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Mal Secco Disease Caused by *Phoma tracheiphila*: A Potential Threat to Lemon Production Worldwide

Lemon (*Citrus limon* (L.) Burm. f) is the most popular acid citrus fruit because of its appealing color, odor, and flavor. World production of lemons was about 7 to 8 million metric tons in 2007. Major producers and exporters of lemon in the world include Argentina, Spain, Iran, the United States, Italy, Turkey, Egypt, Greece, South Africa, Cyprus, Morocco, and Israel (www.cga.co.za). Argentina and Italy are major suppliers of lemon juice, and Spain, Argentina, Turkey, the United States, South Africa, Italy, Chile, Egypt, Uruguay, India, Jordan, Cyprus, China, and Iran are the leading exporting countries of fresh fruit.

Mal secco of citrus (Fig. 1) is a highly destructive vascular disease of lemon, presently confined to the Mediterranean basin, which has a relevant economic impact on the lemon industry in this geographic region. Mal secco is caused by the mitosporic fungus *Phoma tracheiphila* (Petri) Kantschaveli & Gikachvili (syn. *Deuterophoma tracheiphila* Petri). The name of the disease stems from the Italian words *male* (disease) and *secco* (dry). The term "malsecco," referring to nonspecific symptoms, was initially used in a broad sense to indicate citrus diseases of various origins (129). Later, Petri (101) used the term "mal secco of citrus" in a more strict sense to indicate the tracheomycotic disease that was spreading in lemon orchards in Sicily. Mal secco first appeared affecting lemon trees on the islands of Chios and Poros (Greece) near the end of the nineteenth century. In 1929, Petri (101) described the fungus causing mal secco as a new species and named it *Deuterophoma tracheiphila*, which he proposed as the type-species of the new genus *Deuterophoma* Petri. The species was transferred to *Phoma* by Kantschaveli and Gikachvili in 1948 (15). Later, Ciccarone and Russo (31) and Ciccarone (30), who amended the description of the fungus, confirmed this binomial as the correct name.

Disease Symptoms

In lemon orchards, symptoms of mal secco usually first appear in spring as leaf vein (Fig. 2) and shoot chlorosis as well as epinasty of young leaves followed by both shedding of leaves and phylloptosis, wilt and dieback of twigs and branches (Fig. 1) (45,46,97,148). Typically, phylloptosis starts

Fig. 1. Top: Collapse of a lemon tree (cv. Sfusato di Favazzina) affected by mal secco disease in Calabria (southern Italy). Bottom: Wilting and defoliation of twigs in a young lemon tree affected by mal secco in a commercial orchard in Israel. Note the typical sectorial pattern of symptoms on the canopy.

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in the apical parts of branches and sprouts and resembles frost damage (Fig. 3). The progress of the disease depends also on the age of the tree, and the pathogen may kill a citrus plant within a few months or years. The disease course is related to the rate of colonization of the vascular system by the pathogen (44,77). Root infections usually result in more rapid progress of the disease than foliar infections, as the inoculum is spread more efficiently. On the affected withered twigs, immersed, flask-shaped, or globose pycnidia appear as black spots within lead-gray or ash-gray areas (Fig. 4). The ash appearance of withered twigs is due to the presence of pycnidia that lift the epidermis, thus allowing air to infiltrate. Acervuli of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., a secondary invader of withered twigs, are often associated with the pycnidia of *P. tracheiphila*. The acervuli of *C. gloeosporioides* are easily visible and appear arranged in concentric rings, whereas the pycnidia of *P. tracheiphila* are scattered and cannot be distinguished easily with the naked eye, as they are immersed in the cortical tissues of the twig, under the epidermis. If pycnidia are present, they can be mounted in distilled water or lactophenol blue and observed under the microscope. Withered twig pieces may be incubated in a humid chamber for 12 to 24 h to facilitate the detection of pycnidia. After incubation, spore tendrils (cirrhi) protruding from pycnidia can be easily observed under the stereomicroscope. Growth of sprouts from the base of the affected branches, and of suckers from the rootstock, is a very common response of the tree to mal secco. Often a single branch is affected and therefore symptoms appear in one sector of the canopy (Fig. 1). Gradually, the infection progresses basipetally, affecting the entire tree, which eventually dies (Fig. 5). Upon cutting into the twigs or after peeling off the bark of branches or trunk of infected trees, typical salmon-pink or orange-reddish discoloration of the wood can be seen (Fig. 6); this internal symptom is associated with gum production within the xylem vessels.

Although most of the symptoms of mal secco are not specific, the syndrome of the disease is quite characteristic and allows the disease to be diagnosed confidently in the field. In addition to the more common form of mal secco, chronic infections on mature trees, very likely originating from the roots, may cause a brown discoloration of the heartwood without any external symptoms at first. However, when the pathogen invades the outer functional xylem, infected trees collapse suddenly. This form of the disease is known as "mal nero" from the Italian words *male* = disease and *nero* = black, referring to internal wood browning (34) (Figs. 7 and 8). Another form of the disease, commonly distinguished with the name "mal fulminante"

Fig. 2. Top: Vein clearing and chlorosis of lemon leaves affected by mal secco disease. Bottom: Clearing of secondary veins of a sour orange leaf affected by mal secco disease.

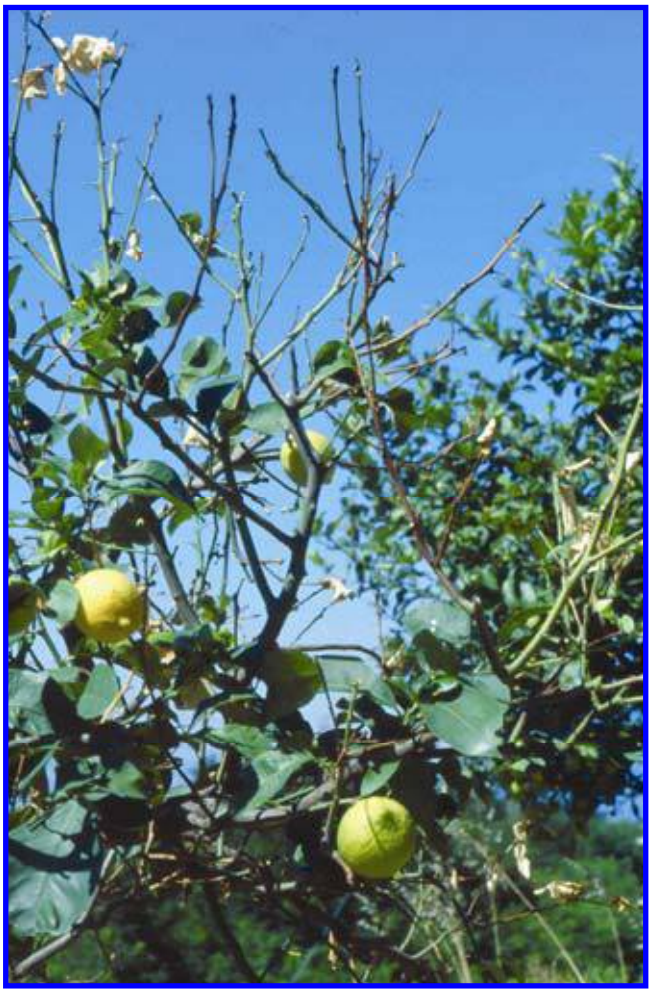

Fig. 3. Leaf shedding and defoliation of apical twigs in a 'Femminello' lemon tree affected by mal secco disease.

(*male* = disease, *fulminante* = withering), is a rapid fatal form that is apparently caused by root or stem infection, which leads to a systemic invasion of the functional xylem by the pathogen and sudden wilting of branches or the whole tree (97,130,133).

Hosts

The principal host of mal secco is lemon, but the fungus has also been reported on many other citrus species, including those in the genera *Citrus*, *Fortunella*, *Poncirus*, and *Severina*, and interspecific as well as intergeneric hybrids (45). The relative susceptibility of citrus selections to mal secco has not always been rated experimentally in comparative pathogenicity tests; in fact, most of the information we have is based on field observations. The disease is highly destructive on lemon, and citron (*C. medica* L.), lime (*C. latifolia* Tan.), and bergamot (*C. bergamia* Risso) also have been reported to be very susceptible to natural infections. Different degrees of resistance are shown by other species and hybrids. Sweet orange (*C. sinensis* (L.) Osbeck), grapefruit (*C. paradisi* Macf.), clementine mandarin (*C. clementina* Hort.), tangerine (*C. reticulata* Blanco), and mandarin (*C. deliciosa* Tenore) are affected sporadically by the disease and are considered tolerant. In some cases, however, mandarins and related hybrids were severely affected (91). Among rootstocks, sour orange (*C. aurantium* L.), the most widespread lemon rootstock in Italy, Greece, and Turkey, rough lemon (*C. jambiri* Lush.), volkamer lemon (*C. volkameriana* Ten. & Pasq.), and alemow (*C. macrophilla* Wester) are very susceptible (39,135). In Italy, severe infections of mal nero have been observed in commercial orchards of sweet orange 'Tarocco nucellar line' and clementine mandarin on sour orange rootstock as well as on 'Fortune' mandarin and 'Tacle' hybrid (*C. sinensis* 'Tarocco' × clementine) on alemow rootstock (7,76). Similarly, the occurrence of mal nero disease on 'Cassar' mandarin and 'New Hall' sweet orange on sour orange rootstock was observed in commercial orchards in Tunisia (66). Conversely, other rootstocks such as Cleopatra mandarin (*C. reshni* Hort. *ex* Tanaka), trifoliate orange (*Poncirus trifoliata* (L.) Raf.), and to a lesser extent, Troyer citrange (*C. sinensis* × *P. trifoliata*) have been reported as tolerant (45). There is evidence indicating that the rootstock influences the susceptibility of the scion to mal secco (89,138). Reports on the degree of susceptibility of citrus species to mal secco are sometimes contradictory, especially for rootstocks. Sour orange, for example, is considered to be very susceptible in Italy, but only moderately affected in Israel (91). Moreover, in pathogenicity tests, the susceptibility of sour orange decreases with the age of the plant (44). In general, on the basis of the authors' experience, it can be stated that almost all citrus species are susceptible to *P. tracheiphila* when artificially inoculated by wounding.

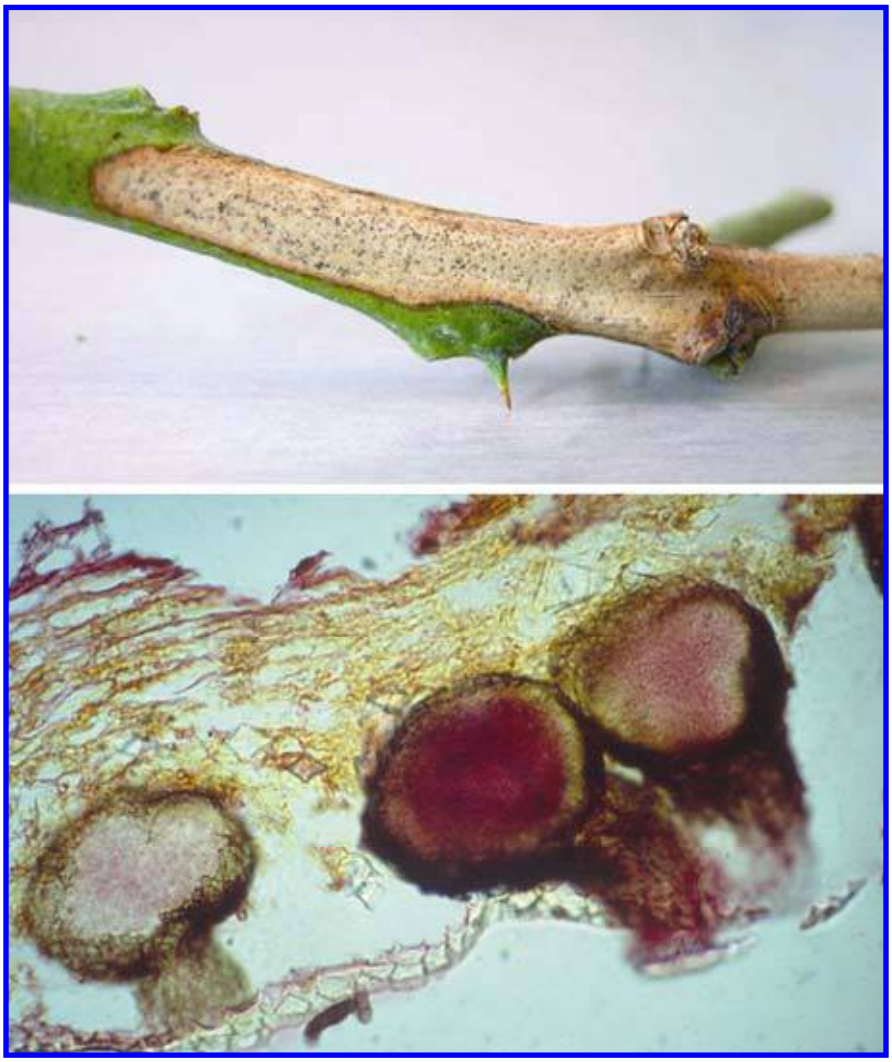

Fig. 4. Top: Withered twig of lemon with pycnidia of *Phoma tracheiphila*. Note the ash appearance of the dried apical portion of the twig on which pycnidia appear as scattered black spots. Bottom: Tangential section of a withered twig of lemon showing pycnidia of *P. tracheiphila* immersed in the cortex under the epidermis (optical micrograph). Note the necks of pycnidia emerging through the epidermis. (Courtesy S. Grasso.)

Geographical Distribution

Mal secco is present in all the citrus-producing countries in the Mediterranean and Black Sea areas with the exception of Spain, Portugal, Morocco, and some areas of the Arabian Peninsula. The disease is not known to occur in the citrus-growing countries of the Americas or Oceania (46,71,97,102), even though there is no obvious climatic or cultural factor limiting the establishment of mal secco disease in uninfested areas. 'Eureka', a major lemon cultivar in Argentina, Australia, Chile, South Africa, and the United States, as well as 'Verna', a major lemon cultivar in Spain, proved to be very susceptible in other countries where the disease is ubiquitous, such as Italy (121) and Tunisia (K. Khaled, *personal communication*). *P. tracheiphila* was reported from Uganda (Central Africa) and Colombia (South America), but such records were not confirmed (17,102). Consequently, South America, which has been included in the present distribution area of mal secco (71), should be removed from the list of countries and continents where the disease has been reported. Presently, *P. tracheiphila* is on the list of A_2 quarantine pests of the European and Mediterranean Plant Protection Organization (EPPO). Moreover, it is a quarantine pathogen on the lists of most other regional plant protection organizations, such as APPPC (Asia and Pacific Plant Protection Commission), CPPC (Caribbean Plant Protection Commission), COSAVE (Comité Regional de Sanidad Vegetal para el Cono Sur), PSC NAPPO (North American Plant Protection Organization), and IAPSC (Interafrican Phytosanitary Council).

The intensive study of mal secco disease started when it was first reported in Sicily

in the eastern province of Messina in 1918. Although it had been present in Greece since the end of the nineteenth century, the causal organism was not determined until 1929 (101). There is no sound evidence to trace back the area of origin of *P. tracheiphila,* although it was suggested that it could be central Asia (Persia, Afghanistan, and northwestern India), which would coincide with the center of diversity of the principal host-species, *C. limon* (142).

It has been assumed that mal secco was introduced into Sicily accidentally with lemon plants imported from Greece (119). The disease in lemon orchards was destructive because the most common cultivar, Femminello, was very susceptible. The disease then rapidly extended south and west to the neighboring provinces of Catania and Palermo. The first report in the Catania province was in 1922, and it was

Fig. 5. Left: Dead lemon tree on sour orange rootstock affected by mal secco. Note the suckers underneath the grafting point (recognized by overgrowth of lemon stock) and the ash-gray color of withered suckers and twigs on which pycnidia of *Phoma tracheiphila* are produced. Right: Orange-reddish discoloration of the wood of the sour orange rootstock in a mal secco–infected 'Femminello Siracusano' lemon tree topworked with 'Monachello' lemon in an attempt to cure the tree. Bark was peeled off to show symptomatic wood.

Fig. 6. Left: Transverse section on an orange twig with the typical orange-reddish discoloration of the wood. Right: Orange-reddish discoloration of the wood in a branch of 'Fortune' mandarin tree grafted onto alemow affected by mal secco. Bark was peeled off to show symptomatic wood.

first reported in Palermo in 1938 (5). By 1947, mal secco appeared in the regions of Latium and Calabria, which are both located in northern Sicily; however, it took about 50 years for epidemics to develop in the lemon-growing areas of the provinces of Syracuse and Ragusa in southeastern Sicily (36). Currently, the disease is present in all Italian lemon-growing regions, including Liguria and the island of Sardinia (Fig. 9).

Fig. 7. Longitudinal section of the stem of a 'Fortune' mandarin tree grafted onto alemow affected by mal nero disease, showing the typical brown discoloration of the wood.

Fig. 8. Transverse section of the stem of a 'Fortune' mandarin tree affected by mal nero disease, showing the typical brown discoloration of the wood.

Mal secco was first reported in Israel and neighboring countries in 1930, although the typical symptoms of the disease in lemons and citrons were noted by growers many years earlier (29).

Economic Impact

Mal secco is a highly destructive disease with a significant negative impact on the lemon industry in areas where it is endemic. Direct damage caused by the disease can lead to low yield related to the reduction of canopy volume, or can lead to the death of the tree. Disease progress in lemon orchards depends on several factors, which are only partly known, but there is evidence to indicate that it increases after hail storms or frosts, probably because numerous wounds on the tree canopy could favor pathogen penetration. It was estimated that in 15 years after being first noticed in Sicily, about 3,000 ha of lemon orchards were destroyed (26,129). According to Cutuli (36), in the 1980s the mean fruit yield of lemon orchards in Sicily (the region that accounts for more than 90% of Italian lemon production) was about 20 metric tons/ha, whereas in lemon orchards not affected by mal secco, yield could reach 60 to 80 metric tons/ha. The same author estimated that 5% of the lemon trees were killed and 50% were infected each year by mal secco on a total lemon orchard hectarage of 20 million (36). In 1956, in the district of Mersin (Turkey), about 20,000 lemon trees were reported to have been killed by mal secco within 15 years (Karel cited in Cutuli et al. [37]). In Greece, 60 to 100% of the trees were affected within 20 to 25 years after planting, depending on the cultivar (147). In 1991, the incidence of mal secco in Greece was roughly 30 to 40%, accounting for yield decreases of 20 to 30%. In more conducive conditions, yield losses up to more than 60% were reported in some lemon and citron orchards (147). It was estimated that in Sicily and in years with a high disease incidence, mal secco disease may have caused losses of up to 50% (35).

In a survey of 49 'Femminello Siracusano' lemon groves in the Syracuse province (Sicily), disease incidence was 36 to 38% in trees under 20 years old and up to 49% in older trees (75). Conversely, the lethality index (number of dead trees/number infected trees × 100) was higher in trees under 8 years old, indicating that young trees are more susceptible than adult trees. The mean reduction in volume of the tree canopy was 34% in the over-20-year-old trees and varied from 24 to 29% in younger trees. Assuming that fruit yield is correlated with canopy volume, these figures are a circumstantial estimate of the losses in fruit yield due to mal secco infection (75). Damage caused by mal secco

includes the presence in the same orchard of trees of a different age and size following the substitution of dead plants, the cost of pruning withered branches and twigs, and reduction in quality of fruit as a consequence of the use of low-fruit-quality resistant cultivars such as 'Monachello'. Mal secco limits the use of susceptible lemon species or cultivars and discourages the introduction of new cultivars, such as virus-free nucellar clones, that are more productive than the old lines but very susceptible to the disease. Moreover, lower fruit production due to the reduction of nitrogen fertilizers can be considered to be indirect damage of the disease.

Mal secco is among the reasons that Sicilian growers quit using the practice known as the "Verdelli process", which consists of withholding water in summer for 35 to 60 days until the trees begin to wilt. The trees are then abundantly irrigated and provided with nitrogen fertilizer which induces the plant to produce a second bloom in August or early September and additional fruit the following summer when lemons are scarce and prices are higher. Lemon trees forced in this way proved to be extremely susceptible to mal secco.

Pathogen

P. tracheiphila is a mitosporic fungus that produces subepidermal black, thick-walled, scattered or serially arranged pycnidia (60 to 165 × 45 to 150 µm in diameter) in the bark of infected twigs and branches and around leaf scars and bark cracks (Fig. 4). Pycnidia are initially subspheric, but at maturity develop a long neck, 45 to 70 µm diameter and up to 250 µm length, with an apical ostiole that protrudes after perforating the epidermis (Fig. 4). Mature pycnidia contain conidiogenous cells (phialides) lining the internal cavity; conidia produced in the pycnidium, usually named pycnoconidia, are hyaline eguttulate or biguttulate, unicellular, mononucleate and sometimes binucleate, subcylindrical, straight or slightly curved, with dimensions of 2.0 to 4.0 × 0.5 to 1.5 µm. At maturity with high relative humidity, conidia are extruded through ostioles in whitish cirrhi. Larger conidia (1.5 to 3 × 3 to 8 µm), usually named phialoconidia (Fig. 10), are produced by phialides (12 to 30 × 3 to 6 µm) borne on free hyphae grown on exposed wood surfaces, including wood debris on soil, wounded plant tissues, and within the xylem elements. Phialoconidia are hyaline, unicellular, uninucleate (sometimes binucleate or trinucleate), straight or curved with rounded apices. Pycnidia and phialides are also differentiated on artificial culture media. Ovoid subpyriform blastoconidia (15 to 17 × 7 to 9 µm) are produced inside the xylem vessel of the host and in culture on liquid media (102). Pycnidia are differentiated in vitro between 5 and 25°C with an optimum at 20 to 25°C; pycnoconidia germinate between 5 and 28°C with an optimum of 25°C (123). In vitro, the optimum temperature for mycelium growth is 20 to 25°C. In artificially inoculated sour orange seedlings, the optimum temperatures for xylem colonization by the pathogen were 15 and 22°C, whereas at or below 10 and above 28°C colonization of the xylem was inhibited (G. Magnano di San Lio, *unpublished results*).

Even though the sexual stage of *P. tracheiphila* has been observed neither in vitro nor in nature, recent molecular evidence demonstrated that this species is phylogenetically related to *Leptosphaeria*, the teleomorphic stage of numerous *Phoma* species. In particular, a neighbor-joining analysis of internally transcribed spacer (ITS) sequences of *P. tracheiphila* in comparison with those of other *Phoma* species, as well as with alignable sequences from anamorphic and teleomorphic taxa retrieved in BLAST searches, revealed a close relationship between *P. tracheiphila* and *Leptosphaeria congesta* (8).

P. tracheiphila produces anthrachinonic pigments in common artificial media, such as potato dextrose agar, carrot agar, and Czapek Dox agar (46), and on the basis of this ability, chromogenous and nonchromogenous variants have been distinguished in culture. Chromogenic isolates show a high phenotypic instability in culture, often forming variant sectors differing in color from the rest of the colony (e.g., they produce albino variants) (Fig. 11). Both chromogenic and nonchromogenic isolates on artificial media soon lose their ability to form pycnidia (46).

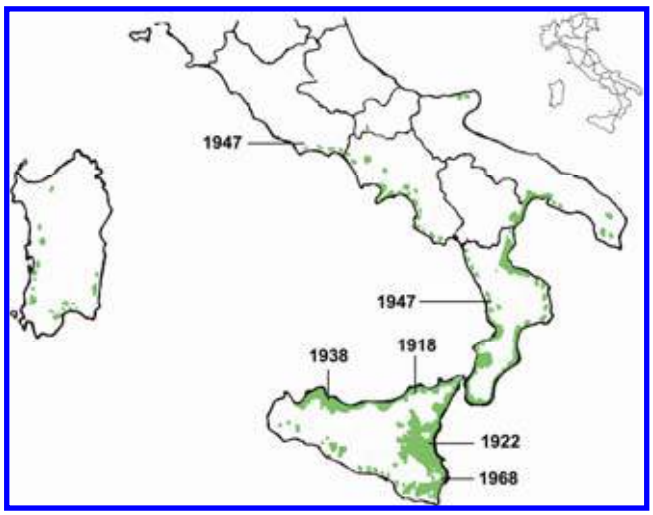

Fig. 9. Geographical distribution of citriculture in southern Italy (shaded areas) indicating the date of first reports of mal secco disease in different lemon-growing areas.

Fig. 10. Differential interference contrast optical micrograph. Phialoconidia and phialides of *Phoma tracheiphila* on free hyphae produced in culture.

Variability of the Pathogen

The morphological and genetic variability of *P. tracheiphila* populations in different lemon-growing areas showed that isolates from various geographical origins were variable in colony morphology and virulence (23,126,144). Chromogenic and nonchromogenic isolates were distinguished on the basis of the ability to produce red pigments in culture (4,103). Five anthraquinone pigments, chrysophanol, helminthosporin, cynodontin, emodin, and islancidin, were identified in culture filtrates of *P. tracheiphila* (6,103,141). Only

the chromogenic strains were able to produce the entire spectrum of pigments when grown on a medium containing coconut flesh (Fig. 12). In nature, chromogenic isolates are more common than nonchromogenic ones and show higher virulence. Similarly, the inoculation of albino strains on sour orange failed to produce disease symptoms (62,80); however, the relationship, if any, between the ability to produce pigments and virulence is still unsubstantiated. It was also demonstrated that the virulence of UV-induced *nit* mutants as well as benomyl-resistant mutants of chromogenic and nonchromogenic isolates of *P. tracheiphila* was no different from that of the wild parental isolates (20). It has been assumed that the occurrence of *P. tracheiphila* variants could be related to the heterokaryotic condition of the thallus. The finding of bi- and trinucleate conidia mixed with normally uninucleate ones and the frequent occurrence of both plurinucleate hyphal elements and anastomoses between hyphae and germinating conidia seem indeed to support this hypothesis (62,78). Biochemical and genetic studies indicated that in Italy, Israel, and Greece, the variability of *P. tracheiphila* populations is very low (8,24,50,72). This genetic uniformity may be the consequence of the lack of sexual reproduction and corroborates the hypothesis that the pathogen was introduced into the Mediterranean basin from other regions of the world.

Epidemiology

Conidia are produced in pycnidia found on withered twigs 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; birds and insects have been suspected as vectors but have never been demonstrated as such. Most probably the pathogen can also be transmitted to other trees through contaminated pruning tools. Twigs and leaves lying on the soil may be 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 (149).

De Cicco et al. (40) investigated the ability of the pathogen to survive in soil artificially inoculated with wood material. In sandy soil, inoculum had lost its infectivity by 30 and 60 days after placement in a growth chamber or in a tunnel of polyethylene shadow net (shade 50%), respectively; in loam soil, inoculum was infective 30 and 60 days in a growth chamber and under net shelter, whereas in clay it maintained infectivity for 120 days. In a field experiment in loam soil, infectivity was retained for at least 120 days. Furthermore, it was observed that in orchards with natural infections, the fungus may remain infectious up to 1 year, depending on the soil type (40). Thus, replanting of susceptible plants in a previously contaminated orchard is not recommended.

The role of infected citrus fruit and seeds in the spread of mal secco could have relevance to restrictions of fruit exportation to disease-free countries. However, infected fruits fall to the ground prior to harvest or are discarded because of their low quality (68). Spread through seeds is not a serious concern because it was found that the fungus colonizes seed coats but not embryos, and the treatment of contaminated seeds with water at 50°C for 10 min is effective against *P. tracheiphila* (68).

P. tracheiphila penetrates the host through wounds via both conidia and mycelium (12). Penetration through stomata was hypothesized by Petri (101) but never demonstrated (91,157). Although the optimum temperature for pathogen growth is about 25°C, optimum temperature for symptom expression and xylem colonization is 20 to 22°C. Infection occurs between 14 and 28°C, whereas at temperatures above 28°C, fungal growth ceases and symptoms are not expressed. Disease progress is temporarily inhibited during the hot or cold temperature extremes. The pathogen was not detected by a PCR-based specific assay in vegetation flushes produced in summer by artificially inoculated lemon plants that had shown symptoms during the previous spring months (S. O. Cacciola, *unpublished data*). In the Mediterranean region, the infection period depends on climatic and seasonal conditions. In Sicily, infections occur from September through April (61,118,139,140). In Israel, mid-November to mid-April was the most conducive time for infection, coinciding with the rainy season, although no correlation was found between the amount of rain, the number of rainy days, and the percentage of infected plants (133). No infection was observed after the rain ceased, so it appears that the rain affects inoculum dissemination rather than infection, as dew is common in groves during this time (133).

Length of the incubation period varies according to season, and in young trees it ranges between 2 and 7 months (64,139), whereas it can last several years in the mal nero form of the disease (36) because this chronic infection could remain confined to the heartwood over a long time. Expression of symptoms is therefore a poor selection criterion for phytosanitary inspection of propagation material.

Identification Methods

Identification of *P. tracheiphila* is currently based on morphological and molecular methods as described in the OEPP/EPPO standard (46).

Diagnosis of mal secco is therefore confirmed when the fungus is isolated on agar media and identified on the basis of cultural and morphological characters or, in the absence of sporulation, by either a molecular method or the analysis of mycelial proteins by polyacrylamide gel electrophoresis (PAGE).

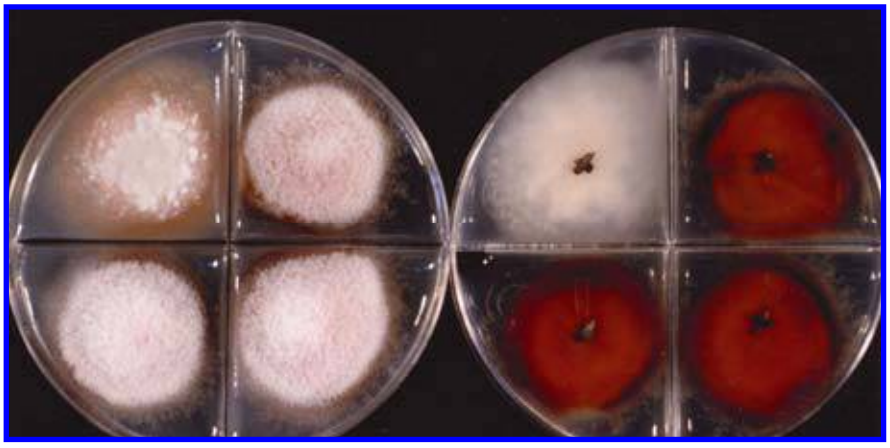

Fig. 11. Single-conidium subcultures of a chromogenic strain of *Phoma tracheiphila* on Czapek Dox agar. Front and back side of the petri dish are shown on the left and the right, respectively. Note an albino variant on the top left.

Fig. 12. Colonies in coconut agar of chromogenic and nonchromogenic variants of *Phoma tracheiphila*. The chromogenic isolate can be distinguished by the red pigmentation; aerial mycelium of the nonchromogenic isolates was scraped to show yellow pigment secreted into the substrate.

An enzyme-linked immunosorbent assay (ELISA) has also been developed, enabling the detection of *P. tracheiphila* antigens in crude plant extracts (82). Despite its potential for early diagnosis of mal secco in epidemiological studies and for quarantine purposes, ELISA has not been widely applied due to high levels of nonspecific reactions and was therefore not included in the OEPP/EPPO standard (46).

Molecular diagnosis can be considered positive when an amplicon of the predicted size is obtained with the PCR-based specific assay of DNA extracted directly from symptomatic twigs and fruits (8,50). More recently, additional techniques based on real-time polymerase chain reaction (PCR) were developed, allowing fast and sensitive quantification of *P. tracheiphila* from infected tissues (44,72).

Isolation in culture. Isolation on agar media remains the most widely used method for routine diagnosis. The disadvantage of this approach is that detection of the pathogen is only possible at a late stage of the infection, when it is already too late for any management decisions to be taken and the spread of the disease can no longer be controlled. In the field, samples for isolation can be taken at any time of year. If nursery plants are grafted onto a susceptible rootstock (e.g., sour orange), the rootstock should also be inspected and tested (46). Samples must be processed within a few days of being collected and should be stored at 8 to 10°C wrapped in damp towels or paper in plastic bags.

P. tracheiphila can be isolated from pieces of infected tissue excised from discolored wood on potato dextrose agar (PDA), carrot agar (CaA), or malt extract agar (MEA) amended with chloramphenicol (1 μg ml^{-1}). Czapek-Dox agar may be used solely as a culture medium. Twig sections (5 to 10 mm thick) should be dipped in sterile distilled water for 30 to 60 min, dried on sterilized filter paper, and placed on agar medium. Alternatively, twig sections (2 to 5 mm thick) may be surface-sterilized with 0.5 to 1% NaOCl or 50% ethanol for 40 s to 5 min depending on the thickness and diameter of the sections, rinsed in sterile distilled water, and plated on agar medium. Petri dishes should be incubated in the dark at 23 ± 2°C for 6 to 12 days. Optimal temperature for growth on PDA is 23 ± 2°C, which allows a growth rate of 3.8 to 6.0 mm/day. Mycelium is initially hyaline, then turns brown or pinkish-red after a few days. Phialoconidia are usually produced after 10 to 12 days. Colonies of the secondary invader *C. gloeosporioides* are often co-isolated from lignified tissues or from withered twigs, and care should be taken in the early identification and accurate monosporic culturing of *P. tracheiphila* before it can be overgrown by *C. gloeosporioides*. Moreover, cultures of *P. tracheiphila* may be confused with *Epicoccum* sp., developing a brown-red to orange, relatively dense aerial mycelium. The agar medium gradually becomes orange-yellow to brownish. Chromogenous and nonchromogenous variants can be distinguished in culture and may be occasionally isolated from the same tree (50,77). Under in vitro conditions, all isolates lose their ability to produce pycnidia and differentiate only phialoconidia. After repeated subculturing, some isolates also lose the ability to produce phialoconidia (46). In the absence of sporulation, isozyme analysis by PAGE or molecular methods should be used.

Analysis of mycelial proteins by PAGE and isozyme analysis. Proteins extracted from mycelium of pure cultures grown for 8 to 9 days on liquid medium (24) may be used to assist in the identification of strains that differ in their capacity to produce pigments or that do not form pycnidia (46). Total proteins may be visualized after Coomassie blue staining. Alternatively, esterase (EC 3.1.1.1 or EC 3.1.1.2) and glucose phosphate isomerase (EC 5.3.1.9) are suggested as diagnostic isoenzymes for *P. tracheiphila*. A reference strain of *P. tracheiphila* should always be included, and other species of *Phoma*, such as *P. medicaginis*, may be used as a negative control (46).

Molecular methods for the identification of *P. tracheiphila*. The first attempt to develop a molecular method of identifying *P. tracheiphila* in pure culture or diseased tissues was reported by Rollo and coworkers (114,115), who designed a dot-blot assay and, subsequently, a PCR assay targeted at a 102-bp randomly cloned fungal DNA sequence. More recently, the primer pair developed by Rollo et al. (115) was shown to generate a series of nonspecific amplimers when tested with DNA extracted from several *Phoma* species and other citrus-related fungi (8,50). Despite its apparent lack of specificity, this protocol represents one of the first examples of a PCR-based test applied to the diagnosis of plant-pathogenic fungi (67).

Balmas et al. (8) developed a pair of *P. tracheiphila*–specific primers (PtFOR2 and PtREV2) based on the consensus sequence obtained from the alignment of the internal transcribed spacer (ITS) region of the nuclear rRNA genes of 17 *P. tracheiphila* isolates and of single representatives of six additional *Phoma* species (*P. glomerata*, *P. exigua*, *P. betae*, *P. cava*, *P. fimeti*, and *P. lingam*). The PCR assay allowed detection of the specific fragment in 10 pg of total genomic DNA or in 5 fg of the ITS target sequence. It was also used to detect the fungus in symptomless twigs and in the hardwood of trees affected by mal nero (7). The ITS region was also targeted by Ezra et al. (50) to design an alternative pair of *P. tracheiphila*–specific primers. This PCR assay proved useful in detecting the pathogen in infected fruit.

Based on the probe previously cloned by Rollo et al. (115), Licciardello et al. (72) designed a primer pair and a dual-labeled fluorogenic probe that were used in real-time PCR with the Cepheid Smart Cycler II System (Cepheid, Sunnyvale, CA, USA) to detect *P. tracheiphila* in citrus samples. The sensitivity of the real-time approach was estimated as approximately 500 fg of DNA, even though 1 pg of DNA was considered the limit for reproducible DNA quantification (72). Using this assay, approximately 7 pg of DNA of *P. tracheiphila* were estimated in symptomless sections of lemon twigs from infected plants (72).

In 2008, two new real-time PCR assays based on SYBR Green I and TaqMan technologies were developed for the specific quantitative detection of *P. tracheiphila* in infected citrus (44). The alignment of the ITS region sequences of *P. tracheiphila* and of other *Phoma* species revealed several regions with low levels of homology among the different species. A primer pair was designed that specifically amplified an 82-bp-long fragment of the ITS region which was quantified by the TaqMan probe Phomaprobe (44). The real-time assay detected 10 cloned copies of the target rDNA sequence, roughly corresponding to 1/10 to 1/20 of the haploid genome (or mononucleate spore). When the real-time assay was tested with serially diluted total genomic DNA extracted from a titrated spore suspension of the target pathogen, the minimum amount detectable was 15 pg, corresponding to <1 fungal spore per reaction (44).

The real-time approach was compared with conventional isolation by analysis of sour orange seedlings artificially infected with *P. tracheiphila*. Target sequence concentration values were consistent with the results of conventional isolation and standard PCR. There was no significant difference between the TaqMan and SYBR Green I approaches, indicating that the SYBR Green-based assay is as sensitive as the TaqMan assay when tested with the same PCR primers (44).

Demontis et al. (44) addressed the risk of false negatives, which is particularly relevant in regulatory situations and assays aimed at monitoring inoculum of *P. tracheiphila* in plant debris or soil for epidemiological purposes. Naturally occurring compounds, such as humic acids, tannins, and lignin-associated compounds, can interfere with PCR reactions and inhibit amplification (16). Thus, complete inhibition of the reaction was demonstrated when conidia of the target pathogen were mixed with an organic substrate before extraction of total DNA in a standard purification procedure (44). An alternative extraction and purification protocol through commercial spin columns was demonstrated to decrease sensitivity as it increased the minimum amount of target DNA to be accurately quantified from 15 to 950 pg (44). Therefore, the adoption of

a method for the prior assessment of DNA quality is essential, despite recent improvements in soil DNA extraction protocols (105). This aspect is particularly important for quarantine pathogens such as *P. tracheiphila*, for which the results of a molecular analysis could impact upon large-scale eradication schemes or trade. Both of the real-time PCR assays have a similar degree of sensitivity. They have also been reported as highly reproducible and should now be carefully validated.

Host–Pathogen Interaction

After invading the vascular system, *P. tracheiphila* slowly colonizes the parenchyma and differentiates pycnidia beneath the epidermis of the twig, which eventually dies. As in other tracheomycoses, there is a direct correlation between the susceptibility to mal secco as determined by symptom severity and colonization of the xylem (22,77). Transformants of *P. tracheiphila* expressing the green fluorescent protein (GFP) gene from *Aequorea victoria* have been useful in studying the colonization process of the xylem by the pathogen (D. Ezra, *unpublished results*). Due to the passive transport of conidia, distribution of the fungus in the xylem is often sectorial (Fig. 13), and clusters of vessels invaded by hyphae corresponding to leaf traces can be observed (Fig. 14). This distribution pattern correlates with the progression of symptoms in the tree canopy. Systemic invasion of the xylem by the pathogen leads to the impairment of water transport in the plant, as can be demonstrated by the increase of hydraulic resistance of the stem and leaves (104), and consequent wilting, the most typical symptom of tracheomycosis.

Raimondo et al. (104) demonstrated that the earliest site of attack by *P. tracheiphila* in citrus leaves is veins. Vein conduits are first damaged through enzymatic digestion of interconduit pits, and later, wider areas of conduit walls are dissolved. These cell wall alterations cause diffuse vascular cavitation with severe irreversible impairment of the vein network, leading to a limitation of gas exchange that was likely to accelerate shedding, and damage to leaf hydraulics, which apparently causes stomatal closure in chlorotic leaf areas. The infected xylem is progressively clogged by both gums and mycelium (12,79,98,99,128,155). Later, digestion of the walls of neighboring conduits constitutes an easier and faster pathway for fungal diffusion.

The lysis of pit membranes provides circumstantial evidence for the production of pectic enzymes by the fungus during the colonization process. These inducible enzymes, produced by the fungus in vivo and in vitro (18,21,48,62,90), may be virulence factors, as the ability of the fungus to colonize the xylem depends on their production.

It is also generally assumed that oligogalacturonides released through cell wall degradation may act as elicitors in the signaling pathway of the host–pathogen recognition system even though this mechanism has not been investigated in the mal secco disease (42,43,47,112,113). *P. tracheiphila* is a necrotrophic pathogen that produces both hydrolytic enzymes and toxins during infection, and several extracellular lipophilic and hydrophilic phytotoxic compounds have been isolated from culture filtrates of the fungus (9,62,93–96,117,145,150). A partially purified preparation of extracellular hydrophilic substances from *P. tracheiphila* was shown to contain a nonselective phytotoxic compound called malseccin, which induced chlorosis and necrosis when injected into leaves of rough lemon (*C. jambhiri* Lush), or when absorbed by cuttings of lemon, tomato, and other nonhost plant species (83). Glycoproteins of 93 Kd and 60 Kd (called Pt60) belonging to the malseccin complex have been isolated from culture filtrates of *P. tracheiphila*, and it was demonstrated that both toxins were able to reproduce symptoms of leaf vein chlorosis when injected into citrus leaves (51,52,84). Pt60 is a highly glycosylated protein, and the carbohydrate sheath protects the protein moiety from enzyme degradation. Pt60 did not show sequence homology with any known protein and is thought to be the most toxic component of the malseccin complex, which probably results from a mixture of several compounds, each differing from the others in the extent of glycosylation (52). Pt60 toxin has been isolated also from the leaves and

Fig. 13. Aerial mycelium of *Phoma tracheiphila* emerging from the xylem of a transverse section of an infected sour orange twig after 14 days incubation at 24°C and 95% RH. Note the sectorial distribution of the mycelium.

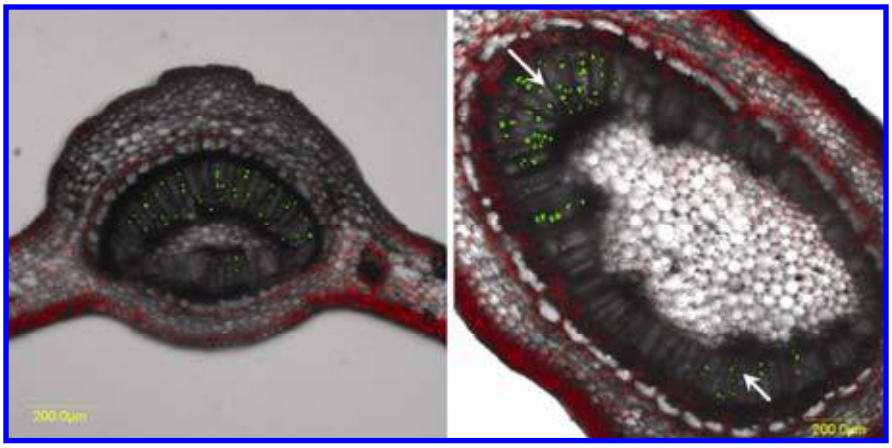

Fig. 14. Localization of a *Phoma tracheiphila* transformant expressing the green fluorescent protein observed with confocal microscopy, after penetration into the leaf. Left, transverse section of the leaf petiole of an inoculated leaf. Vessels colonized by fungus hyphae are sparse in the whole section. Right, stem sections beneath inoculated leaves. Fungus is located only within vessels of the traces of inoculated leaves (arrows).

the xylem of naturally infected lemon trees showing symptoms of the disease including leaf vein chlorosis, the typical salmon-orange wood discoloration, and leaf drop. These findings substantiate the hypothesis that the toxin plays a role in the etiology of mal secco. Furthermore, the activity of Pt60 toxin has been shown to be affected by light: on leaves infiltrated with 1 to 5 µg of the toxin, purified from culture filtrates of *P. tracheiphila,* symptom severity was dramatically reduced when the leaf blade was shaded with a strip of aluminum foil (107,111). A similar effect was observed by inoculating sour orange seedlings with *P. tracheiphila* conidia; plants did not show any visible foliar symptoms until 100 to 120 days after inoculation if they were grown under a very low light intensity. However, symptoms appeared 35 to 50 days after inoculation when the seedlings were grown under natural or artificial light.

A similar effect of light on the expression of symptoms has been observed in other pathosystems in which toxins produced by the pathogen are also involved. Victorin, a host-selective toxin produced by *Cochliobolus victoriae* Nelson, the causal agent of Victoria blight of oat, causes foliar chlorosis under light only and is associated with a loss of chlorophyll followed by photo-oxidative stress, leading to cell death. The glycine decarboxylase complex (GDC), a key component of the photorespiratory cycle, has been indicated as a possible target of victorin (87,88). It might be supposed that toxin Pt60 inhibits GDC as well. However, unlike victorin, which is a primary determinant of pathogenicity for *C. victoriae*, toxin Pt60 would act as a virulence factor for *P. tracheiphila*, because foliar symptoms of chlorosis appear only after the xylem of infected citrus plants has been invaded by the pathogen (77). In previous studies, it has been shown that in leaves of infected sour orange seedlings, an impairment of photosynthesis and an increase of intercellular CO_2 concentration were detectable before the appearance of foliar symptoms, while stomatal conductance decreased significantly only in advanced disease (22,106). Similarly, in wound-inoculated sour orange leaves, the earliest symptoms of chlorosis indicating successful infection appeared 5 to 7 days after inoculation while leaf conductance to water vapor (gL) significantly decreased only 24 days after inoculation (104). These findings are consistent with the hypothesis that the decrease in photosynthetic rate in citrus plants affected by mal secco is primarily due to metabolic disturbances of the photosynthetic process caused by the toxin. Although the primary structure of malseccin has never been elucidated, some of its biological effects have been studied (83–85), and the results have raised the possibility of using this toxin complex to select resistant clones of citrus cultivars. In vitro selection of several cell lines of lemon for tolerance to malseccin was performed using partially purified preparations of the toxin (10,57–59,86).

Citrus plants infected by virus and viroids have been shown to be more tolerant to *P. tracheiphila* infections, and such tolerance was correlated with phenolic substances (28,125,127,134,137). However, the role of phenolics in resistance to mal secco was not confirmed (38). Solel et al. (134) suggested that induced systemic resistance could explain the tolerance of citrus plants infected by *Citrus exocortis viroid* (CEVd) to mal secco. In 1962, Ben-Aziz et al. (13) reported the presence of inhibitory compounds extracted from resistant tangerine varieties. The main compounds identified in the resistant varieties that were absent from lemon and from other susceptible citrus were nobiletin and tangeritin (14). Nobiletin had a strong inhibitory effect on the growth of *P. tracheiphila* in both in vitro and in vivo experiments, while the effect of tangeritin was substantially lower. Recently, Reverberi et al. (111) demonstrated that oxidative stress may play a role in the interaction between *P. tracheiphila* and *C. limon*, including cultivars Femminello, Interdonato, and Monachello. This study showed that the synchronous presence of hydrolytic enzymes, toxic compounds, oxidative stress inducers, and membrane transporters in the fungus, and the differential ability to modulate the lipoperoxidative pathway in the host, could play a key role in the interaction between *P. tracheiphila* and its hosts.

Disease Management

Quarantine measures. There are strict regulations in many countries to prevent the introduction or spread of *P. tracheiphila.* In Italy, a government decree (DM 17 April 1998 published in Gazzetta Ufficiale della Repubblica Italiana 1 giugno 1998, n. 125) has made the eradication of the disease compulsory by ordering pruning and uprooting of symptomatic trees, and burning of resulting infected plant material, including the stump. The European and Mediterranean Plant Protection Organization (OEPP/EPPO) has included *P. tracheiphila* in the A2 list of quarantine pests and diseases. *P. tracheiphila* is of quarantine concern to most other regional plant protection organizations, such as APPPC, CPPC, COSAVE, IAPSC, and NAPPO (45,46). Preventive measures based on early diagnosis are the most effective to limit the introduction and further spread of the pathogen. Official methods for the inspection of propagation materials and the identification of *P. tracheiphila* are described in the OEPP/EPPO standards. However, official methods of diagnosis are being updated and the current standard protocol of OEPP/EPPO is under revision as new, more practical and sensitive molecular diagnostic methods based on real-time PCR were published recently (44,72). *P. tracheiphila* has also been included in the list of animal and plant pathogens with potential biological warfare applications (73).

Cultural practices. Since its first appearance in lemon orchards, mal secco has been controlled by the costly practice of pruning diseased twigs (91,124). Careful pruning of withered shoots, removal of suckers, and burning of pruned branches are recommended to reduce inoculum. Pruning is performed in spring and early summer when symptoms are more easily recognized. As long as the main trunk is not colonized by the fungus, removal of the diseased branches can limit spread and sometimes cure the tree of the disease. Conversely, if a tree is found to be infected in the trunk, it must be uprooted and burned. Pruning cuts should be done at least 50 cm beneath symptoms or wood discoloration. However, this method is often unreliable, as pruners make cuts in order to eliminate only twigs and branches with symptoms of pink-salmon discoloration. When infected wood is left on the plant during pruning, large cuts may induce the tree to produce new vegetation flushes which in turn lead to a systemic and rapid spread of the disease. Diseased trees can sometimes be saved by topworking pollarded plants by grafting with resistant cultivars or species (Fig. 5). Pruning and topworking should not be carried out on cloudy and rainy days. Similarly, soil cultivation in late autumn and winter or in rainy periods enhances the risk of root infections. Severe outbreaks of mal fulminante were observed in lemon orchards after prolonged no-tillage combined with chemical control of weeds, most probably because in no-tilled soil undisturbed rootlets grow near the soil surface and therefore come into contact more easily with the inoculum produced in the tree canopy.

The use of clean, healthy plants from certified nurseries is recommended to prevent the spread of the disease. Windbreaks and hail nets also reduce injuries and, consequently, the risk of infection through wounds (110,148).

Chemical control. The application of copper-based fungicides and ziram may reduce infection by *P. tracheiphila* (148). These fungicides must be applied every 2 to 4 weeks during the rainy season (roughly from October to February), when temperatures and conditions are favorable to infection; however, many treatments are not cost effective in commercial lemon groves. Conversely, spraying of trees is recommended in nurseries, especially immediately after hail or frost damage. Systemic fungicides such as benomyl, carbendazim, and thiophanate methyl have been tested experimentally, but they were found to be ineffective on mature trees and never applied in lemon orchards (132,136).

Host genetic resistance. The most effective means to control mal secco on a

large scale would be to use resistant cultivars or clones and to graft them onto resistant rootstocks, but unfortunately at present this strategy is not feasible. In Italy, the susceptible lemon cultivar Femminello has been replaced in several areas of Sicily either by the cultivar Monachello, a putative spontaneous hybrid between lemon and citron (121), which is resistant but has reduced yield, or by other less desirable cultivars. Santa Teresa Riva, a Sicilian selection of Femminello Ovale, is moderately tolerant compared with other clones of Femminello but produces poor-quality fruits outside the area where it was developed. Interestingly, the parent tree of Santa Teresa Riva was an old disease-free tree found in a Femminello orchard that had almost been destroyed by mal secco (120). Other mal secco–tolerant Femminello clones include Zagara Bianca and Continella. However, their tolerance to mal secco is not comparable to that of Monachello, and their adoption has been limited. In Greece, Thanassoulopoulos (146) described a new cultivar of lemon that originated from cultivar Maglini. This new cultivar was named Ermioni and was found to be highly tolerant to mal secco, with commercially acceptable fruit quality but slightly reduced productivity (10 to 20% less than Maglini). Interdonato, a spontaneous hybrid between lemon and citron (121), is a cultivar selected on the Ionian coast of Sicily that has been successfully introduced also into Turkey because of its tolerance to mal secco. However, it has a poor yield, does not bloom several times, and its juice has low acidity.

Nucellar clones obtained in Italy from commercial lemon cultivars proved to be more susceptible to mal secco than the originals (100). Tolerant inter- and intraspecific hybrids obtained by conventional methods produced fruits with very poor commercial value (25,122). Triploids, hybrids between Lisbon lemon (4n) and Trovita sweet orange (2n), showed tolerance to mal secco but poor yield (60).

Somatic and cybrid hybridization has also been used to obtain varieties of lemon tolerant to mal secco (152–154). An example was a somatic hybrid of Valencia sweet orange with Femminello lemon and two Femminello cybrids (151). The plants regenerated from these fusions were tested for their tolerance to mal secco and were found to be more tolerant than Femminello but less so than the tolerant Monachello variety (152). This study opened up the potential for using hybrids with the resistant Valencia as a fertile parent for crosses with lemons in order to obtain tolerant lemon cultivars. Moreover, it emphasized the role of the nuclear and cytoplasmic background in contributing to the tolerance character. No commercial cultivar obtained through this means is currently available.

A triploid hybrid named Lemox (European patent number 20040073) was obtained by crossing a tetraploid male parent (Doppio Lentini lemon) and a hybrid of Femminello × Pera del Commendatore, which in turn is a natural hybrid with lemon citron and pummelo characteristics. Plants of this new hybrid growing in a diseased area were reported to be tolerant to mal secco (109).

A mutant nucellar clone of Femminello Siracusano, named 2Kr, with high yield potential was obtained with cobalt γ-radiation and has been included in the official lists of lemon cultivars whose use is recommended in Italy, although it was found to be highly susceptible to mal secco. Presently, none of the lemon cultivars included in these lists, with the exception of Monachello, are tolerant. Recently, Gulsen et al. (65) reported the development of seedless and mal secco–tolerant mutants of Kutdiken lemon from γ-irradiated buds. Field performance of these new mutants has not yet been tested (65).

Other biotechnological approaches have been used in breeding programs aimed at obtaining mal secco–resistant varieties. In vitro selection of several cell lines of lemon for tolerance to malseccin was performed with partially purified preparations of the toxin (86). It has also been reported that malseccin-treated nucellar embryogenic calli of lemon over-secreting fungal cell-wall hydrolytic enzymes, such as chitinase and 1,3-β-glucanase, showed tolerance to the toxin (57–59), and the cell line obtained by this approach was named Femminello-s. However, plants regenerated from these treated calli were very susceptible to the pathogen (19). Moreover, one of these somaclones (FS01) selected in vitro from nucellar embryogenic calli of lemon for its resistance to malseccin, and which was claimed to be as tolerant as Monachello to *P. tracheiphila* (55), proved to be less susceptible to mal secco than Femminello but more than Villafranca, a widely grown lemon cultivar in Israel known to be very susceptible to the disease (A. Sadowsky, *personal communication*).

In conclusion, although in vitro selection was originally claimed as an innovative and promising technique to obtain lemon clones tolerant to the disease (55), no commercial lemon cultivar obtained with this breeding method is presently available and no detailed information on yield, fruit quality, or agronomic characteristics has been reported (70).

Host resistance to *P. tracheiphila* infection was studied with the aim of understanding the genetic mechanisms of resistance to mal secco. Reforgiato Recupero et al. (108) hypothesized that resistance to the disease is due to three alternative genes (A, B, and C) determining dominant resistance. The presence of a single dominant allele can therefore confer resistance. In addition, they suggested the existence of a fourth dominant gene (D) which is able to cancel the dominance of the B allele. This hypothesis relies on the analysis of segregation of progenies obtained from crosses between the monoembryonic species *C. latipes* (Swing.) Tan, as female parent, with polyembryonic sour orange (*C. aurantium*), trifoliate orange (*P. trifoliata*), and volkamer lemon (*C. volkameriana*) as male parents. The response to the pathogen was inferred from the production of chitinase, a pathogenesis-related (PR) protein reported to be present in great quantities in mal secco–resistant *Citrus* genotypes. However, the actual role of PR proteins in cell tolerance to *P. tracheiphila* has been questioned (10).

Genetic transformation of commercial varieties has been used as an alternative approach to obtain lemon plants tolerant to mal secco. The *Chit42* gene of *Trichoderma harzianum* encoding an endochitinase was inserted into Femminello Siracusano lemon by *Agrobacterium tumefaciens*–mediated transformation in order to regenerate plants resistant to fungal diseases (54,56,69). Foliar extracts of transgenic plants inhibited in vitro conidial germination as well as mycelial growth of *P. tracheiphila*. These trials indicated that the *Chit42* transgenic clones can potentially control mal secco disease in lemons, but results are not conclusive and do not correlate with field resistance. The transgenic clone is currently under evaluation and subjected to regulations concerning GM organisms (Italian Biosafety Clearing House. Legislative Decree n. 224 8.7.2003. Notification Number, B/IT/04/03).

Like other biotechnological methods used for the improvement of lemon cultivars, genetic transformation is far from being adopted routinely. A drawback in using genetically modified lemons could be the refusal of the public to purchase the fruit and the opposition of environmentalist movements around the world to genetically modified crops. This attitude constitutes a major problem for the development and use of transgenic plants, and will probably prevent this potential solution from becoming a primary approach to manage and control mal secco, as well as other fruit tree diseases. Selection and genetic improvement of lemon rootstocks for mal secco tolerance may be worthy of further research.

Biological control. In other experimental models, nonpathogenic mutants prevented the establishment and development of disease in the host plant (53). The first experiments in biological control of mal secco were, indeed, based on preinoculation with either a nonpathogenic fungal species, such as *Verticillium dahliae*, or a weakly virulent isolate of the pathogen (63,92,116). Effective protection from infection by *P. tracheiphila* was observed in sour orange seedlings preinoculated with a weakly virulent isolate of the pathogen (41). Induced systemic resistance

could be the mechanism underlying tolerance of preinoculated citrus plants. The same mechanism was advocated by Solel et al. (134) to explain the tolerance of citrus plants infected by CEVd to mal secco. The great variability in virulence of *P. tracheiphila* isolates that easily lose pathogenicity when cultured on artificial media is an obstacle for the characterization of stable, weakly pathogenic isolates that can be used in biological control strategies. Recently, *Agrobacterium*-mediated mutagenesis of a virulent strain of *P. tracheiphila* was attempted in order to give a better insight into the genetics of host–pathogen relationships in mal secco disease. Independent transformants (n = 2,263) were tested for pathogenicity on rough lemon seedlings, and 20 of them showed reduced pathogenicity. Further characterization of the mutants is in progress and the role of the mutated gene(s) in the pathogenicity process is being studied. These nonpathogenic mutants of *P. tracheiphila* are being tested for their biocontrol potential against mal secco on lemon plants (D. Ezra, *unpublished results*).

Another approach to biological control of mal secco has been the use of endophytes, microorganisms that spend most of their life cycle inside plant tissues without causing visible damage or defense reaction in the host plants (2,3). The concept of using endophytes for the control of diseases in plants has been demonstrated by the introduction of naturally and genetically modified fungi and bacteria into plants (1,33,53). Many endophytes isolated from trees secrete secondary metabolites ranging from low molecular weight molecules to complex glycoproteins (11,27,49, 81,156). A biorational approach for finding and isolating beneficial microorganisms to be used as biological control agents of citrus diseases is to select symptomless plants in a diseased orchard, with the assumption that these plants may be harboring beneficial endophytes that have helped them to remain healthy (143). Endophytic bacteria were tested as possible biological control agents of mal secco on citrus plants by Lima et al. (74) for the first time in Italy. Other attempts to use endophytic species of *Pseudomonas* as biological control agents against citrus mal secco are more recent (32). Although promising, all these experiments were confined to the laboratory or under greenhouse conditions. Both bacterial and fungal endophytes were recently isolated from different types of citrus in Israel, and their ability to inhibit and kill *P. tracheiphila* was tested in vitro. Some of these endophytes have demonstrated strong antifungal activity against *P. tracheiphila* and were introduced into rough lemon seedlings. Their influence and ability to protect the plant from infection and fungal development in the plants is being evaluated (D. Ezra, *unpublished results*).

Conclusions

Although the use of host genetic resistance is the most desirable option to control citrus mal secco, the goal of obtaining tolerant cultivars with competitive yields and satisfactory bioagronomic characteristics has not been achieved. This, therefore, remains one of the primary objectives of lemon-breeding programs. As a consequence, citrus mal secco disease remains a limiting factor to lemon production in the Mediterranean region. Moreover, this disease is a potential threat for other lemon growing areas of the world. Countries such as China are especially at risk when they introduce propagation materials from other countries. Recently, *P. tracheiphila* has been isolated from surface-sterilized achenes of the invasive plant *Centaurea stoebe* L. (spotted knapweed) collected in Germany (131). Endophytic survival within a nonhost plant suggests a chilling scenario for the potential spread of mal secco disease beyond its current limits. This finding also has intriguing implications for determination of the center of origin and the possible shift of *P. tracheiphila* from an endophyte to a pathogen.

At present, mal secco disease is a problem for lemon and for other minor citrus, such as lime, citron, and bergamot. However, almost all citrus species proved to be susceptible to *P. tracheiphila*, at least when artificially inoculated. Therefore, this pathogen may cause problems when introduced into new areas where citrus species other than lemon are growing or susceptible rootstocks are used. In this respect, even though the potential of *P tracheiphila* as a biological weapon has probably been over-emphasized, its presence in the lists of quarantine pathogens of almost all citrus-growing countries is fully justified. The presence of regulation measures is useful even in areas where mal secco is already present because control strategies, including the use of tolerant lemon cultivars, cultural practices, and chemical treatment, may be at least partially effective when inoculum levels are low.

Apart from the impact on the lemon industry, the presence of mal secco represents a serious limit to the trade of citrus plants and the expansion of the citrus nursery industry, as citrus trees cannot be exported to countries where the disease has not been reported. Moreover, it could be an obstacle for the development of international breeding programs for the genetic improvement of citrus due to restriction in the exchange of propagation material.

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Additional Web Resources

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http://www.gazzettaufficiale.it/
http://www.eppo.org/STANDARDS/standards.htm