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15. Root and crown rot of olive caused by *Phytophthora* spp.

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Abstract. *Phytophthora* root and crown rot has been traditionally considered a minor disease of olive. However, in recent years it has been recognized as an emerging problem in several olive-growing countries such as Australia, Italy and Spain probably as a consequence of the expansion of plantings in new areas with heavy soils and the more intensive use of irrigation in both olive nurseries and commercial groves. The disease has been reported from most olive-growing countries and is caused by several soil-borne species of *Phytophthora*, including *P. cinnamomi*, *P. citricola*, *P. cryptogea*, *P. drechsleri*, *P. gonapodyides*, *P. inundata*, *P. megasperma*, *P. nicotianae* and *P. palmivora*. Diagnosis is currently based on the isolation and identification of isolates by both traditional and molecular methods. New molecular techniques are currently available that could be applied for both the identification of isolates and *Phytophthora* infections directly in host-tissues as well as in

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soil and water samples. A number of dedicated databases could improve the efficiency of these techniques. Moreover, DNA analysis has greatly contributed to phylogenetic studies of *Phytophthora*. Control of *Phytophthora* root and crown rot of olive is mainly based on preventive measures.

1. Introduction

Phytophthora de Bary is a cosmopolitan genus of plant pathogens formerly referred to as alga-like fungi. Currently, this genus is assigned to the Order *Pythiales* in the Phylum *Oomycota* of the Kingdom *Straminipila* that includes also the diatoms, brown algae and golden brown algae [1]. The placement in this kingdom was supported by several characteristics, including peculiar metabolic pathways, the presence of β-glucans rather than chitin in cell walls, production of motile heterokont zoospores and predominance of the diploid stage in the life cycle. At present there are at least 98 valid *Phytophthora* species, including new species described formally very recently, such as *P. multivora* P. M. Scott & T. Jung, *P. plurivora* T. Jung & T.I. Burgess, *P. rosacearum* E.M. Hansen & W. F. Wilcox and *P. sansomeana* E.M. Hansen & P. W. Reeser [2, 3, 4], as well as species whose formal description is in progress, such as *P. niederhauserii* Z.G. Abad & J.A. Abad and *P. morindae* Z. G. Abad & S. Nelson (Gloria Abad, personal communication). Most of these species infect a wide range of plants and some, such as *P. cinnamomi* Rands, *P. infestans* (Mont.) de Bary and *P. ramorum* Werres, de Cock & Man in't Veld, are responsible for historical devastating plant diseases. Tree diseases incited by *Phytophthora* are common in temperate as well as in wet tropical regions and appear in the form of root rot, collar rot, stem canker, leaf blight, and fruit rot. In the traditional taxonomy, *Phytophthora* species, like the True Fungi (*Eumycota*), were discriminated mainly on the basis of morphology [5]. The characteristics that Waterhouse [6] used to differentiate the species into six main groups (I-VI), which were also adopted in the tabular keys of Newhook *et al*. [7] and Stamps *et al*. [8], are: apical thickening (papilla) of sporangia and width of exit spore; caducity of sporangia and pedicel length; type of antheridia (amphyginous or paragynous) and mating system. These keys have been made mostly for convenience of identification, but do not necessarily correspond to a natural classification [9]. An outstanding contribution for the identification and characterization of *Phytophthora* species, especially those with similar or overlapping morphological features, has arisen from the use of biochemical and molecular techniques. Total mycelial proteins and isozyme electrophoretic banding patterns showed excellent discrimination power but, with few exceptions, they were used to provide confirmatory

evidence in the identification of species or as an aid in the morphological diagnosis [5, 10, 11, 12]. Conversely, techniques based on the analysis of DNA have become the most powerful and widely used tool in the taxonomy of *Phytophthora* and for the detection and identification of species [13]. The separation of species in recent years has been supported with phylogenies inferred from sequence analysis of both nuclear and mitochondrial loci. Multi-locus phylogeny based on molecular markers indicates the presence of 10 clades [14] within the genus that only partially correspond to the classical Waterhouse grouping [14, 15] (Fig. 1).

The internal transcribed spacer (ITS) regions of genomic ribosomal DNA (rDNA) gene repeat regions have proven particularly useful for the separation of fungal taxa, because the rate of accumulation of mutations in these regions often approximates the rate of speciation. Sequence analysis of ITS regions has confirmed its value in distinguishing morphologically similar *Phytophthora* species and is currently used as a routine method for the identification of isolates [16].

According to the etymology of *Phytophthora*, which in ancient Greek literally means “plant destroyer”, almost all species belonging to this genus are aggressive plant pathogens. Several factors contribute to the efficiency of these micro-organisms as plant pathogens including the ability to produce different types of propagules, the rapid sporulation on host-tissues in favorable environmental conditions resulting in polycyclic infections, the ability of zoospores to actively reach the infection sites by chemotaxis and electrotaxis, the ability to survive as chlamydospores and oospores for long periods. *Phytophthora* species produce oospores as sexual propagules and three types of asexual spores, sporangia, zoospores and chlamydospores (Figs. 2, 3).

Chlamydospores can be distinguished from hyphal swellings as they are delimited by a continuous cell-wall. Sporangia may germinate directly by the production of a germ tube or indirectly by the production of motile biflagellate zoospores. Up to more than 30 zoospores may burst from a single sporangium in saturated soil and move with the surface water. Zoospores can swim for hours but encyst within 30 minutes in the presence of host tissues. Only some species, such as *P. cinnamomi* and *P. nicotianae*, produce a large number of chlamydospores. Chlamydospores like oospores are resting spores and spread the inoculum when they are moved about with soil.

As far as sexual reproduction is concerned, some species are self-fertile (homothallic), whereas others are self-sterile (heterothallic). In heterothallic species, oospores are produced when two opposite mating types, A1 and A2, grow together on a suitable medium and in a favorable environment. Isolates with opposite mating types of different species are often able to reciprocally

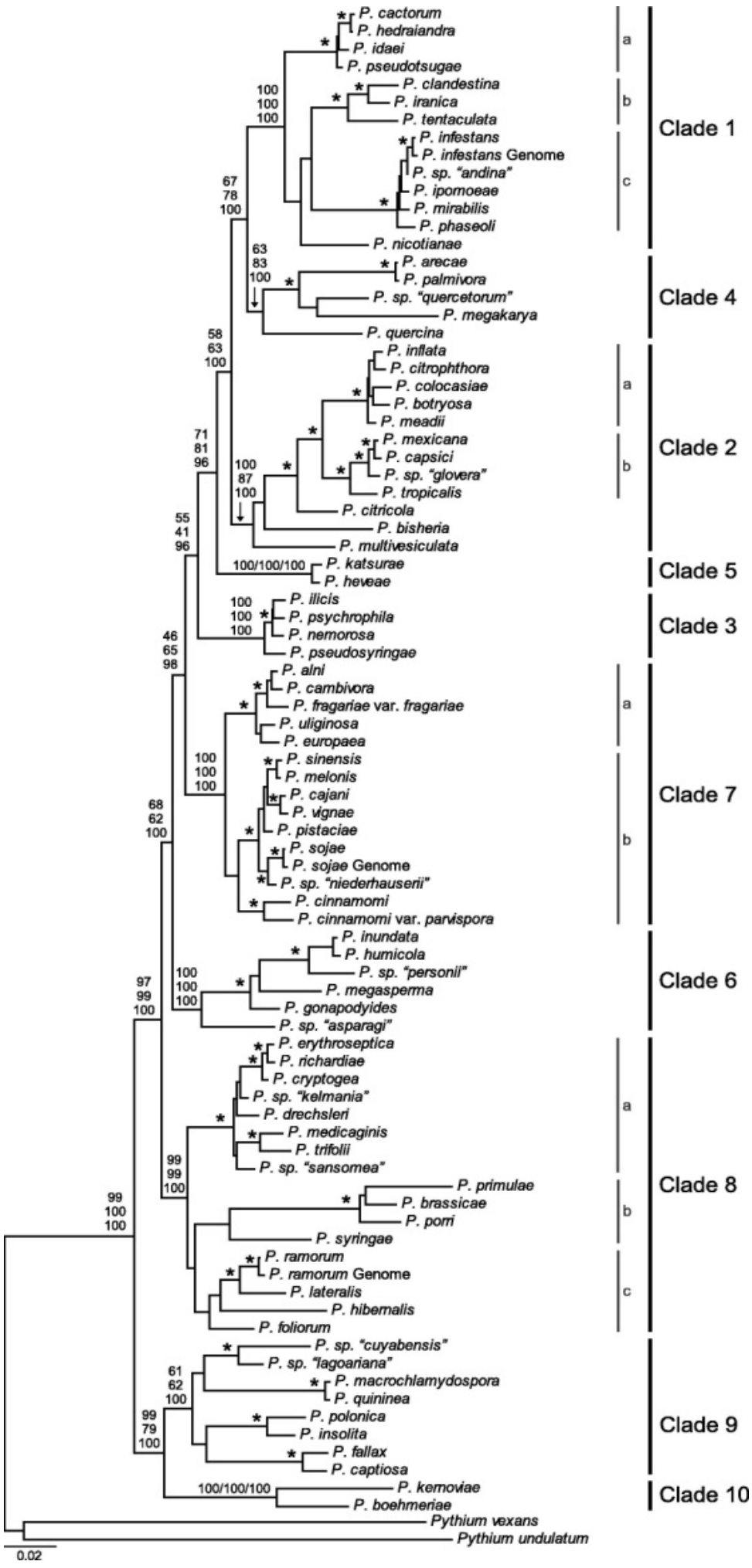

Figure 1. A genus-wide phylogeny for *Phytophthora* using seven nuclear loci (about 8700 nucleotides). Numbers on nodes represent bootstrap support values for maximum likelihood (top) and maximum parsimony (middle), and Bayesian posterior probabilities presented as percentages (bottom). Nodes within clades receiving unambiguous (100%) support in all three analyses are marked with an asterisk (*). Scale bar indicates number of substitutions per site (after Blair *et al.* [14]).

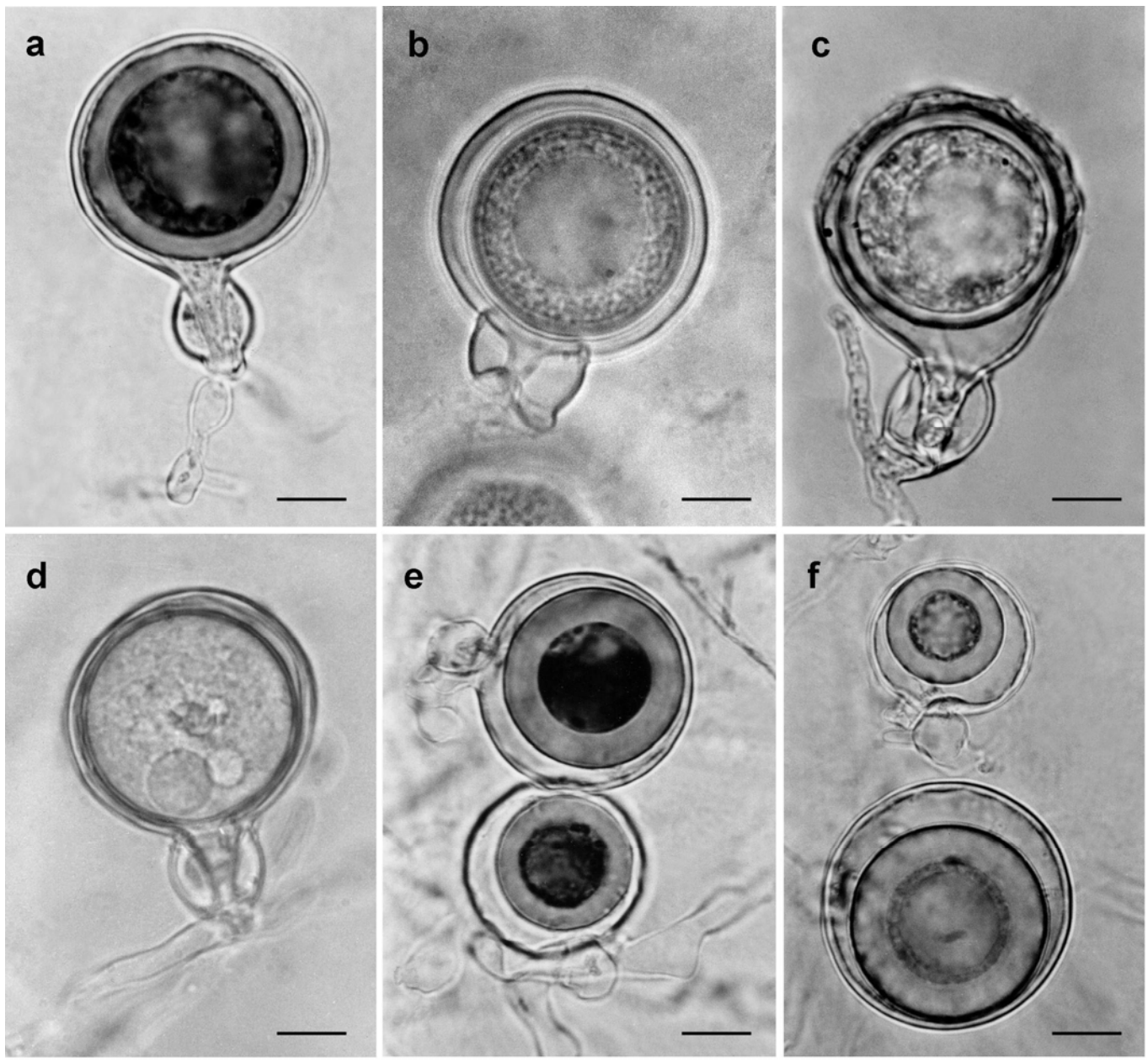

Figure 2. Oogonia with amphigynous antheridia of *Phytophthora cryptogea* (a), *P. nicotianae* (b), *P. cinnamomi* (c) and *P. palmivora* (d). Oogonia of *P. megasperma* with paragynous antheridia and thick-walled oospores (e and f). (Bars correspond to 10 μm)

induce the production of gametangia. The mating system of a *Phytophthora* species determines its ability to outbreed: homothallism favors selfing, whereas heterothallism increases the frequency of outbreeding. In both homothallic and heterothallic species, oospores act as resting propagules enabling the survival of the species for long periods in the soil in the absence of a host plant or in host tissues when environmental conditions are adverse. Although the relative importance of sexual reproduction in the life cycle of most *Phytophthora* species is not known, the crossing between A1 and A2 mating types can be a source of genetic variability in the progeny. Both asexual and sexual spores are potentially infective. *Phytophthora* is basically a soil-borne microrganism and almost all species have a soil-borne resting stage. However, some species, such as *P. palmivora*, are adapted to attack above-ground parts of plants as they produce caducous sporangia that may be aerially dispersed (Fig. 4).

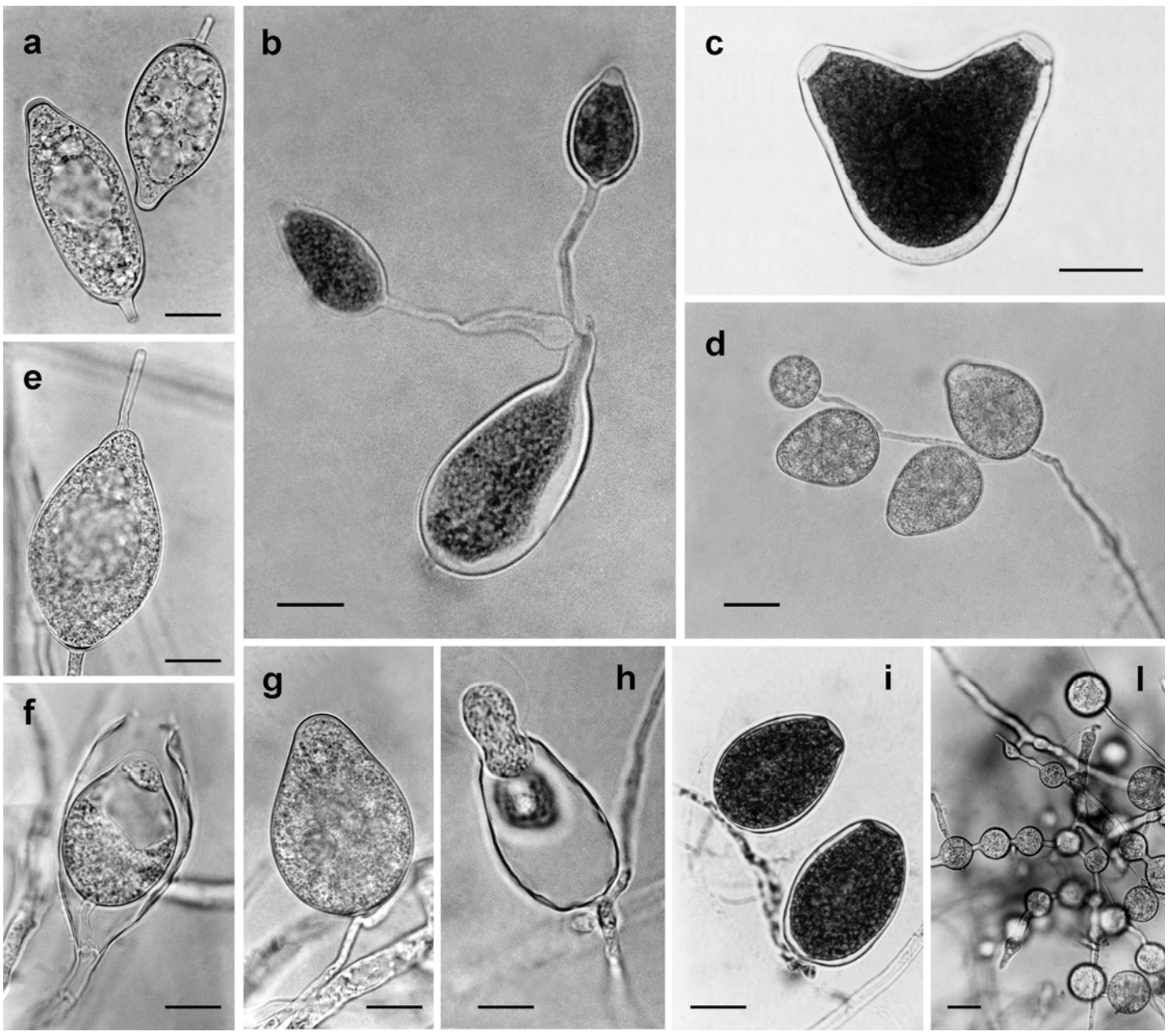

Figure 3. Typical caducous papillate sporangia of *Phytophthora palmivora* with a short (5 μm) pedicel. Note the basal plug corresponding to the insertion of the pedicel (a). Direct germination of a sporocyst of *Phytophthora palmivora* producing two small papillate sporangia (b). Bilobate semi-papillate sporangium of *P. citrophthora* (c). Simple sympodium with non-papillate sporangia of *P. cryptogea*. Note the undifferentiated spherical sporangium at the terminal position (d). Direct germination (e) and internal proliferation (f) of non-papillate sporangia of *P. cinnamomi*. Non-papillate persistent (g) and indirectly germinating (h) sporangia of *P. cryptogea*. Non-papillate persistent sporangia of *P. drechsleri* (i). Hyphal swellings of *P. cryptogea* (l). (Bars correspond to 10 μm).

Primary inoculum, which survives as mycelium, oospores and chlamydospores in infected tissues, starts epidemics when environmental conditions are favorable and the presence of a host plant stimulates spores to germinate. Conditions conducive to the development of *Phytophthora* root and collar rot may vary according to climates; e.g. a continuous disease cycle is typical of the wet tropical conditions. However, the season when a given disease is most active depends on the biology of the pathogen, the host, and the environment. Zoospores, swarming from sporangia, swim toward the

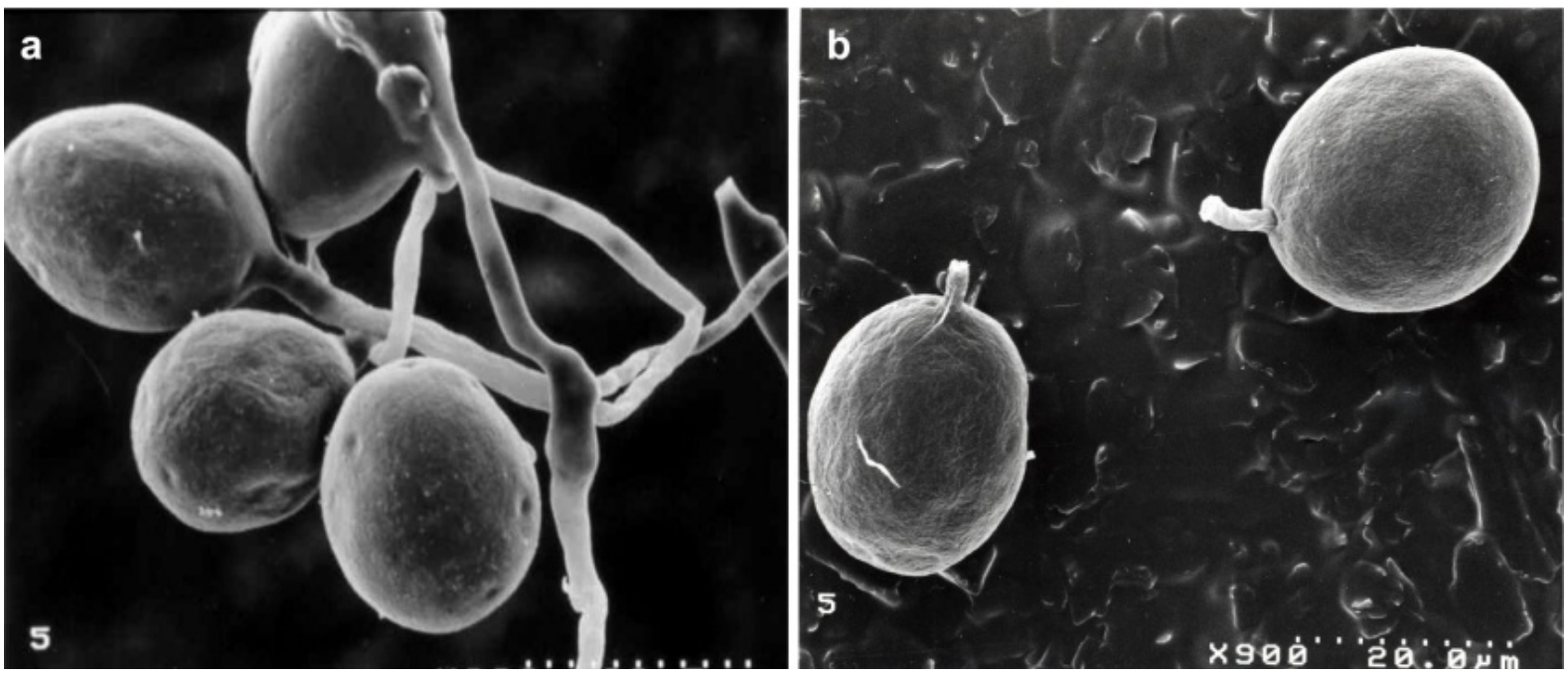

Figure 4. Scanning electron micrographs of sporangia of *Phytophthora palmivora* produced in axenic culture on potato dextrose agar (PDA). Simple sympodium sporangiophore bearing sporangia (a). Sub-globose non-papillate caducous sporangia (sporocysts) with a short (5 µm) pedicel (b). Usually sporangia of *P. palmivora* are papillate, however on PDA this species often produces both typical papillate and sporocysts (see Erwin and Ribeiro [5]) (photos courtesy by G. Magnano di San Lio).

growing host root attracted by chemicals and aggregate either just behind the root tip, in wounds, or in areas where roots branch off. They encyst just before infecting the root; the encystment includes dropping of flagella and deposition of cell wall. The cyst germinates and begins to differentiate the hyphae that grow inside the host. Some species of *Phytophthora* may also germinate directly from oospores or chlamydospores to form a germ tube (Fig. 5).

Although soil saturation is necessary for infection, once *Phytophthora* is inside plant tissues, it can continue to colonize the root even if the soil is not saturated and grows through the root system. At first, only a few fine roots are damaged. The pathogen grows through the root system into larger roots until it reaches the root crown area where it kills the cambium. Once the root crown is girdled, water and nutrients cannot reach the leaves and the transfer of photosynthates from the canopy to the roots is impaired. The upper portions of the plant begin to wilt and die back and the root rotting is accelerated. Affected olive trees show reduction of growth and leaf dieback resulting in a drastic thinning of the canopy, and trees eventually die [17]. It is not unusual that infected plants are right next to apparently healthy plants. Symptoms may develop quickly when the water demand of the plant increases during the first dry periods of summer. The success of primary infection leads to the differentiation of secondary inoculum on the surface of

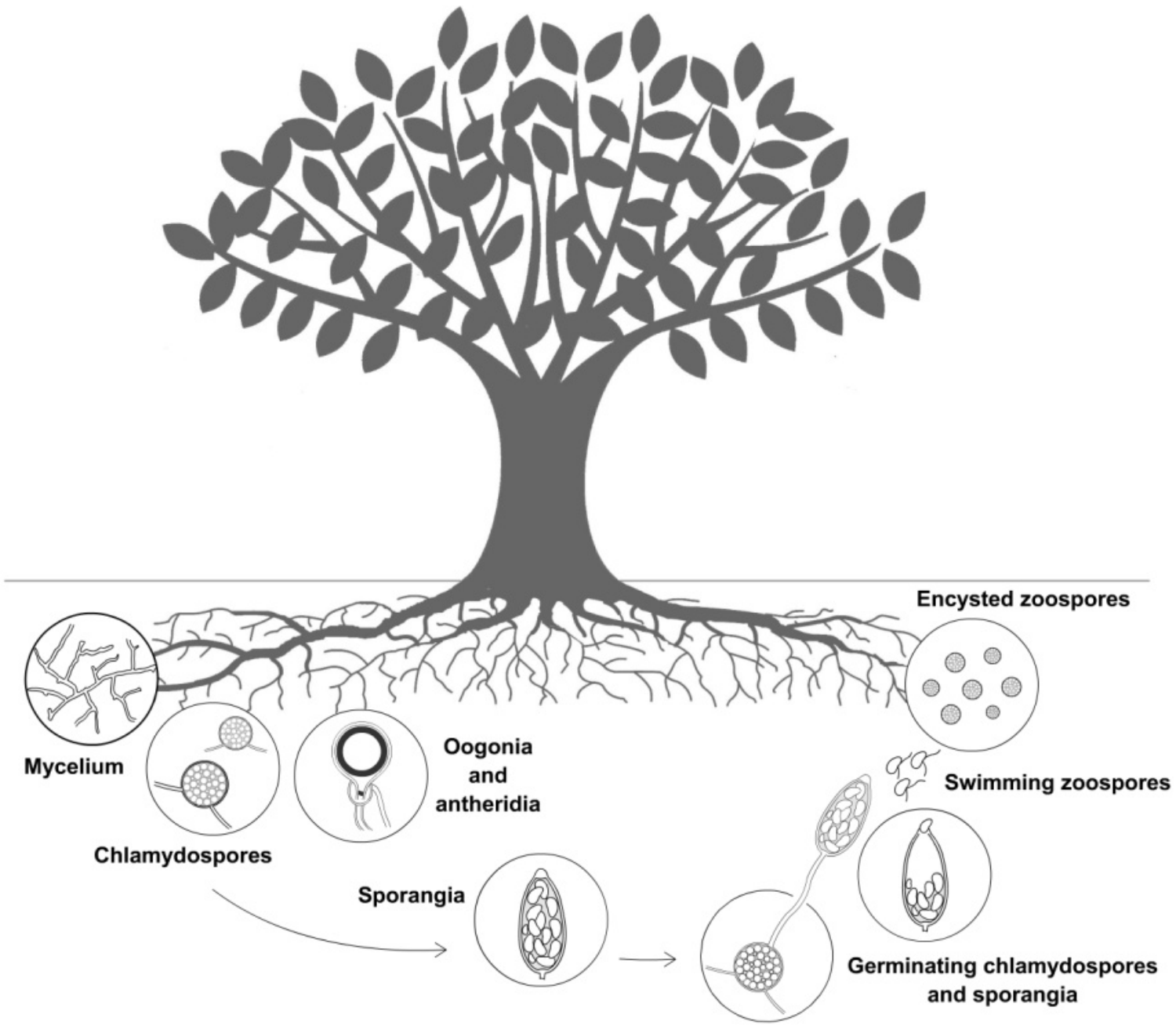

Figure 5. Diagrammatic representation of the disease cycle of Phytophthora root rot of olive.

rotting roots. Zoospores can swim to nearby roots of the same plant or can be moved by running water to suitable penetration sites of a healthy plant. In general, *Phytophthora* depends on free water for spread and infection, and on human activity for long-distance spread. Explosive epidemics are caused by the rapid increase of secondary inoculum and the slope of the disease progress curve depends upon the rate of propagation success of these propagules. Oospores differentiate in infected roots. While in or on the ground this tissue decomposes or withers away, leaving the oospores behind. Dead plants may be removed, but infected portions may remain. If new, susceptible plants are planted in the same area, and then all that is needed is soil saturation to start the disease cycle once again.

In Mediterranean regions, temperatures during winter may limit the development of root infections of species with a higher optimum temperature such as *P. nicotianae* and *P. palmivora.*

The disease cycle of *Phytophthora* is complex as it involves numerous sources of primary and secondary inoculum and several modes of dissemination; all these features confer to this organism a high plasticity in adapting to different environmental conditions and, therefore, they should be taken into account in the development of integrated disease management strategies.

Phytophthora root and crown rot of olive (*Olea europaea* L.) usually occurs in young trees where long-term water-logging of the soil has taken place; only occasionally, however, this disease has been found also on trees growing in well-drained soils. Symptoms on infected trees include chlorosis and premature drop of the leaves, progressive thinning and dieback of the whole canopy. The tree often shoots from dormant buds in the lower part of the stem. An overproduction of small and parthenocarpic drupes may occur (Fig. 6). Above-ground symptoms are caused by the extensive necrosis of roots and by the consequent reduction of the active root system. When soil conditions are conducive to the infection, a crown and basal stem rot girdling the tree, which can be better noticed by unearthing the tree, may occur. Infected trees decline progressively over several years or die suddenly. Chronically infected trees appear stunted.

Figure 6. Decline symptoms on young olive trees caused by simultaneous infections of *Phytophthora palmivora* and *Verticillium dahliae* in Sicily. In Spain, this syndrome has been referred to as “seca”, which means drying. Healthy tree (left). Affected tree showing chlorosis, thinning of the canopy as a result of leaf drop, unusual fruiting and wilt (right).

Phytophthora root and crown rot was first reported in Greece [18] but has been considered a minor disease of olive for a long time. Since then, the disease has been recorded in various olive-growing regions throughout the world, including Argentina, Australia, California and the Mediterranean region [19, 20, 21, 22, 23, 24, 25, 26]. Recent and almost contemporary reports from Spain and Italy [11, 12, 21, 27, 28, 29] would indicate that it can be recognized as an emerging phytopathological problem in new olive plantations in the Mediterranean region. In Australia, Phytophthora root and crown rot is considered a major problem probably due to the recent expansion of plantings in new areas with heavy soils [30]. The disease was found to be correlated with high summer rainfall and most of the affected trees were 6- to 10- years old; apparently, it did not occur in nurseries (Greg O'Sullivan, personal communication). In a national survey on olive pests and diseases [31], Australian growers indicated Phytophthora root and crown rot as the most frequent root disease and the second most common disease of olive, after peacock spot. Conversely, in California, Phytophthora root and crown rot is not considered a serious threat to the olive industry [23].

2. Causal agents

Several species of *Phytophthora* have been reported to cause root and crown rot of olive and other species of the Family *Oleaceae* (Table 1). In California, *P. citricola* Sawada and *P. drechsleri* Tucker have been indicated as the causal agents of this disease in olive groves [19, 23].

In Australia, several species have been reported to be associated to rotten roots of olive or recovered from the soil around the symptomatic plants, including *P. cinnamomi*, *P. citricola, P. cryptogea* Pethybr. & Laff., *P. drechsleri*, *P. nicotianae* v. Breda de Haan, *P. inundata* Brasier, Sànchez-Hernández & Kirk and *P. palmivora* [31]; however, their role as causal agents of olive root rot has not yet been experimentally demonstrated. *P. inundata*, which was formally described recently [32], is common in river water as well as in pond debris in natural and forest ecosystems and proved to be highly pathogenic to young olive trees (www.fishingforphytophthora.murdoch.edu.au/). *P. cinnamomi* is a polyphagous pathogen, infecting both cultivated fruit trees such as avocado and numerous native plants in forest ecosystems. Although the first report of this species on olive dates back to 1948 (Anonymous, 1949 cited in Erwin and Ribeiro [5]; Vera Sergeeva, personal communication), little is known of its significance for the olive industry [33]. Recently two *Phytophthora* isolates (identification number DAR 76532 and DAR 76533) obtained from olive in New South Wales (Australia) by M. Priest and kindly provided by V. Sergeeva were identified as

Table 1. *Phytophthora* species reported on olive worldwide.

Species	Taxonomic group[1]	Clade[2]	Sporangia	Antheridia	Mating system
P. cinnamomi	VI	7	non-papillate[3]	amphigynous	heterothallic
P. citricola	III	2	Semi-papillate often bi-lobed	paragynous	homothallic
P. cryptogea	VI	8	non-papillate	amphigynous	heterothallic
P. drechsleri	VI	8	non-papillate	amphigynous	heterothallic
P. gonapodyides	VI	6	non-papillate	amphigynous	heterothallic
P. inundata[4]	-	6	non-papillate	amphigynous	heterothallic[5]
P. megasperma[6]	V	6	non-papillate	both paragynous and amphigynous	homothallic
P. nicotianae	II	1	mono- and bi-papillate occasionally caducous	amphigynous	heterothallic
P. palmivora	II	4	papillate caducous	amphigynous	heterothallic

[1]Waterhouse [6]. [2]Cooke *et al.* [15]; Blair *et al.* [14]. [3]In general, non-papillate sporangia are persistent. [4]This species was formally described in 2003 (Brasier *et al.* [32]). [5]Some A1xA2 combinations of this species fail to mate; others are unable to produce gametangia but induce gametangial formation in the opposite sexual compatibility type of another species (Brasier *et al.* [32]). [6]*P. megasperma* in a strict sense (BHR type).

as *P. cryptogea* on the basis of ITS sequences amplified by PCR using the ITS6/ITS4 universal primers. The ITS sequences of these isolates were deposited in GenBank (Santa Olga Cacciola, personal communication).

In Greece, *P. citricola* and *P. megasperma* Drechsler were reported as occasional pathogens of olive [5, 18].

In Spain, following unusually high rainfall during fall and winter, new plantations of olive trees (1- to 10-years old) were severely affected by a syndrome locally named ‘seca’ (drying) that was observed as a consequence of prolonged soil water-logging. Symptoms included foliar wilting, dieback and death of trees which often died rapidly, with or without previous

yellowing or defoliation. Besides some insect damage and agronomic problems, the `drying syndrome' was initially reported to be associated with Verticillium wilt, winter frost and root rot fungi [21]. However, subsequently, *Verticillium dahliae* Kleb. could not always be isolated, whereas *Phytophthora* spp. were consistently obtained from infected root tissues, thus suggesting a prominent role of these Oomycetes in this syndrome [28]. *P. megasperma* "BHR-type" *sensu stricto* [34, 35] appeared to be the most common species associated with the syndrome. Another species less frequently isolated from rotten roots was *P. inundata*. The pathogenicity of both species was confirmed in tests with several isolates on 6-month-old rooted cuttings of olive, under both weekly watering and water-logged conditions [27]. A third species, *P. palmivora* (Butler) Butler, has been reported from southern Spain to be associated with 'seca' and as an agent of root rot of young olive trees in nurseries [21].

A syndrome similar to 'seca' has been observed in 3- to 5- year-old irrigated olive orchards in Sicily (southern Italy). *P. palmivora* and *V. dahliae* were both isolated from rotted roots and the incidence of positive isolations was 30 and 80% respectively (Santa Olga Cacciola, personal communication). *P. palmivora* has been also associated with root rot and wilt of 1- to 2-year-old olive trees in new plantations [11]. Up to 40% of the trees were affected and symptoms were noticed 4 to 9 months after being transplanted from the nursery to the field [36].

In an extensive survey of olive nurseries and groves in Sicily, five species of *Phytophthora*, including *P. citricola*, *P. inundata*, *P. megasperma* "BHR-type", *P. nicotianae* and *P. palmivora*, have been associated with root rot and decline of olive trees [25]. Like in Spain, *P. megasperma* was the most frequent species in commercial groves and was isolated from basal stem cankers of young trees and from roots of both young and mature trees. This species had been previously reported for the first time as the causal agent of olive root rot and basal stem cankers in 1999 in Italy when it was isolated from 3-year-old trees showing symptoms of decline [12]. P. *citricola* occured only occasionally, whereas *P. palmivora* (A1 mating type) and *P. nicotianae* (both A1 and A2 mating types) were frequently associated with the rot of fine roots. *P. nicotianae*, which had been previously reported in Italy as causal agent of root rot of *Forsythia viridissima* Lindl. [37], an ornamental plant of the *Oleaceae* family, was sporadically recovered from olive trees with symptoms of root rot. *P. palmivora* was found to be widespread in both nurseries and new olive plantations, thus suggesting that this species is spreading with nursery-trees. *P. inundata* was associated with root and collar rot of mature olive trees after prolonged flooding of the soil. In pathogenicity tests, *P. inundata* and *P. megasperma* were the most virulent species and

caused both root and crown rot. Irrespective of the *Phytophthora* species only plants subjected to flooding after root inoculation through infested soil showed symptoms of decline. This is in agreement with the observation that in the field Phytophthora crown and root rot is a problem primarily in soils where prolonged water-logging has occurred [11, 21]. According to Sanchez-Hernandez *et al.* [21] the association of flooding with *Phytophthora* infections would indicate that the high sensitivity of olive to root asphyxiation may be more properly regarded as root-rot caused by *Phytophthora* spp.

Surveys carried out in the Apulia region, southern Italy, indicated that Phytophthora root rot is widespread in olive orchards and nurseries [29]. Three species were identified, including *P. citricola*, *P. megasperma* and *P. palmivora*, the last one being the most frequent in this Italian region (http://www.olviva.it). In Apulia, there is also an occasional record of a rot of mature olive drupes cv Coratina caused by *P. nicotianae* and a *Phytophthora* sp. [38]. The disease affected mostly olives in the lower part of the tree canopy and its incidence was up to 60% of drupes.

In Argentina, the drying syndrome has been observed in different varieties of young olive plantings under modern irrigation technology and different pathogens including *Fusarium*, *Phytophthora* and *Verticillium* were associated to this syndrome [24]. Recently, both *P. nicotianae* and *P. palmivora* were reported as causal agents of root rot of olive trees in Argentina [39, 40]. Moreover, *P. nicotianae* has been isolated in Nepal from roots of *Olea cuspidata*, an olive species that is native to Asia [41].

3. Diagnosis

Accurate detection and diagnosis of Phytophthora root rot and identification of species involved are useful both in the nursery to prevent pathogen spread and in the orchard to manage the disease.

Phytophthora root rot of olive, like Phytophthora root rot of other trees and horticultural crops [42], has been unnoticed and probably its incidence and diffusion in olive orchards and nurseries have been underestimated for a long time because it does not show specific symptoms and cannot be diagnosed without specific detection methods. Moreover, the course of this disease is chronic and the roots of olive trees can be infected months to years before the appearance of above-ground symptoms.

Phytophthora species cannot be isolated on common agar media from soil, roots and decayed tissues colonized by secondary invaders since they have limited saprophytic ability and are not able to compete with other microrganisms. Since *Phytophthora* species are difficult to isolate from

necrotic plant tissues and soil, the bait method has been widely utilized as the only effective method to isolate *Phytophthora* before the advent of selective media in the early 1960s. Baiting techniques are still in use for large-scale surveys as an early warning system to check if *Phytophthora* is present in an area, to increase the frequency of successful isolation from infested soil and water as well as to isolate *Phytophthora* species whose growth is inhibited by chemicals added to selective media [5]. The bait consists of a highly susceptible host-plant that is readily infected by *Phytophthora*. Leaves of banksia, pittosporum, hakea and oak, for example, were used in a recent survey for monitoring waterways in Western Australia (http://www.fishingforphytophthora.murdoch.edu.au). To our knowledge, no specific bait material has been used in surveys of olive orchards and nurseries.

Generic media could be utilized to isolate *Phytophthora* from host tissues and/or baiting tissues; however, the use of selective media has markedly increased the success of isolation. These media contain chemicals such as polyene antibiotics that inhibit bacteria and fungi but have little or no effect on the mycelium growth or on the propagule germination of *Phytophthora* [43, 44]. Among numerous existing selective substrates for isolating *Phytophthora,* the BNPRAH [45], the PARPH [46, 47, 48] and the PARPNH [49] media have been used to isolate *Phytophthora* species from infected olive tissues [11, 12, 21, 28, 38, 39]. All these media are composed with a universal culture medium or basal medium, such as corn meal-agar (CMA) or potato-dextrose-agar (PDA), amended with selective chemicals, including antibiotics and antifungal compounds. PARPH, the most widely used selective medium for isolating *Phytophthora*, is CMA supplemented with pimaricin, ampicillin, rifampicin, pentachloronitrobenzene (PCNB) and hymexazol. Pimaricin is a broad-range polyene antibiotic active against most fungi; ampicillin is an antibiotic active against Gram-positive bacteria; rifampicin is a macrocyclic antibiotic mainly active against mycobacteria and Gram-negative bacteria; PCNB is a fungicide effective against soil-borne fungi and complements polyene antibiotics in selective media. Hymexazol is a fungicide added to suppress the growth of *Pythium* and *Mortierella* (Zygomycetes) that are common soil inhabitants; however, it can also inhibit some *Phytophthora* species and is not active against some soil-borne *Pythium*, such as *P. irregulare* and *P. vexans*. A comprehensive list of both selective chemicals and their properties is reported by Erwin and Ribeiro [5].

Species of *Phytophthora* causing olive root rot differ significantly as far as their ecological characteristics are concerned, thus their accurate identification may have practical relevance for the development of control strategies. Identification of *Phytophthora* isolates has largely relied on synoptic keys of Waterhouse [6], Newhook *et al.* [7] and Stamps *et al.* [8],

which are based on morphological criteria. However, these keys proved to be inadequate because of the low number o morphological features used to distinguish the species, great intra-specific variability and overlapping characters among species. The biochemical approach based on total mycelial proteins and isozyme electrophoretic patterns proved to be a valuable complementary tool for the identification of *Phytophthora* species infecting olive [11, 12, 36]. Nucleic acid technology has been provided with a series of diagnostic tools that have proved invaluable for detecting and monitoring these pathogens. *Phytophthora* isolates from olive have been identified by PCR amplification of ITS regions of rDNA using the universal primers ITS6 and ITS4 followed by either digestion with restriction enzymes (ITS-RFLP) or sequencing and comparison of sequences with local and Genbank available databases [39, 40, 50].

In general, DNA sequence analysis is the most accurate method for the identification of *Phytophthora* isolates to species level. Besides nuclear encoded ITS regions of rDNA, several other nuclear and mitochondrial loci could be used to separate species and applied to design primers for diagnostic purposes. Species-specific diagnostic markers have been developed from specific genes and they include translation elongation factor 1 alpha, *β-tubulin* [51, 52], *elicitin* [52, 53, 54], enolase, heat shock protein 90 genes, *tigA* gene fusion, 60S ribosomal protein L10 and the large subunit of the rDNA [14], *cina* [55], *lpv* [54], *rpb1* [56], *gpa1*, *ras*-like and *trp1* [57]. While these sequences are available for almost all *Phytophthora* species, an additional nuclear locus, the *ypt1* ras-related protein, is available for some species only [58, 59]. Mitochondrial genes include cytochrome oxidase (*cox*) I and II regions, *nad1*, *nad9, rps10,* and *secY* genes [14, 15, 51, 60, 61, 62].

If DNA sequence analysis is not feasible, there are several other molecular techniques such as RFLP (Restriction Fragment Length Polymorphism Analysis) and SSCP (Single Stranded Conformation Polymorphism) which could be applied for the identification of isolates [15, 63, 64]. Even though these molecular markers require more data interpretation than DNA sequence analysis, they are an alternative option when cost and/or availability of an automated sequencer are a limiting factor. RFLPs techniques include the digestion of the ITS regions of the rDNA as well as the *cox1* and *cox2* gene cluster of the mitochondrial DNA [60]. RFLPs, the earliest DNA fingerprinting method has been used extensively. They are robust and co-dominant and can be useful to distinguish homozygotes from eterozygotes for an allele. This advantage can be especially important for the study of organisms with a dominant diploid phase, such as oomycetes. In addition, RFLPs give an indication of gene copy number and presence of gene families. This could be useful information

because the number of gene copies can be different even within a genus. However, in order to perform genomic RFLPs, a large amount (e.g., 5-10 µg) of high-quality DNA for each assay and pure culture is needed [65].

SSCP analysis of the ITS regions of the rDNA could be useful for the identification of isolates to species level. Recently, a key for the genus *Phytophthora* integrating a classical morphological approach and modern fingerprinting technology based on SSCP migration patterns has been published by Gallegly and Hong [13].

Fingerprinting techniques, such as RAPDs (Random Amplified Polymorphic DNAs) generate DNA profiles from template DNAs. They are fast, simple, cheap, and require only a small amount of DNA (e.g., 10-25 ng). Furthermore, RAPDs do not necessitate information on genome sequences. However, they are not always reproducible and generate mostly dominant markers [66].

Basically, all molecular diagnostic methods include the following steps: DNA extraction from infected tissues, soil or water, the amplification of target-DNA by PCR with specific primers and the visualization of amplified products. DNA amplification allows the use of a very small amount of starting DNA for the analyses. Post-amplification procedures that represent a drawback for the use of conventional PCR as a routine diagnostic method could be overcome by the real-time PCR approaches. Further improvements in the diagnosis of *Phytophthora* have been made with the development of multiplex PCR methods that imply the use of several PCR primers in the same reaction. The advent of these techniques allows the detection of several pathogens simultaneously, thus reducing time and costs. Multiplex real-time PCR has the potential to quantitatively detect different pathogens at the same time using multiple primers in a single reaction [16].

If the identification of *Phytophthora* at the genus level is required, genus-specific primer pairs can be used; they have been designed on both nuclear and mitochondrial loci, including the ITS regions, the Ras-related protein *ypt1*, and the spacer regions between the mitochondrially encoded *cox1* and *2* genes [59, 61].

For the direct diagnosis of *Phytophthora* from infected plant tissues, both genus- and species-specific PCR markers are available, including random clones of genomic DNA and SCAR (Sequence Characterized Amplified Regions) as well as individual genes. A very interesting technique that does not require a thermal cycler to carry out the assay or collect the data is the loop-mediated isothermal amplification (LAMP) that was applied for the detection of *P. ramorum*; however, this method is less sensitive than PCR [67].

Macro-arrays are being developed for the identification of *Phytophthora* to species level. Although macro- and micro- DNAarrays are more

technically challenging to perform than RT-PCR or DNA sequencing, they have an almost unlimited multiplexing capability to detect many different species from environmental samples [68, 69, 70].

Microsatellites also known as SSR (Simple Sequence Repeat) have become the marker system of choice for the analysis of populations because they are co-dominant, very repeatable, and provide many alleles per locus. They are costly to develop but very affordable once developed [71]. Recently, Schena *et al.* [72] screened available uni-gene datasets from the sequenced genome of *P. infestans*, *P. sojae* and *P. ramorum* for candidate SSR markers with the aim of applying them to a wide range of *Phytophthora* species.

Each type of molecular marker has qualities and limitations and the choice of a marker system depends on the target to be achieved, the amount of available DNA and pre-existing genome information [65, 73, 74].

Perhaps the most exciting aspect of DNA amplification methods is the possibility to diagnose *Phytophthora* directly in environmental samples such as tissues, infested soil or water, and to provide a powerful tool for studying the ecology of these pathogens and the epidemiology of the diseases they cause. In general, these methods have several advantages with respect to the conventional culturing and microscopic examinations, including rapidity, sensitivity, ability to explore biodiversity and variability of *Phytophthora* species, and the possibility to detect a target-DNA in a complex mixture. However, higher yields of DNA with fewer inhibitors are essential when different types of environmental samples have to be handled. A very promising and innovative molecular method for the study of the ecology of *Phytophthora* in agricultural and natural ecosystems has been recently published [75]. This method for *in situ* diagnosis is based on a nested PCR approach with genus-specific primers and allows the detection of “molecular species”, i.e. species referred to as ‘Operational Taxonomic Units’ or ‘Phylotypes’ defined according to sequence similarity [76,77], which are defined and named in the absence of a culture [16]. This phylogenetic approach could also be used to study biodiversity and co-evolution, while providing a better and more complete insight into the *Phytophthora* populations associated with olive roots in different environments as, in general, culture-based methods reveal only partly the microbial community in rhizosphere soil.

Additional and more detailed information on the isolation, detection and identification of *Phytophthora* may be found in monothematic books and comprehensive reviews [5, 13, 16, 78]. Because this subject is evolving rapidly and needs continuous updating, it is advisable to refer to

Phytophthora-dedicated databases, such as the "Phytophthora Database" published on line (www.phytophthoradb.org/).

Although molecular techniques have been continuously and rapidly developing, their potential has not yet been fully exploited in the diagnosis of *Phytophthora* diseases of olive.

4. Management and control

Prevention is the best strategy for the management of soil-borne diseases of plants in general. Many fundamental studies demonstrate the crucial role of the soil-water status on both the production of sporangia and the occurrence of root infections by *Phytophthora* species. These studies show that sporangia are produced when the matric potential value in soil is slightly more negative than zero (free water) and that zoospores, which are the infectious propagules, form and disseminate when soil is saturated, i.e. the matric potential value equals zero [5]. As asphyxiation due to soil water saturation predisposes the roots to the infection of these pathogens, Phytophthora root and crown rot of olive is chiefly associated with both heavy soils and prolonged periods of rainfall [23, 25, 79]. In fact, although olive trees tolerate a large range of soil conditions, they are very sensitive to soil asphyxiation, and soil water saturation for as little as one day, particularly when combined with higher temperatures, can cause root death [31]. Consequently, the management of soil water is basic for the control of this disease. Careful selection and preparation of the planting site could help in preventing soil water-logging problems. Similarly, cultural practices that prevent prolonged saturation of soil such as planting on mounds, soil drainage and proper management of irrigation may reduce root and crown rot. Irrigation technologies involving the use of emitters that do not wet the trunks and instruments that measure the water status in soil, such as tensiometers and neutron probes, may be valuable for the development of an integrated disease management approach. Growers who receive positive identification of *Phytophthora* should first consider cultural practices before turning to chemical control and, if plants still do not show signs of recovery after these practices, a fungicide application may be warranted. In Australia, foliar applications of potassium phosphonate are commonly used for the control of Phytophhtora root and crown rot [31], mainly in young olive plantings to protect the trees until they are well established. Potassium phosphonate, which is also referred to as phosphite or phosphorous acid, is available under a number of trade names and is effective as a preventive treatment because it enhances the plants' defence mechanisms. However, neither potassium phosphonate nor other chemicals such as metalaxyl and its

isomer mefenoxam, commonly used in other crops to control diseases caused by Oomycetes, are registered on olive in most olive-growing countries. In experimental trials Ridomil 5G and Ridomil 25WP proved to be very effective in controlling Phytophthora root rot on 7-month-old olive plants potted in soil infested with *P. megasperma* propagules [80].

Results from recent surveys of new olive plantings in Italy and Spain would indicate that some *Phytophthora* species, including *P. palmivora* and *P. megasperma,* are frequently associated with planting stocks. Nursery accreditation and sanitation protocols provide useful tools to ensure high quality and disease-free planting material (www.olviva.it) as well as to prevent the spread of these pathogens in commercial olive groves. The diagnosis of Phytophthora root rot in olive nurseries can be more efficient by using molecular methods now available to detect and quantify *Phytophthora* species in soil, water and tissues [16]. Soil amendments or the use of suppressive substrates as possible options in olive nurseries for the control of Phytophthora root rot have not yet been fully explored.

The increasing importance of Phytophthora root rot in new plantings has stimulated the search for genetic resistance as a control means against this disease (cited in Trapero and Blanco [79]) but as yet no olive cultivars or rootstocks tolerant to *Phytophthora* are available. On the other hand, breeding programs to improve the genetic resistance of olive to diseases are mainly focused on Verticillium wilt which, besides being more common and widespread, is also a severe soil-borne disease (G. Bubici and M. Cirulli this book; [23, 81]).

5. References

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Additional web resources

- http://www.olviva.it
- http://www.fishingforphytophthora.murdoch.edu.au
- Phytophthora Database http://www.phytophthoradb.org