

Probes are designed with 5′ FAM (6-carboxyfluorescein) as the reporter and 3′ TAMRA (tetra-methylrhodamin) as the quencher; all primers and probes are synthesized by MWG Biotech **(Table 3).**

Table 3. Sequences of primers and probes

Target	Primer/Probe	Orientation	Assay	Sequence 5′–3′	Target region (nucleotides)	EMBL accession
Colletotrichum acutatum	ACUT-F1	Forward	TaqMan®	CGG AGG AAA CCA AAC TCT ATT TAC A	122–146	EF694675
	ACUT-R1	Reverse		CCA GAA CCA AGA GAT CCG TTG	192–212	EF694675
	ACUT-PB	Probe		CGT CTC TTC TGA GTG GCA CAA GCA AAT AAT TAA A	150–183	EF694675
Colletotrichum gloeosporioides	GLOE-F1	Forward	TaqMan®	GGC GGG TAG GGT CYC CG[a]	52–68	AJ536226
	GLOE-R2	Reverse		ACT CAG AAG AAA CGT CGT TAA ATC AG	128–153	AJ536226
	GLOE-PB	Probe		CTC CCG GCC TCC CGC CYC[b]	74–91	AJ536226
Colletotrichum spp.	COL GEN-F1	Forward	TaqMan®	TGC CTG TTC GAG CGT CAT T	320–338	EF694675
	COL GEN-R2	Reverse		CTA CGC AAA GGA GGC TCC G	413–431	EF694675
	COL GEN-PB	Probe		AAC CCT CAA GCW CYG CTT GGY KTT GG[a]	341–366	EF694675

Colletotrichum acutatum- and *C. gloeosporioides*-specific primers and TaqMan® probes **(Table 3)** are designed within the most divergent area of the ITS1 region of the aligned fungal species. Multiple sequence alignment between 307 sequences belonging to 21 species of *Colleotrichum* and the sequences of other related plant pathogens allowed the design of primers and a TaqMan® probe specific for the genus *Colletotrichum* within the divergent sequences of the ITS2 region of the r RNA gene (Garrido et al., 2009).

Management
The control of the disease must be based on an Integrated Pest Management (IPM) program

preventing infections. Prevention is especially important in AA because *Colletotrichum* spp. are established slowly in the orchard over years, showing up when environmental conditions are favorable for disease outbreaks. Once disease symptoms appear, the control of AA would be difficult if the inoculum pressure in the field is high. Thus, preventive measures including cultural practices, cultivar resistance and biologic control must be considered to face *Colletotrichum* infections. Fungicide treatments should be used only to prevent economic losses in years of epidemics when preventive measures are not enough to control the disease or orchards with a disease history suggest that preventive treatments are required. All these management strategies as well as an update on the research studies concerning this topic are described in López-Moral et al., 2020.

References

1. Adaskaveg, J.E.; Gubler, W.D.; Duncan, R.; Stapleton, J.J.; Holtz, B.A.; Trouillas, F.P. UC IPM Pest Management Guidelines: Almond UC ANR, 2017. Publication 3431.
2. Förster, H.; Adaskaveg, J.E. Identification of subpopulations of Colletotrichum acutatum and epidemiology of almond anthracnose in California. Phytopathology 1999, 89, 1056–1065.
3. Freeman, S.; Katan, T.; Shabi, E. Characterization of Colletotrichum species responsible for anthracnose diseases of various fruits. Plant Dis. 1998, 82, 596–605.
4. Garrido, C., Carbú, M., Fern´andez-Acero, F.J., Boonham, N., Colyer, A., Cantoral, J.M., Budge, G., 2009. Development of protocols for detection of Colletotrichum acutatum and monitoring of strawberry anthracnose using real-time PCR. Plant Pathol. 58, 43–51.
5. López-Moral, A.; Agustí-Brisach, C.; Lovera, M.; Arquero, O.; Trapero, A. Almond Anthracnose: Current Knowledge and Future Perspectives. Plants 2020, 9, 945.
6. López-Moral, A.; Raya-Ortega, M.C.; Agustí-Brisach, C.; Roca, L.F.; Lovera, M.; Luque, F.; Arquero, O.; Trapero, A. Morphological, pathogenic and molecular characterization of Colletotrichum acutatum isolates causing almond anthracnose in Spain. Plant Dis. 2017, 101, 2034–2045.
7. Palacio-Bielsa, A.; Cambra, M.; Martínez, C.; Olmos, A.; Pallás, V.; López, M.M.; Adaskaveg, J.E.; Förster, H.; Cambra, M.A.; Duval, H.; et al. Almond Diseases, In Almonds. Botany, Production and Uses; Socias-Company, R., Gradziel, T.M., Eds.; CAB International: Boston, MA, USA, 2017; pp. 321–374.
8. Palazón, I.J.; Palazón, C.F. Estudios sobre Gloeosporium amygdalium Brizi en los almendros españoles. Anuario INIA Serv. Prot. Veg. 1979, 11, 29–43.
9. Peres, N.A.; Timmer, L.W.; Adaskaveg, J.E.; Correl, J.C. Lifestyles of Colletotrichum acutatum. Plant Dis. 2005, 89, 784–796.

Almond dieback - Dépérissement de l'amande – Disseccamento del mandorlo *Neoscytalidium dimidiatum*

The Almond dieback by *Neoscytalidium dimidiatum* (Family: *Botryosphaeriaceae*) is a canker disease causing severe decline and dieback to almond trees (*Prunus dulcis* (Mill) D. A. Webb); the syndrome is widespread in California (Nouri et al., 2018); recently, it has been also reported from many orchards in Tunisia, with an incidence of up to 20% of affected plants.
Almond trunk and branch canker diseases are a major concern to the almond industry worldwide because they can affect young trees and become more prevalent as orchards age. Trunk disease pathogens generally attack woody parts of the host, causing symptoms such as cankers, dieback, and internal symptoms, including wedge-shaped necrosis as well as discoloration of the vascular tissues (Gramaje et al. 2012). Among some of the most common canker diseases that can affect almond are those caused by fungi in the *Botryosphaeriaceae* family (English et al. 1966, Olmo et al. 2016). Most species within the *Botryosphaeriaceae* family have a worldwide distribution and occur on a wide variety of plant hosts as saprobes, parasites, and endophytes (Slippers and Wingfield 2007).

Diagnosis

***In field* - symptoms and signs**
Plant symptoms are non-specific. They include shoot blight with scorching of leaves, branch wilt, decline and death of trees. Cross-sections of the branches of infected trees showing necroses and brown vascular discolorations are reported on **Figure 1.**

Figure 1 Dieback and wilting of branches in Almond trees in Tunisia.

***In laboratory* – isolation, characteristics of cultures and molecular identification of the pathogen**
Isolation of the pathogen can be carried out directly from necrotic tissues. For the isolation, small pieces (4-5 mm) of necrotic wood has to be surface sterilized (1% NaClO solution for 2 minutes, 70% ethanol for 30 seconds), washed twice with sterile distilled water, plated onto oatmeal agar (OMA) and incubated at 25 °C for seven days. After incubation, colonies of *N. dimidiatum* on OMA are characterized by a dense and hairy aerial mycelium that gradually turned dark grey to olive green. This kind of colonies produce arthroconidia; they are typically dark brown, thick-walled, and one-celled, 6.3 to 14.2 × 2 to 4.5 µm, and ovate to rectangular.

The identity of the pathogen is confirmed by traditional PCR targeting the internal transcribed spacer region (ITS1-5.8S-ITS2); the amplification can be carried out by using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990).

References

1. English, H., Davis, J. R., and De Vay, J. E. 1966. Dothiorella canker, a new disease of almond trees in California. Phytopathology 56:146.
2. Gramaje, D., Agust´ı-Brisach, C., P´erez-Sierra, A., Moralejo, E., Olmo, D., Mostert, L.,Damm, U., and Armengol, J. 2012. Fungal trunk pathogens associated with wooddecay of almond trees on Mallorca (Spain). Persoonia 28:1-13.
3. Nouri, M.T., Lawrence, D.P., Yaghmour, M.A., Michailides, T.J., Trouillas, F.P., 2018. Neoscytalidium dimidiatum causing canker, shoot blight and fruit rot of almond in California. Plant Disease 102, 1638-1647.
4. Olmo, D., Armengol, J., Le´on, M., and Gramaje, D. 2016. Characterization and pathogenicity of Botryosphaeriaceae species isolated from almond trees onthe Island of Mallorca (Spain). Plant Dis. 100:2483-2491
5. Slippers, B., and Wingfield, M. J. 2007. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: Diversity, ecology and impact. Fungal Biol. Rev. 21:90-106
6. White, T.J., Bruns, T., Lee, S., and Taylor, J.W. 1990. "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in PCR Protocols: a Guide to Methods and Applications, eds M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (San Diego, CA: Academic Press Inc), 315–322.

Citrus Black Spot - Tache noire des agrume - Macchia nera degli agrumi *Phyllosticta citricarpa*

The Citrus Black Spot (CBS) is a foliar and fruit decay affecting the majority of Citrus species (Baldassari et al. 2008; EFSA 2014; EPPO 2020; Kotzé 1981; Paul et al. 2005). CBS is present in Asia, Australia, South Africa and South America and recently has been reported from Tunisia (Boughalleb-M'Hamdi et al. 2020).

The causative agent of the disease is *Phyllosticta citricarpa* (teleomorph: *Guignardia citricarpa*) a quarantine pathogen listed in the Annex II Part A of the Commission Implementing Regulation (EU) 2019/20724. The Commission Delegated Regulation (EU) 2019/17025 also listed *P. citricarpa* as a priority pest. It is included in the EPPO (European and Mediterranean Plant Protection Organization) A1 List (EPPO 2020). *Phyllosticta citricarpa* is also a quarantine organism in the USA (Schirmacher et al. 2019).

Spread of the pathogen responsible for CBS occurs via spores **(Figure 1e)**, including both wind borne ascospores produced in pseudothecia (ascocarps, see after) and waterborne conidia produced in pycnidia (see after) (Guarnaccia et al. 2017).

Ascospores are considered the primary source of inoculum in the CBS disease cycle, while conidia in rainwater are mostly responsible for the short downward dispersal of the pathogen (Guarnaccia et al., 2017; Spósito et al. 2011). Alternate wetting and drying cycles of the leaves combined with mild to warm temperature fluctuations are favorable conditions for maturation of pseudothecia and ascospore (Fourie et al. 2013; Guarnaccia et al. 2017; Hu et al. 2013).

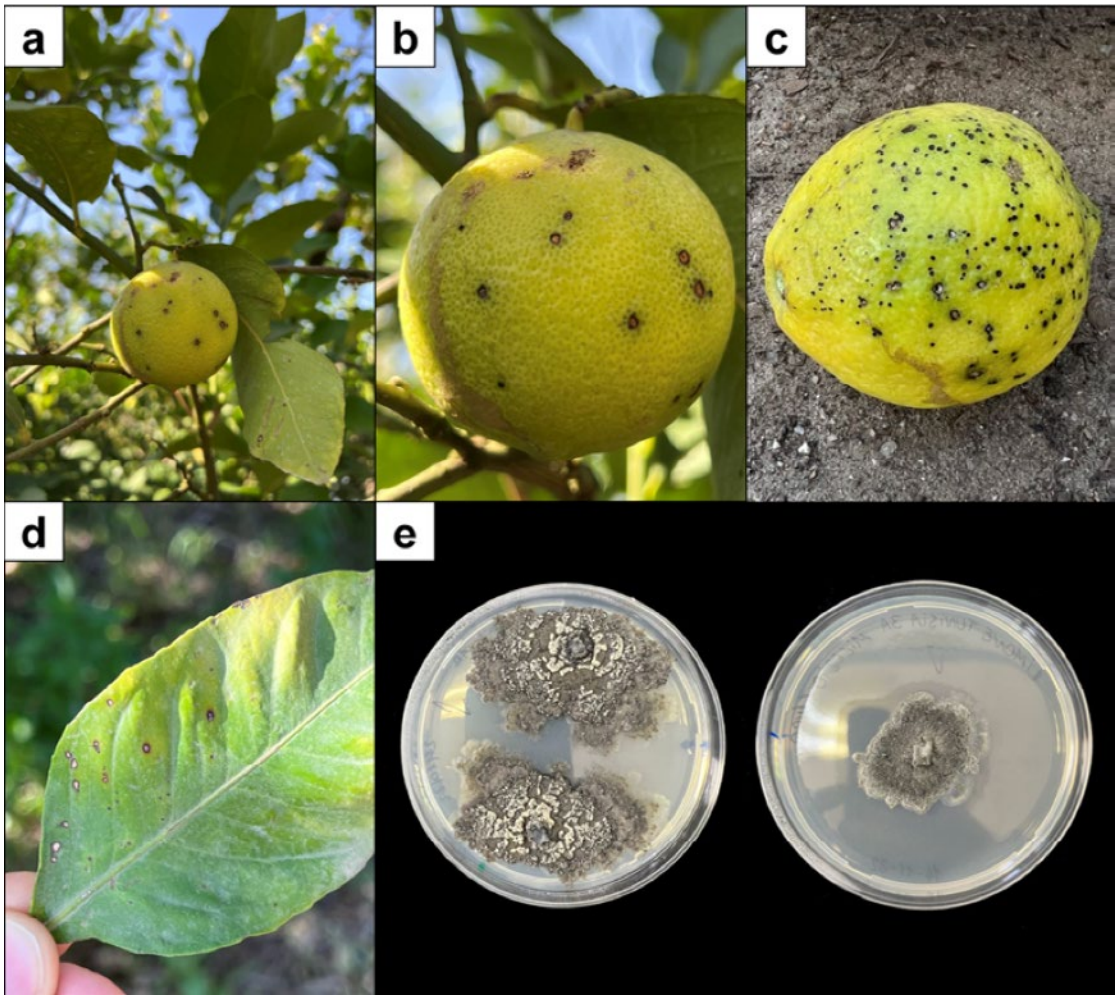

Figure 1 a, b and **c,** hard and freckle spots by *Phyllosticta citricarpa* on peel of mature lemon fruits from a commercial orchard (Tunis, Tunisia); **d,** hard spot lesions on lemon leaves; **e,** seven-day-old sporulating cultures of *Phyllosticta citricarpa* grown on PDA medium at 25°C.

Diagnosis

***In field* - symptoms and signs**
CBS is associated with various symptoms on fruits (Guarnaccia et al. 2019; Kotzé 1981). The most commons are 'hard spots' **(Figure 1a and b)**, which are characterized by sunken, pale brown necrotic lesions with a dark reddish brown raised border, often containing pycnidia (EPPO 2020; Guarnaccia et al. 2017, 2019). Further symptoms are: (i) virulent spots, which are sunken necrotic lesions without defined borders mostly on mature fruits; (ii) false melanose, consisting of small black pustules usually in a tear stain pattern; (iii) freckle **(Figure 1c),** cracked or speckled spot (Guarnaccia et al. 2019).

Leaf and twig symptoms rarely occur on orange, mandarin and other commercial citrus species, but they are frequently reported on lemons. They appear as round, small, sunken necrotic lesions with a yellow halo **(Figure 1d)** (Guarnaccia et al. 2019; Kotzé 1981).

Temperature is a crucial factor for disease development. Rapid temperature increments, typically from 20° to 27°C), when fruits are ripe stimulate the appearance of Citrus Black Spot symptoms and lead to formation of a significant number of fruit lesions (Guarnaccia et al. 2019; Lee and Huang 1973). High light intensity can further facilitate fruit lesion development, thus the side of the canopy more exposed to sunlight typically shows more symptoms (Guarnaccia et al. 2019). Old age and physiological stress also appear to facilitate the development of CBS (Guarnaccia et al. 2019; Kotzé 1981).

In field a precise diagnosis of *P. citricararpa* is complicated by the fact that necrotic spots are generic symptoms that can be caused by other *Phyllosticta* species (Guarnaccia et al. 2019; EFSA 2014; EPPO 2020) and even by other ascomycetes (e.g. *Septoria citri* and *Cytosporina citriperda*).

***In laboratory* – isolation and characteristics of cultures**
Isolation from lesions from fruits and infected leaves proceeds as it follows. The infected plant material has to be disinfected with a filter paper soaked in a 96% or 70% ethanol. Then, lesions are excised (by a cork borer or a scalpel), transferred either on cherry decoction agar (CHA) or malt extract agar chloramphenicol (MALTCHL), or potato dextrose agar (PDA) amended with streptomycin sulphate (250 mg/L) and incubated at 22-23°C for 48-72 hours. After incubation, outgrowing hyphas are transferred on PDA plates and let to growth at 22-23°C for 10-12 days. Then, the final isolates should be obtained by monoconidial culture.

On PDA, colonies of *P. paracitricarpa* are flat, rather regular and slow growing, initially white-grey mycelium, gradually becoming greenish to dark green, with white hyphae at the margin; additionally, they have irregular margins lined by a much wider translucent zone of colorless submerged mycelium **(Figure 1e).** The center of the colony is dark with grey to glaucous aerial mycelium, often with numerous small tufts. The reverse of the colony is very dark in the center and surrounded by areas of grey sepia and buff (Baayen et al., 2002). Stromata start to develop after 7–8 days, whereas mature pycnidia with conidia are generally produced within 10–14 days. Because of the wide morphological similarities with species in the genus *Phyllosticta* (e.g. *Phyllosticta citriasiano, Phyllosticta paracapitalensis* and *Phyllosticta paracitricarpa*) (Guarnaccia et al. 2019) the in vitro identification of *P. citricarpa* cannot lead to the precise identification of the pathogen.

***In laboratory* – molecular detection**
Identification of *P. citricarpa* can be performed by sequencing the *tef1* gene will allow unambiguous identification of *P. citricarpa* and especially its distinction from *P. paracitricarpa* (EPPO, 2020; Guarnaccia et al., 2017).
Recently, a Real-Time PCR protocol targeting a portion of the *tef1* gene makes it possible the precise identification of *P. citricarpa* (Zajc et al., 2022).

DNA extraction
The prerequisite to the application of PCR is the DNA extraction from either plant material or

pure culture. To this aim, commercial kits can be used.

Conventional PCR and sequencing

The conventional PCR protocol for the detection of *P. citricarpa* targets the *tef1* region which can be amplified by using the primers forward EF1-728F – 5'-CATCGAGAAGTTCGAGAAGG-3' and reverse EF2 – 5'-GGA(G/A) GTACCAGT(G/C) ATCATGTT-3' (Guarnaccia et al., 2017). The PCR amplification can be carried out by using commercial kits following the manufacturer instructions. The species identification by the obtained PCR product has to be carried out by the bioinformatic analysis of the related sequence.

Real-time PCR

One of the most promising Real-time PCR protocol for the detection of *P. citricarpa* has been developed by Zajc et al. (2022). The method targets a portion of the *tef1* gene and consists of the primer pair Pc-TEF1-F (GAAGGTCAGTTGCCTCACACTTT), Pc-TEF1-R (GTCATATAACCGAGCGCCAAA) and the specific TaqMan Pc-TEF1-Probe (VIC-TTGCGCCTCCACTTG-MGBNFQ); this latter is labelled with a 5' VIC reporter and a 3' nonfluorescent minor groove binder (MGB).

Management

Different chemical and cultural control measures are used for CBS management (Guarnaccia et al., 2017). The most effective includes the fungicide application during the period of fruit susceptibility. The main fungicides used are strobilurins (quinone outside inhibitors, QoI), dithiocarbamates and fixed copper (multisite activity), and methyl benzimidazole carbamate (Guarnaccia et al., 2017).
In addition to chemical control, cultural control measures, such as the removal of leaf litter with machines, the acceleration of leaf litter decomposition with urea, ammonium sulphate, sugarcane bagasse, the mulching with plants that grow between rows of orchards to cover leaf litter, the pruning of dead twigs and irrigation and balanced nutrition are commonly used to reduce the amount of *P. citricarpa inoculum* (Guarnaccia et al., 2017).

References

1. Baayen RP, Bonants PJM, Verkley G, Carroll GC, van der Aa HA, de Weerdt M et al. (2002) Non-pathogenic isolates of the citrus black spot fungus, Guignardia citricarpa, identified as a cosmopolitan endophyte of woody plants, Guignardia mangiferae (Phyllosticta capitalensis). Phytopathology 92, 464–477.
2. Baldassari RB, Wickert E, De Goes A (2008) Pathogenicity, colony morphology and diversity of isolates of Guignardia citricarpa and G. mangiferae isolated from Citrus spp. Eur J Plant Pathol 120:103–110.
3. Boughalleb-M'Hamdi N, Fathallah A, Benfradj N, Ben Mahmoud S, Bel Hadj Ali A, Medhioub L, Jaouadi I, Huber J, Jeandel C, Ioos R (2020) First report of Citrus Black Spot disease caused by Phyllosticta citricarpa on Citrus limon and C. sinensis in Tunisia. New Dis Rep 41:8
4. EFSA PLH Panel (EFSA Panel on Plant Health) (2014) Scientific Opinion on the risk of Phyllosticta citricarpa (Guignardia citricarpa) for the EU territory with identification and evaluation of risk reduction options. EFSA J 12: 3557.
5. EPPO (2020) PM 7/017 (3) Phyllosticta citricarpa (formerly Guignardia citricarpa). Bulletin OEPP/EPPO Bulletin 50: 440–461. https://doi.org/ 10.1111/epp.12700
6. Fourie P, Schutte T, Serfontein S, Swart F (2013) Modeling the effect of temperature and wetness on Guignardia pseudothecium maturation and ascospore release in citrus orchards. Phytopathology 103(3):281-292. http://dx.doi.org/101094/PHYTO-07-11-0194
7. Guarnaccia V, Gehrmann T, Silva-Junior GJ, Fourie PH, Haridas S, Vu D, Spatafora J, Martin FM, Robert V, Grigoriev IV, Groenewald JZ, Crous PW (2019) Phyllosticta citricarpa and sister species of global importance to Citrus. Mol Plant Pathol 20(12):1619-1635. https://doi.org/10.1111/MPP.12861
8. Guarnaccia V, Groenewald JZ, Li H, Glienke C, Carstens E, Hattingh V, Fourie PH, Crous PW (2017) First report of Phyllosticta citricarpa and description of two new species, P. paracapitalensis and P. paracitricarpa, from citrus in Europe. Stud Mycol 87:161–185. https://doi.org/10.1016/J.SIMYCO.2017.05.003
9. Hu J, Johnson EG, Wang NY, Davoglio T, Dewdney MM (2013) qPCR quantification of pathogenic Guignardia citricarpa and nonpathogenic G. mangiferae in citrus. Plant 98(1):112-120. http://dx.doi.org/10.1094/ PDIS-04-13-0465-RE
10. Kotzé JM (1981) Epidemiology and control of Citrus Black Spot in South Africa. Plant Dis 65(12):945-950.
11. Lee YS and Huang CS (1973) Effect of climatic factors on the development and discharge of ascospores of the citrus black spot fungus. Journal of Taiwan Agricultural Research 22, 135–144.
12. Paul I, Van Jaarsveld AS, Korsten L, Hattingh V (2005) The potential global geographical distribution of Citrus Black Spot caused by Guignardia citricarpa (Kiely): Likelihood of disease establishment in the European Union. Crop Prot 24(4):297–308.
13. Schirmacher AM, Tomlinson JA, Barnes AV (2019) Species-specific real-time PCR for diagnosis of Phyllosticta citricarpa on Citrus species. EPPO Bull 49: 306-313.
14. Spósito MB, Amorim L, Bassanezi RB, Yamamoto PT, Felippe MR, Czermainski ABC (2011) Relative importance of inoculum sources of Guignardia citricarpa on the Citrus Black Spot epidemic in Brazil. Crop Prot 30(12):1546–1552. https://doi.org/10.1016/J.CROPRO.2011.08.007
15. Zajc J, Kogej Zwitter Z, Fišer S, Gostinčar C, Vicent A, Domenech AG et al (2023) Highly specific qPCR and amplicon sequencing method for detection of quarantine citrus pathogen Phyllosticta citricarpa applicable for air samples. Plant Path, 72(3), 548-563.

Mal secco of citrus - Mal secco des agrumes - Mal secco degli agrumi *Plenodomus tracheiphilus*

Mal secco of citrus is a tracheomycotic disease caused by *Plenodomus tracheiphilus* (formerly, *Phoma tracheiphila*), a mitosporic fungus classified by the European and Mediterranean Plant Protection Organization (EPPO) as an A2 quarantine pest. To date, mal secco is present in the citrus-producing countries in the Mediterranean and Black Sea areas with the exception of Spain, Portugal and Morocco (EPPO, 2015).

Among affected citrus species, lemon *(Citrus × limon)* is the principal host of the pathogen, although the disease is also common on citron, bergamot, lime, sour orange, and rough lemon (Nigro et al., 2015).

The pathogen spreads itself by dispersion of spores (conidia), which in field are produced inside pycnidia (see after) and from hyphae growing on exposed wood or debris, including branches, leaves, and fruits. Under natural conditions, the inoculum can be dispersed by wind and rain. The pathogen can also be transmitted to other trees through contaminated pruning tools. Twigs and leaves lying on the soil are a source of inoculum for infection through wounded roots. Leaves infected by the fungus fall to the ground during autumn and spring, and the fungus within the leaf tissue is able to sporulate at temperatures ranging from 10 to 25°C (Migheli et al., 2009).

Diagnosis

***In field* - symptoms and signs**
The 'Mal Secco' disease induces a broad array of both specific and non-specific symptoms that can occur singularly or simultaneously in the manifestation of the disease (Migheli et al., 2009) **(Figure 1).**

The first symptom usually appears on the leaves of the uppermost shoots, which display a slight discoloration of the primary and the secondary veins **(Figure 1i);** then, the leaves lose their shine, turn yellow, wither and fall down, mostly without the petioles, which persist on the shoots. The shoots often appear chlorotic on the apical part while maintaining a normal green color in the basal part **(Figure 1d and e);** sometimes they turn brown.

Newly infected shoots show a yellow or pink-salmon to reddish discoloration of the wood, which occurs also in the wood of the main and secondary branches as well as in the trunk, where the pathogen growth is progressing **(Figure 1g and h).** With time, the plant undergoes a progressive basipetal desiccation of shoots, branches, and trunk, which leads the whole plant to die **(Figure 1a-c)** (Abbate et al., 2019; Migheli et al., 2009; Nigro et al., 2011; Russo et al., 2020).

In addition to the more common form of mal secco, two different forms of the disease can be distinguished: 'mal fulminante', a rapid fatal form of the disease apparently due to root infection; and 'mal nero', a consequence of chronic infection of the tree leading to a browning of the heartwood.

An additional specific trait of the 'Mal Secco' disease is the presence, on the surface of infected tissue, of signs of the pathogen (Gentile et al., 2000; Migheli et al., 2009). These are represented by pycnidia, long-lasting structures that can occur, starting from the end of the autumn, in 1- to 2- year-old slowly desiccating shoots or suckers. Their presence elicits the detachment of the epidermis from the underneath tissues, which is followed by penetration of air, resulting in the appearance of long silver-gray stripes on the affected organs (Nigro et al., 2011). The cracking of the epidermis makes possible to observe pycnidia as black spots directly by the naked eye or with a low magnification lens (Nigro et al., 2011).

***In laboratory* – isolation and characteristics of cultures**
The fungus can be isolated by placing pieces of infected tissues taken from discolored wood from living twigs or from symptomatic leaves on Potato Dextrose Agar (PDA) (EPPO, 2015).

Isolation from symptomatic twigs. First, twigs are debarked; then, the infected xilematic tissue is separated from the rest and cut into small pieces (3.0-4.0 mm in diameter); then, a surface sterilization of the infected material is carried out by immersion in 1% NaOCl (1 minute – m), rinsing in sterilized distilled water (sdw) (1 minute – m), immersion in 70% ethanol and final rinsing in sterilized distilled water (sdw) (1 minute – m). Sterilized pieces are blotted dry with adsorbent paper, plated onto PDA amended with streptomycin sulphate (250 mg/L) and incubated at 22-23˚C for 48 hours. After incubation, outgrowing hyphas are transferred on PDA plates and let to growth at 22-23˚C for 10-12 days. Then, the final isolate is obtained by monoconidial culture.

Isolation from symptomatic leaves. leaf veins are separated from the rest of the lamina and are cut into small pieces (2–3 mm); then, the following isolation steps (sterilization, plating, incubation) proceed as above.

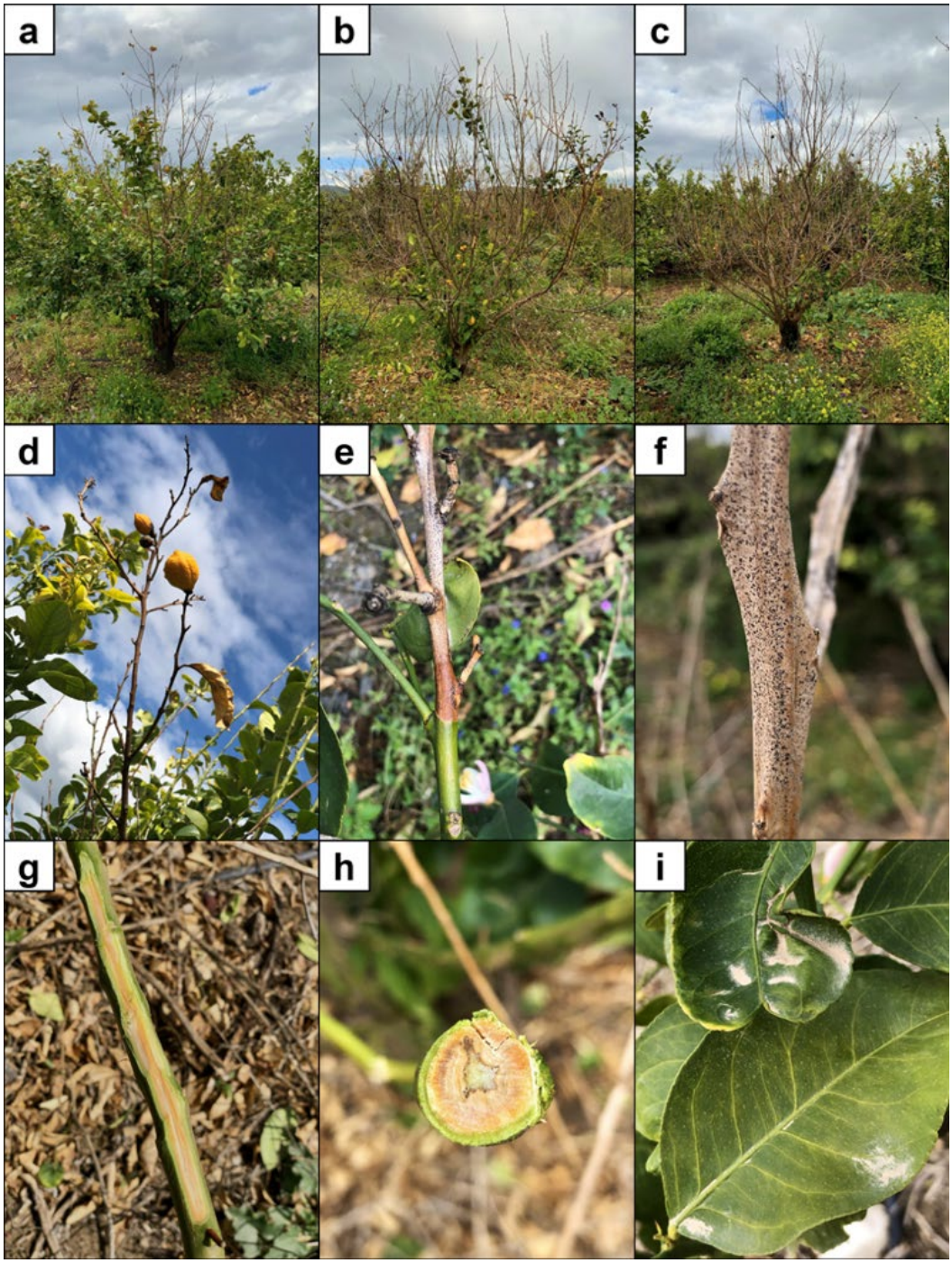

Figure 1 a, **b** and **c**, progression (from left to right) of the severity of wilting and defoliations of twigs in a young lemon tree affected by Mal Secco in a commercial orchard in Sicily (Italy); **d** and **e**, shedding of leaves and defoliation of apical twigs; **f** withered twig of lemon with scattered pycnidia of *Plenodomus tracheiphilus*; longitudinal **(g)** and transverse **(h)** sections on a lemon twig with the typical orange-reddish discoloration of the wood; i, clearing and chlorosis of leaf veins in a lemon tree affected by Mal Secco.

In pure culture *Plenodomus tracheiphilus* presents peculiar traits and structures. In detail, in PDA the pathogen shows a mycelium initially hyaline, that turns brown or pinkish- red after a few days. On PDA, optimal temperature for growth is 23 ± 2°C and the growth rate is 3.8–6.0 mm per day at this temperature. Chromogenous and non-chromogenous variants have been distinguished in culture and isolates differing in virulence have been found to occur in nature **(Figure 2)** (Magnano di San Lio et al., 1992).

The asexual reproduction of *P. tracheiphilus* is realized by conidia; these are produced either on the apex of conidiogenous hyphas **(Figure 3)** (indicated as phialides; phialoconidia of diameter 1.5–3.0 x 3.0–8.0 µm) or within pycnidia **(Figure 4);** these latter are small, black and globose long-lasting resistance structures presenting an inner cavity covered by small hyaline conidia (diameter 0.5–1.5 x 2.0–4.0 µm) which are extruded through ostioles in whitish cirrhi (EPPO, 2015).

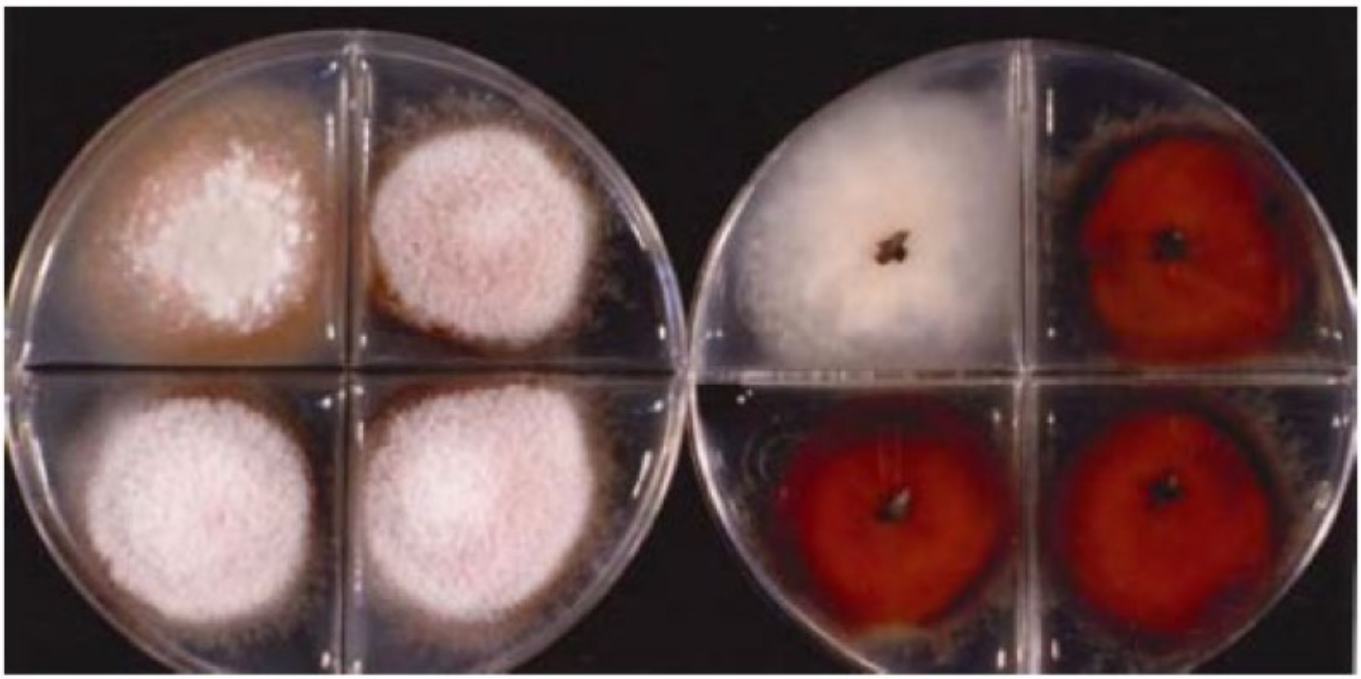

Figure 2 single-conidium subcultures of a chromogenic strain of *Plenodomus tracheiphilus*. Front and back side of the petri dish are shown on the left and the right, respectively. Note an albino variant on the top left.

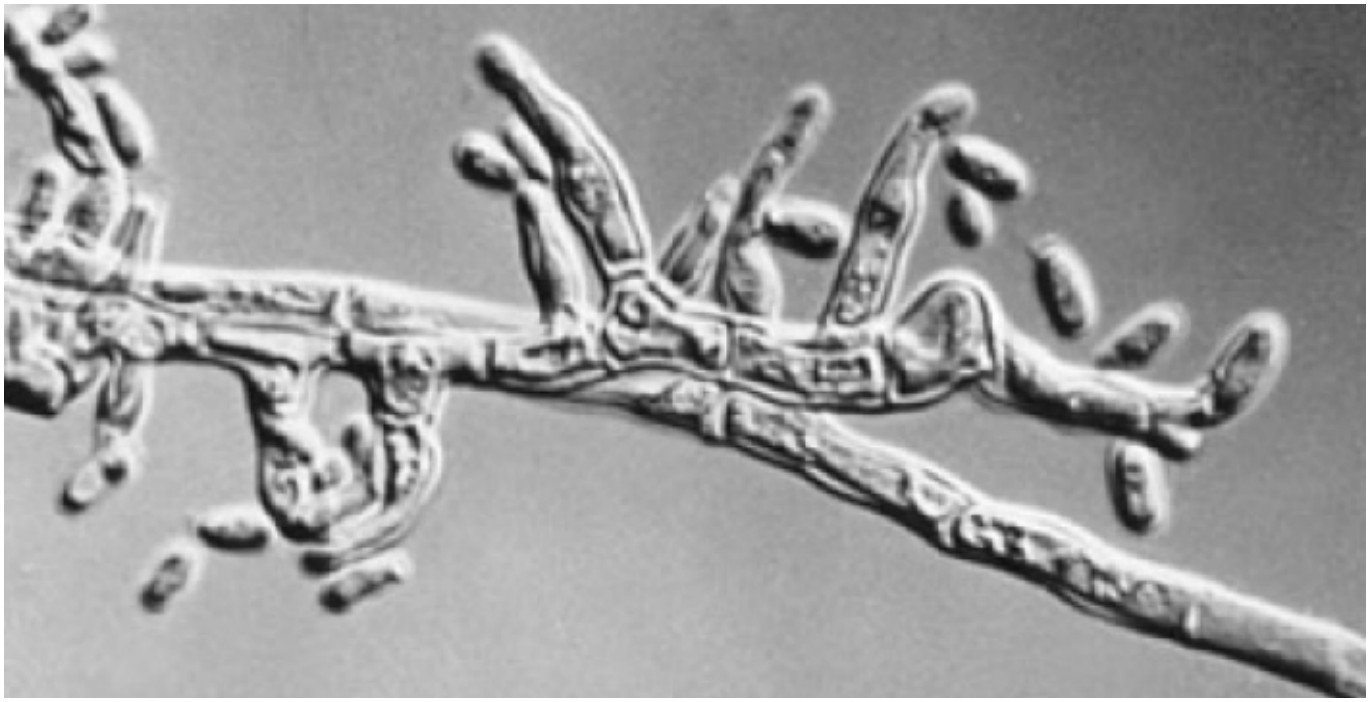

Figure 3 phialoconidia and phialides of *Plenodomus tracheiphilus* on free hyphae produced in culture.

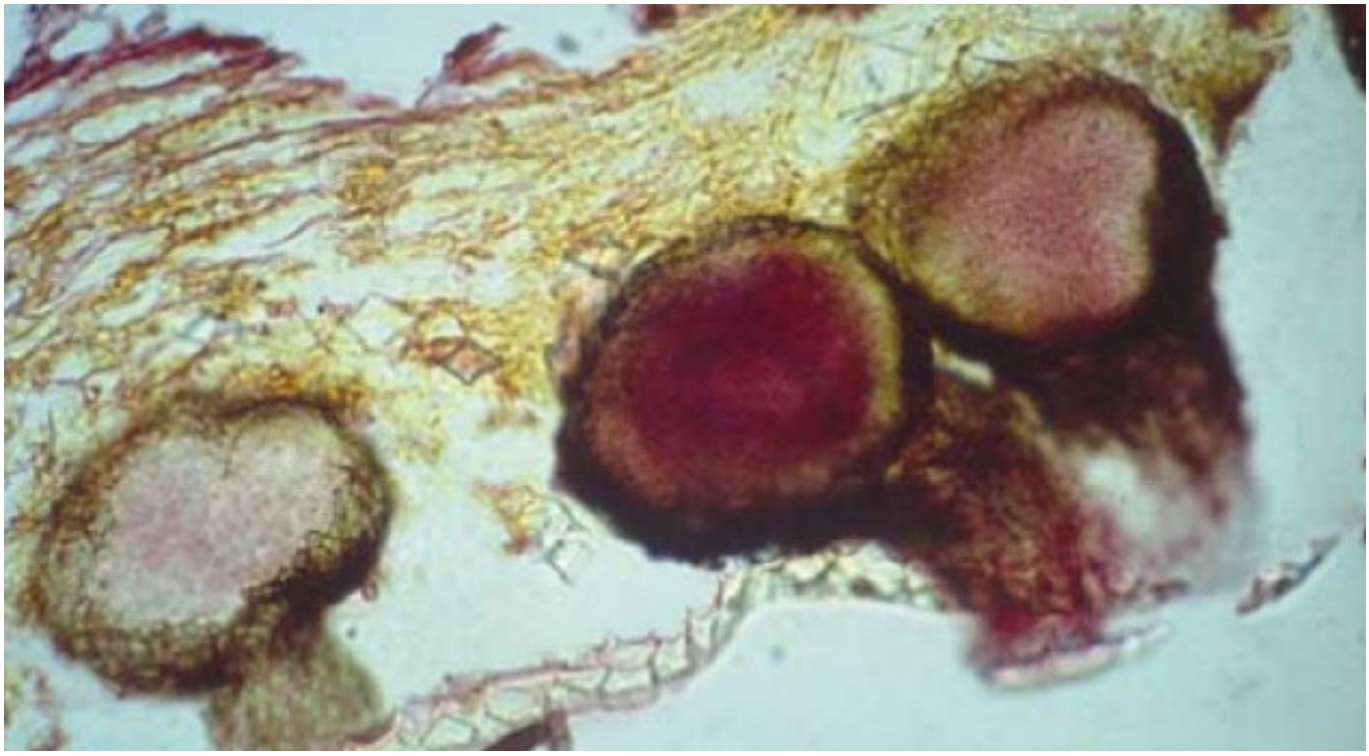

Figure 4 section of a withered twig of lemon showing pycnidia of *Plenodomus tracheiphilus* immersed in the cortex under the epidermis. Note the necks of pycnidia emerging through the epidermis.

Production of phialides and phialoconidia can be observed on 10–12 days old cultures grown on PDA, while the *in vitro* development of pycnidia can be stimulated by a 12-24 hours incubation, in humid chamber, of mal secco symptomatic twig pieces (EPPO, 2015).

***In laboratory* – molecular detection**

To date, the most consolidated methods for the molecular detection of the *P. tracheiphilus* in suspected plant material, as well as to confirm the isolation of the pathogen in pure culture, are all based on PCR techniques (EPPO, 2015).

DNA extraction

The prerequisite to the application of PCR is the DNA extraction from either plant material or pure culture. To this aim, specific laboratory protocols can be followed (Balmas et al., 2005), or commercial kits can be alternatively used.

Conventional PCR

The suggested conventional PCR protocol for the detection of *P. tracheiphilus* was developed by Balmas et al. (2005). The method targets two polymorphic regions of the internal transcribed spacer region (ITS) and is based on the employment of the *P. tracheiphilus*-specific primers Pt-FOR2: 5'-GGATGGGCGCCAGCCTTC-3' and Pt-REV2: 5'-GCACAAGGGCAGTGGACAAA-3'. The PCR amplification can be carried out by using commercial kits following the manufacturer instructions. The obtained PCR products (if any) can be separated on 1.5% agarose gel in TAE running buffer and visualized over a UV light.

Real-time PCR

The most employed Real-time PCR protocol for the detection of *P. tracheiphilus* was developed by Demontis et al. (2008). The method targets the ITS region of the nuclear rRNA and can be applied by using either SYBR® Green I or TaqMan® technologies. Sequences of the primers and probe are the followings: Phomafor: 5'-GCT GCG TCT GTC TCT TCT GA-3', Phomarev: 5'-GTG TCC TAC AGG CAG GCAA-3', Phomaprobe: 5'-FAM CCA CCA AGG AAA CAA AGG GTG CG BHQ-3'.

The PCR amplification can be carried out by using commercial kits following the manufacturer instructions. The PCR amplification plot is monitored in real time by the detection system.

Management

To date, no single method is effective in controlling the Mal Secco. Common strategies of control are based on the application of improved agronomical practices including the reduction of fungal inoculum by pruning symptomatic twigs and branches, particularly withered shoots bearing pycnidia, and the timely removal of rootstock suckers. Spraying with authorized copper-based fungicides are also carried out, especially on young plants from nurseries (Abbate et al., 2019). In order to reduce the environmental contamination by fungicides, a new super absorbent polymer (SAP) has been shown to act as efficient reservoir for the controlled release of copper in the treatment of pruning cuts of lemon twigs affected by the mal secco (El boumlasy et al., 2022). Other environmentally safe strategies for the management of Mal Secco have been pursued through lemon breeding (Migheli et al. 2009) based on the selection of spontaneous lemon genotypes tolerant to infection. Accordingly, in the Ionian coast of Sicily (Italy), the traditional variety 'Femminello' has been in the past replaced with the cultivars 'Monachello' and 'Interdonato', two spontaneous hybrids between lemon and citron. The latter species is highly resistant but produces qualitatively lower and reduced fruit yield (Catalano et al., 2021; Migheli et al. 2009).

References

1. Abbate L, Mercati F, Fatta Del Bosco S (2019) An overview on citrus Mal Secco disease: approaches and strategies to select tolerant genotypes in C. limon. Crop Breed Genet Genom 1:e190018. https://doi.org/10.20900/cbgg20190018
2. Balmas V, Scherm B, Ghignone S, Salem AOM, Cacciola SO & Migheli Q (2005) Characterization of Phoma tracheiphila by RAPDPCR, microsatellite-primed PCR and ITS rDNA sequencing and development of specific primers for in planta PCR detection. Eur J Plant Pathol 111, 235–247.
3. Demontis MA, Cacciola SO, Orru M, Balmas VC, Maserti E, Mascia L et al. (2008) Development of real-time PCR based on SYBR® Green I and TaqMan® technologies for specific quantative detection of Phoma tracheiphila in infected Citrus. Eur J Plant Pathol 120, 339–351.
4. El Boumlasy S, La Spada F, Pane A, Licciardello A, Debdoubi A, Tuccitto N, Cacciola SO, (2022) A super absorbent polymer containing copper to control Plenodomus tracheiphilus the causative agent of Mal Secco disease of lemon. Front Microbiol 13:987056. doi: 10.3389/fmicb.2022.987056
5. EPPO (2015) PM7/048 (3) Plenodomus tracheiphilus (formerly Phoma tracheiphila). Bulletin OEPP/EPPO Bulletin 46: 183-192. https://doi.org/10.1111/epp.12218
6. Gentile A, Deng ZN, Tribulato E, Albanese GR (2000) Evaluation of lemon somaclones for tolerance to Mal Secco disease by artificial inoculation. Acta Hortic 535(535):259-263. https://doi.org/10.17660/ACTAHORTIC.2000.535.32
7. Magnano di San Lio G, Cacciola SO, Pane A & Grasso S (1992) Relationship between xylem colonization and symptom expression in mal secco infected sour orange seedlings. Proceedings of the International Society of Citriculture 2, 873–876.
8. Migheli Q, Cacciola SO, Balmas V, Pane A, Ezra D, Magnano di San Lio G (2009) Mal Secco disease caused by Phoma tracheiphila: A potential threat to lemon production worldwide. Plant Dis 93(9):852–867.
9. Nigro F, Ippolito A, Salerno MG (2011) Mal Secco disease of citrus: A Journey through a century of research. J Plant Pathol 193(3):523–560.
10. Nigro F, Ippolito A, Salerno MG (2015) Searching for citrus rootstocks resistant to Mal Secco disease: A review. Acta Hortic 1065:987–992.
11. Russo R, Caruso M, Arlotta C, Lo Piero AR, Nicolosi E, Di Silvestro S (2020) Identification of field tolerance and resistance to Mal Secco disease in a citrus germplasm collection in Sicily. Agron 10(11):1806. https://doi.org/10.3390/AGRONOMY10111806

Olive tree dieback - Dépérissement de l'olivier - Deperimento dell'olivo *Pleurostoma richardsiae*

The Olive tree dieback by *Pleurostoma richardsiae* (family *Pleurostomataceae*) is a generalized decline of evergreen tree European olive (*Olea europaea* L.) reported from Brazil, Croatia and Italy (Canale et al, 2019, Carlucci et al, 2013, Ivic et al, 2018); recently, this syndrome has been also reported from north and central Tunisia.

The syndrome is characterized by foliar browning and leaf drop, wilting of apical shoots, die-back of twigs and branches, and brown streaking under the bark of the trunk, branches and twigs.

Diagnosis

In field - symptoms and signs

Plant symptoms are non-specific. The syndrome begins with foliar browning and leaf drop, wilting of apical shoots, die-back of twigs and branches, and brown streaking under the bark of the trunk, branches and twigs similar to those produced by *Verticillium* wilt (Canale et al, 2019; Carlucci et al, 2013) **(Figure 1).** Then, bark discolorations evolve in cankers.

Figure 1 Characteristic of dieback symptoms associated with fungal pathogens in olive trees in Tunisia. Severe twig dieback symptoms olive tree and discoloration of wood evolving in cankers.

In laboratory – isolation, characteristics of cultures and molecular identification of the pathogen

Isolation of the pathogen can be carried out directly from necrotic tissues. For the isolation, small pieces (4-5 mm) of necrotic wood has to be be surface sterilized (1% NaClO solution for 2 minutes, 70% ethanol for 30 seconds), washed twice with sterile distilled water, plated onto oatmeal agar

(OMA) and incubated at 25 °C for 21 days. After incubation, colonies of *Pleurostoma richardsiae* on OMA are characterized by a white to off-white cottony appearance in the center, with decreased aerial hyphae extending to the gray, slightly uneven colony margin. This kind of colonies produce brown sub-globose thick-walled conidia and phialides.

The identity of the pathogen is confirmed by traditional PCR targeting the internal transcribed spacer region (ITS1-5.8S-ITS2) and part of the β-tubulin (BT2); amplifications of the two genes can be carried out by using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) / ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) and T1 (5′-AACATGCGTGAGATTGTAAGT-3′) (O'Donnell et al., 1997) / Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (Glass et al., 1995) primer sets, respectively.

References

1. Carlucci, A., Raimondo, M., Cibelli, F., Phillips, A., and Lops, F. 2013. Pleurostomophora richardsiae, Neofusicoccum parvum and Phaeoacremonium aleophilum associated with a decline of olives in southern Italy. Phytopathologia Mediterranea. 52:517-527.
2. Canale, M. C., Nunes Nesi, C., Falkenbach, B. R., Hunhoff Da Silva, C. A., and Brugnara, E. C. 2019. Pleurostomophora richardsiae associated with olive tree and grapevine decline in southern Brazil. Phytopathologia Mediterranea. 58:201-205.
3. Glass, N.L., Donaldson, G.C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied Environmental Microbiology. 61:1323–1330.
4. Ivic, D., Tomic, Z., & Godena, S. 2018. First report of Pleurostomophora richardsiae causing branch dieback and collar rot of olive in Istria, Croatia. Plant Disease. 102(12), 2648-2648.
5. O'Donnell K, Cigelnik E. 1997. Two different intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus Fusarium are nonorthologous. Molecular Phylogenetics and Evolution. 7:103–116.
6. White, T.J., Bruns, T., Lee, S., and Taylor, J.W. 1990. "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in PCR Protocols: a Guide to Methods and Applications, eds M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (San Diego, CA: Academic Press Inc), 315–322.

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Main beneficiary
Università degli Studi di Catania (UNICT)

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P3: Centre Technique des Agrumes (CTA)
P4: Institut National de la Recherche Agronomique de Tunisie (INRAT)
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PROMETEO PROJECT IN NUMBERS	
Duration	**24 months**
Project start-up	**29/10/2021**
Date of completion	**28/10/2023**
N. of project Partners	**8**
Overall budget	**1.459.103,08 €**
EU Contribution	**1.291.659,13 €**

PROMETEO PROJECT ACTIVITIES	
Number of dissemination events and thematic meetings	**5**
Involved participants	**450+**
Project website	**1**
Social media channels	**4**

OUR CONTACTS

Project website: https://www.prometeo-italietunisie.eu

E-mail address: info@prometeo-italietunisie.eu

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