STUDIES ON CORK OAK DECLINE: AN INTEGRATED APPROACH

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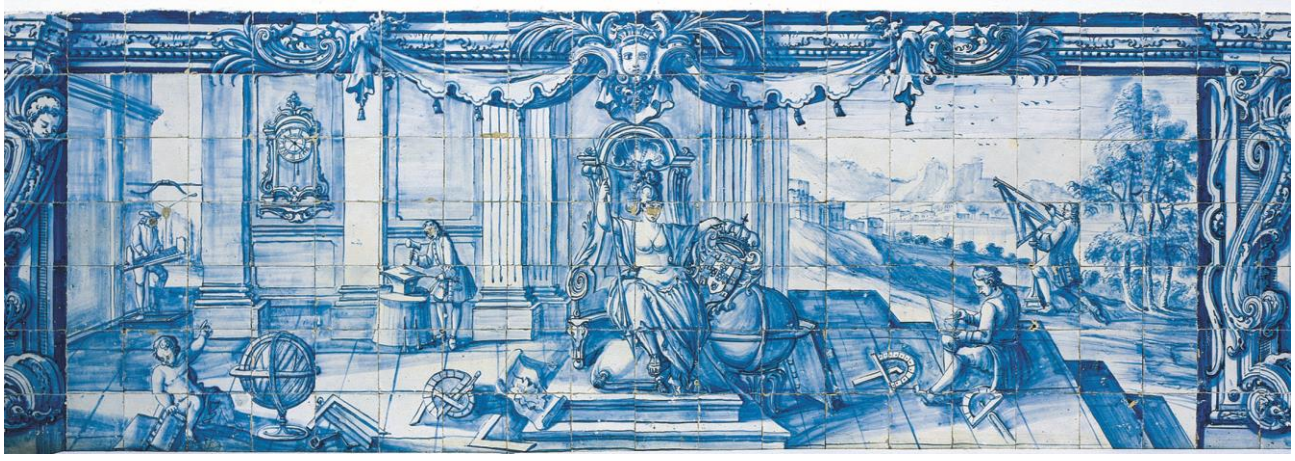

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“The true meaning of life is to plant trees

under whose shade you do not expect to sit.”

Nelson Henderson

RESUMO

Estudos sobre o declínio do sobreiro: uma abordagem integrada

Neste trabalho abordaram-se vários aspectos relacionados com o declínio do sobreiro (*Quercus suber*). Uma análise exaustiva á bibliografia realçou que o sobreiro é moderadamente susceptível a *Phytophthora cinnamomi*, ocorrendo declínio quando há encharcamento do solo e limitações à expansão de raízes. Também foram identificados alguns aspectos que foram alvo de investigação mais detalhada, como a utilização de métodos robustos para detecção de *P. cinnamomi* e conhecimento acerca da fisiologia associada ao declínio. Métodos moleculares para diagnose de *P. cinnamomi* de amostras de solo revelaram-se inapropriados devido à combinação da baixa concentração do inóculo com a reduzida quantidade de amostra passível de analisar. Em relação à fisiologia do declínio, duas respostas ao défice hídrico foram detectadas, uma levando à morte por falta de fotoassimilados, outra por falha no transporte hídrico. Por fim, a utilização de ectomicorrizas em condições naturais mostrou ser uma abordagem a aprofundar para a melhoria sanitária das árvores.

ABSTRACT

Studies on cork oak decline: An integrated approach

This work integrates various aspects related to cork oak decline. A thorough bibliographic analysis stressed that cork oaks (*Quercus suber*) are moderately susceptible to *Phytophthora cinnamomi*, and decline is likely to occur under conditions limiting roots expansion and causing waterlogging. It was also identified some important aspects subjected to detailed research, like the prerequisite of robust methods for *P. cinnamomi* diagnosis and knowledge about the tree physiology associated to decline. Molecular methods for *P. cinnamomi* diagnosis from soil samples proved unsuitable because of the combination of reduced inoculum concentration in the soil and low sample size possible to analyze. In relation to the physiology of decline, two responses to water stress were detected, one leading to mortality caused by lack of assimilates and another caused by failure in water transport. Finally, the use of ectomycorrhizal under natural conditions has proven to be an interesting approach for trees health status improvement.

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1- GENERAL INTRODUCTION

Abnormal episodes of cork (*Querus suber*) and holm oaks (*Q. rotundifolia,* syn. *Quercus ilex* spp. *rotundifolia*, *Q. ilex* spp. *ballota*, *Q. ballota*; Lousã and Fabião 1997) mortality in the Mediterranean basin have been reported since the beginning of the XX century, which were associated to climatic events, and cultural practices in the woodlands (Baeta Neves 1949, 1954; Natividade 1950, Macara 1975). During the 1980s, cork and holm oak mortality was associated to the soilborne pathogenic *Phytophthora cinnamomi* (Brasier 1992a, 1993) and since then it has been considered by many authors the main cause of cork and holm oak decline, though coupled with unfavorable environmental conditions (Cobos et al. 1992; Robin et al. 1998; Gallego et al. 1999; Moreira 2001; Sánchez et al. 2002). In cork and holm oak woodlands there were indications that abiotic factors may also be strong involved in the observed decline (Cabral & Sardinha 1992, Sousa et al. 2007). Ribeiro & Surový (2007) demonstrate, in the first national forest inventory of dead trees in cork oak stands, that limitation in soil depth and high precipitation were statistically linked to high mortality indices. Although the authors did not include in the study biotic factors like *P. cinanmomi* distribution, they reinforce the conclusions that abiotic variables are linked to oak mortality. Such studies raised some concerns about the relative importance of those factors in Mediterranean oak decline. One could question if mortality linked to excess soil moisture was mediated by *P. cinnamomi*, or, on the other hand, trees growing in soils with no depth limitations could co-exist with the pathogen with no expenses of their health. Only after identifying the main actors and their correlations in tree decline will be possible to move for the next step, such as development of approaches to prevent decline. First of all, in order to achieve these objectives, it would be needed a reliable

methodology for *P. cinnamomi* diagnosis. Former studies faced some inherent difficulties in detecting *P. cinnamomi* in the field, leading to a probable underestimation of the pathogen and difficulties in relating its occurrence with abiotic factors and tree health status.

Other aspect should also be included in those studies, namely physiologic responses of trees under decline. In general, the link between the pathogen infection, abiotic factors and the overall tree physiology is poorly understood, hence the development of the disease is unclear (Jönsson 2006). *P. cinnamomi* is primarily a root pathogen of woody species and causes rot of fine feeder roots; secondary symptoms resemble those of drought: the crown thins, foliage becomes chlorotic, wilt and epicormic shoots form (Brasier et al. 1993, Robin et al. 1998), but it is lacking studies towards the comprehension of tree physiologic status associated with decline. Former studies were performed mainly with infected seedlings and centered essentially in plant water relations, were a significant reduction in leaf water potential and stomatal conductance was observed (Maurel et al. 2001, Luque et al. 2002). It is referred that mortality of oak seedlings is strongly related with root pruning by the pathogen, causing impairment in water absorption and, therefore, plant water stress (Robin et al. 2001; Clemenz et al. 2008). However, adult trees have a more complex root system, with sink roots accessing deep groundwater tables and fine-roots mainly distributed in the upper soil layer (Otieno et al. 2006, Surový et al. 2011). Thus, infection in their root system may have different effects on adult trees. Several subjects need answers, like what physiologic responses are associated to pathogen infection and the role of fine-roots in tree decline.

Since *P. cinnamomi* attacks fine-roots, other interesting approaches would be related to the role of ectomycorrhizae in oaks health status and, particularly, in

protection against the pathogen. Ectomycorrhizae (ECM) are symbiotic of plants and widely known to improve their nutritional status (Smith and Read 1997; Quarles 1999a, b, Marx et al. 2002). Moreover, they form hyphal sheaths around roots eventually acting as physical barrier against root attack by pathogens, which was verified on sweet chestnut (*Castanea sativa*) and in shortleaf pine (*Pinus echinata*) roots, where mycorrhizal root tips with complete mantle were not infected by *Phytophthora* (Marx & Davey 1969 a,b; Brazanti et al. 1999). Other mechanisms involved in disease suppression such as a chemical barrier, antibiosis or antifungal compound production, eventually acting in synergism, may also protect mycorrhizal plants against root pathogens (Zak 1964; Marx 1973; Brazanti et al. 1999). There are several studies in controlled conditions relating the protective effect of ECM against root pathogens (ex: Marx & Davey 1969a, b; Brazanti et al. 1999) but, under natural conditions, studies were focused in the differences on natural ECM colonization according to tree health status. In somestudies in declining cork oak sites, root tip colonization was reported to remain substantial in declining trees, but a switch between some ECM species occurred in direction to low-biomass ectomycorrhizae with less abundant extramatrical hyphae, which are presumed to require less carbon from the host tree (Kuikka et al. 2003; Saravesi et al. 2008; Blom et al. 2009; Lancellotti & Franceschini 2013). However, it was suggested that healthy trees may eventually be more selective in ECM symbioses than declining trees that are more dependent on ECM response diversity (Blom et al 2009). If declining trees were more dependent of available ECM diversity, they could be favored by artificial applications of selected ECM inoculum. Additionally, other studies reported less ECM in declining trees (ex: Causin et al. 1996; Scott et al. 2013). Such cases could also be favored by artificial ECM application, providing abundant inoculum of selected species to enhance colonization in declining trees. Former

introduction of ECM and fertilizers in declining adult cork oaks showed an overall improvement in tree health status (Symbio docs), suggesting the potential of ECM application in health improvement and, eventually, on *P. cinnamomi* suppression. There are not regular methods to prevent *P. cinnamomi* infection worldwide besides cultural practices to avoid spread of the pathogen to uninfected forest (Dawson & Weste 1985, Brasier & Jung 2003) and the use of chemicals like metalaxyl and potassium phosphonate. Metalaxyl is highly selective against Oomycetes, however it was reported to progressively loss efficacy against *P. cinnamomi* (Darvas 1983, Gouveia 2004). Potassium phosphonate injections stimulate the defense mechanisms of the trees (Guest & Grant 1991, Fernández-Escobar et al. 1999, Navarro et al. 2004), but results with Mediterranean oaks were unsatisfying (Solla et al 2009). Experiments in natural conditions with ECM application should therefore be performed to analyze their effect in tree health, in order to assess their subsequent usefulness as biocontrol of root pathogens.

1.1- GENERAL OBJECTIVES

In this study it was realized an extensive and deep analysis of the published works related to cork and holm oak decline, namely *P. cinnamomi* surveys, pathogenicity tests, studies about the influence of site characteristics in tree decline and approaches to control the disease (chapter 3). Goals of this chapter were: 1) to identify the main shortcomings on the association between *P. cinnamomi* and oak decline, suggesting, when possible, some approaches to overcome them; 2) to assess the relative

importance of each factor involved in decline and 3) revise the role of *P. cinnamomi* in cork and holm oak decline.

In chapter 4 it was tested and compared two methodologies for *P. cinnamomi* diagnosis, both to be applied in soil rhizosphere under the host trees. The first one, baiting selective-medium method, is widely used but it is also referred to presents some limitations, resulting in the production of false negatives. On the other hand, DNA-based molecular diagnostics have been overwhelmingly developed to identify, diagnose and study *Phytophthora spp.* (Cooke et al. 2007). Recently, it was published a protocol concerning molecular methods to diagnose *P. cinnamomi* from total DNA extracted from soil samples (William et al. 2009). In this chapter both methods were tested, difficulties were identified as well possible solutions to overcome them in order to obtain a reliable diagnosis method for *P. cinnamomi* occurrence.

The next approach in this study was a contribution to the comprehension of cork oak decline, particularly to understand the physiologic status of the trees showing different decline symptoms. Unspecific symptoms like tree defoliation and upper branches dieback, or alternatively a fast drying of the leaves, have been observed in declining trees (Cobos et al. 1992; Tuset et al. 1996; Gallego et al. 1999; Moreira 2001; Ruiu 2006; Sousa et al. 2007). They are usually associated to water stress, caused or not by *P. cinnamomi*. Nevertheless, only after analyses of the main physiologic processes related to decline symptoms it will be possible to generate further studies on the functional relation between pathogen infection and host symptoms. Thus, chapter 5 was focused on 1) the association between visible symptoms of decline and physiologic status of the trees, with emphasis in tree water relations, and, additionally, 2) the study was complemented with an exploratory approach on physiologic status of the trees with *P. cinnamomi* in the rhizosphere.

Finally, last chapter was toward approaches that could eventually contribute for prevention of *P. cinnamomi* infection, at least during the first critical years of the seedlings after reforestation in the field. Thus, it was tested ectomycorrhizal application in cork oak seedlings in nurseries with non-sterile conditions and also in an established young cork oak trees in the field. The objective was to test if application of ectomycorrhizae in natural conditions would be effective in colonizing host trees for further applications regarding inhibition of *P. cinnamomi* infection and/ or improvement of tree health status.

CHAPTER 2

Bibliographic review

2.1- BRIEF DESCRIPTION OF THE SPECIES

2.1.1- The *montado* system

The cork (*Quercus suber*) and holm oaks (*Q. rotundifolia*) are sclerophyllous evergreen species from the family *Fagaceae*, endemic to the Western Mediterranean region. The climate of this region is characterized by summer drought and mildly cold winters. Trees must withstand a long hot and dry summer season when water deficits are associated with high light intensity and temperatures, being alleviated by the autumn rains (Vaz et al. 2010). Those species survive drought thanks in part to their extensive and deep root system that tap water from deeper soil, maintaining water status and xylem conductance above lethal levels throughout the summer drought periods (Pausas et al. 2009). Both species requires high demands in light, but cork oaks dominate the moister areas whereas holm oak occupies the drier inland areas. This geographical distribution seems to reflect different tolerances to drought between species (David et al. 2004). Cork oaks has a most distinctive characteristic, a secondary meristem, the phellem, that produces an outer coat of insulation consisting of corky bark of continuous layers of suberized cells that may have evolved as an adaptation to fire; a tree continuously generates cork that can be stripped without severe damage at regular intervals, usually each nine years, to provide commercial cork (Pausas et al. 2009, Toribio et al. 2005). Cork oaks are modest in regard to soil requirements, growing sandy and clayey soils of granitic and schistic origin, but free of calcium carbonate; on the other hand, holm oaks are adapted to a wide range of edaphic conditions growing in all type of soils, consequently occupying a bigger area than cork oaks, though being ousted by them on the richer and wetter sites (ATLAS 1987, Gonzalez 1993).

Cork and holm oak woodlands are of high conservation and socioeconomic value within their areas of geographic distribution around the Mediterranean basin: Portugal, Spain, southern France, Sardinia, Algeria, Morocco and Tunisia (DGRF et al. 2007). They dominate the forestry layer of the agro-silvo-pastoral system called ‘‘*montado*’’ in Portugal or ‘‘*dehesa’’* in Spain, forming a savannah-like landscape (fig. 2.1).

Figure 2.1: Montado system with cork oaks (*Quercus suber*), some stone pine (*Pinus pinea*) native pasture and cattle, Herdade da Machoqueira do Grou, Coruche, Portugal

It occupies approximately 5.3 million hectares of woodland in Spain (Sánchez and Garcia 2007) and 1.2 million hectares in Portugal (DGRF 2007). The herbaceous layer is comprised of either cultivated cereals (oats, barley, wheat) or, more commonly, native vegetation dominated by annual species, which are used as grazing resources (Joffre et al. 1999); the livestock layer includes sheep, goats, Iberian pigs that graze the seasonal acorn production, and cattle. *Montado*s also support productions of natural pasture, mushrooms, honey, and are natural habitat for hunting species, and many more

(Pinheiro et al. 2008). Diversity of production—forage, acorn, wood, cork, charcoal—is the characteristic of these systems and the long-term ecological sustainability derives from the sub-optimization of the resources for many centuries (Joffre et al. 1999). *Montados* also have a significant environmental value related to biodiversity preservation, carbon sink, soil conservation, microclimate maintenance and energy conservation (anonymous 2013).

In Portugal, permissions to cut down cork and holm oaks, independently of the health status of the trees, must be granted by the ‘‘Autoridade Nacional Florestal.’’

2.1.2- *Phytophthora cinnamomi*

According to recent classifications based on molecular phylogeny, *P. cinnamomi* is classified in the kingdom Chromista, phylum Oomycota, order Peronosporales, family Peronosporaceae (Hardham 2005), though for some authors *Phytophthora* species are considered as members of the “union of fungi”, reflecting their fungus-like hyphae and nutrient acquisition (Anderson 2006). Together, fungi and Oomycetes cover the majority of eukaryotic plant pathogens and their convergent evolution seems to have forced the development of similar infection strategies, but their significant physiologic and biochemical differences are reflected in the large variations observed in sensitivity to conventional fungicides; some fungicides act by inhibition of the ergosterol biosynthesis pathway on fungi; others inhibit chitin synthase on fungi cell walls, however, oomycetes do not have the ergosterol pathway or chitin in their cell walls (Latijnhouwers et al. 2003).

P. cinnamomi is a soilborne oomycete believed to be of tropical or subtropical origin that is widely distributed in temperate and tropical regions. It is suggested to have spread throughout Europe in the nineteenth century, when sweet chestnut (*Castanea sativa*), a highly susceptible host was found affected by the so called ink disease in Portugal and Spain (Brasier 1996; 2000). Zentmyer (1980) listed approximately 950 host species and since then that number has increased considerably (Hardham 2005). *P. cinnamomi* is the most widespread of *Phytophthora spp.*, has the largest host range and causes extensive economic losses in agriculture, horticulture and forestry, being a major threat to natural ecosystems and biodiversity (Zentmyer 1983; Hardham 2005).

P. cinnamomi produces three types of spores: zoospores, chlamydospores and oospores. Somatic hyphae form multinucleate sporangia that, under lower temperatures and in the presence of free water, cleave and release 10–30 uninucleate biflagellate wall-less zoospores which are attracted to the zone of elongation of the host root tip, where they encyst forming walled cysts that germinate and penetrate the plant. (Zentmeyer 1980; Ribeiro 1983; Hardham 2005). Contrary to most *Phytophthora spp.*, *P. cinnamomi* is more attracted to hosts than to nonhosts (Carlile 1983). In susceptible plants like avocado, brown lesions in small roots appear in 24-36 hr after inoculation and mycelium is found throughout the root within 72 hr (Zentmeyer 1980). Within 2 or 3 days in a susceptible host, sporangia will form on the plant surface and the asexual cycle may be repeated many times in quick succession, rapidly amplifying the inoculum potential in the infected area (Hardham 2005). During warm and moist conditions the mycelia produce asexual sporangia, however, under less favorable conditions the production of asexual chlamydospores predominates (Cahill et al 2008). *P. cinnamomi* hypha readily forms clusters of thin-walled chlamydospores that germinate through germ tubes; they can persist in dead roots and in the soil, thus making them one of the

survival forms of this pathogen (Zentmeyer 1980). In fact, the role of chlamydospores is dynamic with a saprobic life cycle and can germinate independently of a host and in limited short term competition with other soil organisms (Weste 1983a). Oospores are sexual spores produced after fertilization of oogonia by antheridia. *P. cinnamomi* is usually regarded as heterothallic species, where two mating types, designated as A1 and A2, are required for oospore production. However, the A2 type can act as homothallic, forming oospores by selfing under special conditions, such as colony aging and specific chemicals produced by *Trichoderma spp.* or avocado roots (Zentmeyer 1980; Brasier 1992b). A1 type has limited distribution whereas A2 type is widespread geographically and is associated to severe outbreaks on crops and ornamentals in southern Australia, Europe, and America (Zentmeyer 1980; Brasier 1992b).

P. cinnamomi parasitizes living roots, however, it has some saprophytic ability in soils with low microbial activity, and particularly in saturated soils were it can compete with other soil microorganisms (Weste 1983b; Zentmeyer 1980; McCarren 2006). It persists in soil or infected plant material and when conditions favoring mycelium growth prevail, the pathogen enters the asexual sporulation cycle (Hardham 2005). This pathogen is known to survive for as long as 6 years in moist soil. Moisture is the key factor in the establishment, spread and longevity of the pathogen (Zentmyer 1980). *P. cinnamomi* is primarily a root pathogen of woody species and causes rot of fine feeder roots; larger roots are only occasionally attacked (EPPO 2004). Secondary symptoms resemble those of drought: foliage becomes chlorotic, wilt and, depending on the severity of the root rot, dies back and the crown thins, epicormic shoots are formed but wilt, turn brown and die; the pathogen may cause also stem cankers which often result in sudden death (EPPO 2004). Host susceptible reactions vary from rapid mortality following infection to field tolerance (Zentmyer 1980). Initiation of plant

infection involves zoospore attraction by root exudates, encystment and cyst germination in the root cap cell zone and development of mycelium in the cortical cells, phloem and xylem of the infected roots, although the pathogen is not able to hydrolyze lignified cell walls (Davison et al 1994).

P. cinnamomi was first described as a root pathogen of cork oak in 1944 by Lopes Pimentel (1946) although it was first misidentified as *P. cambivora* (in Carvalho 1993). This pathogen was also isolated from cortical cankers in cork oak trees in Russia in the 1950's (Globa-Mikhailenko 1960) and in California in the 1970's (Mircetich et al 1977). Two different populations were detected in southern Iberia following molecular studies, though there were no differences in pathogenicity between both populations when artificial inoculations were performed (Caetano et al 2007). Although it has been reported differences in virulence of isolates from different origins to some hosts (Robin & Desprez-Loustau 1998), no significant differences between three *P. cinnamomi* isolates were found in respect of the frequency of mortality, wilting and leaf necrosis of holm and cork oak seedlings (Robin et al 2001).

2.2- Mortality events of cork and holm oak trees in Mediterranean basin

Abnormal episodes of cork oak (*Quercus suber*) mortality with unknown etiology have been reported since the end of the nineteenth century and consistently throughout the twentieth century, in Portugal and Spain (Baeta Neves 1949, 1954; Natividade 1950; Macara 1975; Cabral and Sardinha 1992; Brasier et al. 1993; Carvalho 1993; Sousa et al. 2007). Natividade (1958) refers that in 1951 about 246,000 dead or injured cork oaks were cut down in Portugal. A diachronic analysis based on aerial photographs of the

southwest Portugal indicated that between 1958 and 1987 the area of cork oak distribution remained stable, though there was a noteworthy reduction in their density (Carvalho et al. 1992). No holm oak (*Quercus rotundifolia*) mortality was referred during that period (Carvalho 1993). During the 1980s, there was another mortality outbreak in the Iberian Peninsula, increasing its severity by the end of the decade, and this time affecting both cork and holm oaks (Brasier 1992a, 1993; Cobos et al. 1992; CAMA 2001; Moreira 2001). For example, in Portugal, between 1990 and 1992, there was a substantial increase in the defoliation level of cork and holm oak trees and authorization to land owners for cutting down dead or injured cork trees increased about 70 % (DGRF 2007; Sousa et al. 2007). In France and Italy, cork and holm oak mortality was perceived after 1989 (DFCI 1991; Ruiu 2006). Following this outbreak, mortality in south Portugal and Spain was investigated with regard to the possible presence of the fungus causing North American oak wilt (*Ceratocystis fagacearum*, Brasier et al. 1993); these authors found no evidence of this disease, however, observations of decline symptoms and its distribution in the field suggested a root disease caused by a soil and waterborne oomycete organism. Affected trees were found to have undergone loss of fine feeder roots, and some showed extensive lesions on major roots. Brasier and collaborators isolated the oomycete *Phytophthora cinnamomi* in six out of the nine surveyed declining sites in Spain and suggested that the pathogen was a major factor in the rapid oak mortality in both Spain and Portugal (Brasier 1992a, 1993). Following Brasier et al. (1993) survey, several others' prospections were carried out in declining stands in Portugal, Spain and France, where *P. cinnamomi* was isolated from the rhizosphere with relative success (Cobos et al. 1992; Robin et al. 1998; Gallego et al. 1999; Moreira 2001; Sánchez et al. 2002). Several other pathogens and pests have been associated with cork and holm oak decline, varying in their aggressiveness to the trees

(Macara 1974, 1975; Ferreira & Ferreira 1989; Luque & Girbal 1989; Gallego et al. 1999; Riziero et al. 2002; Sicoli et al. 2002; Sánchez et al. 2003b; Santos 2003; Jiménez et al. 2005; Machado 2005; Romero et al. 2007; Sousa et al. 2007; Corcobado et al. 2010, Torres-Vila et al. 2012). Although their involvement in tree mortality may be locally relevant, with emphasis for *Botryosphaeria spp.* in Catalonia, Spain (Luque & Girbal 1989), only *P. cinnamomi* was associated with the overall mortality outbreaks occurring in South Europe since the 1980s (Brasier 1992a; Cobos et al. 1992; Robin et al. 1998; Moreira 2001;Sánchez et al. 2002). In North Africa, a serious decline in cork and holm oak stands has also been reported, however, to our knowledge *P. cinnamomi* was not recovered in any of the surveyed stands, and loss of vitality appears to be associated with climate, other diseases, pests and human intervention (Ben Jamaâ et al. 2002; Bouhraoua et al. 2002; Chakali et al. 2002; Benia et al. 2005; Bouhraoua & Villemant 2005; Hasnaoui et al. 2005; Assali & Falca 2007; Habib 2007; Sid Ahmed 2007; Ben Jamaâ & Piazzetta 2010; Ferka-Zazou et al. 2010; Ghaioule et al. 2010; Khouja et al. 2010; Linaldeddu et al. 2010; Mannai et al. 2010).

2.3 - Symptoms of cork and holm oak decline

Two main types of syndromes associated with decline have been observed (Cobos et al. 1992; Tuset et al. 1996; Gallego et al. 1999; CAMA 2001; Moreira 2001; Ruiu 2006; Sousa et al. 2007):

1) a sudden death of the tree, characterized by the fast drying of the crown followed by tree death in one or two seasons, particularly in early summer after the winter rains and

in early autumn following the dry season; yellow or brown leaves may remain attached to the tree for some time (fig. 2.2 a).

2) a progressive decline and gradual loss of foliage, where the first symptoms are drying of the top of the tree, sprouting of epicormic shoots, a more intense leaf drop which may affect the whole crown or only some branches (fig. 2.2 b).

Affected trees occur either in groups of variable size within a forest that appears to be healthy, or dispersed throughout the forest (Cobos et al. 1992; Gallego et al. 1999). Observation of the root system showed many dead fine roots, even in trees with low defoliation levels (Moreira 2001), and particularly in affected trees in moister soils (Brasier et al. 1993). Other symptoms not so frequent are tarry exudations on trunks and inner bark lesions or cracks in the stem bark and low branches (Brasier et al. 1993; Gallego et al. 1999; Sánchez et al. 2003a). Robin et al. (1998) observed bleeding cankers at the base of some cork oak trees not severely declining and *Phytophthora spp.* were recovered with a high frequency from canker tissue samples. Other recovered pathogens from canker or exudations in upper branches were *Brenneria quercina* and *Hypoxylon sp*. (CAMA 2001). Sánchez et al. (2003a) refer that decline symptoms are very unspecific. Chlorosis and wilting, defoliation, branch lesions, the absence of feeder roots can be ascribed to drought, insect defoliators and pathogens like *Botryosphaeria spp.* (anamorph: *Diplodia ssp*) or *Biscogniauxia mediterranea* (de Not) Kuntze (syn. *Hypoxylon mediterraneum* (Gallego et al. 1999; CAMA 2001; Santos 2003; Machado 2005; Franceschini 2007).

Figure 2.2: Symptoms of decline in cork oaks (*Quercus suber*) at Herdade da Machoqueira do Grou, Coruche, Portugal

A) Sudden Death; B) Chronic decline

CHAPTER 3

Decline of Mediterranean oak trees and its association with *Phytophthora cinnamomi*: Analysis of the bibliography

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3.1- ABSTRACT

Mortality events in cork and holm oaks have occurred in the Mediterranean basin since the beginning of the XX century, but severity of decline increased during the 1980s. By that time, the exotic soil borne pathogen *Phytophthora cinnamomi* was often recovered from declining stands and since then it has been considered the main factor associated with decline. This work analyses data concerning *P. cinnamomi* surveys in cork and holm oaks trees, pathogenicity tests carried out in controlled experiments, studies about the influence of site characteristics in tree decline and approaches to control the disease. Results of field surveys showed that the pathogen is widespread and pathogenicity tests suggested that host susceptibility to the pathogen is moderate when seedlings are in appropriate watering conditions, particularly cork oaks. Occurrence of decline is also associated with soil characteristics that interfere with root expansion and water retention. We assessed the relative importance of each factor involved in decline and revised the role of *P. cinnamomi* in cork and holm oak decline.

3.2- AIM OF THE PAPER

Although *P. cinnamomi* isolation was frequently recovered from declining sites in some studies (Brasier et al. 1993; Sánchez et al. 2002, 2003a; Romero et al. 2007), in other studies, pathogen detection was not so successful (Cobos et al. 1992; Robin et al. 1998; Moreira and Martins 2005). Moreover, pathogenic tests with seedlings in controlled conditions indicated that *P. cinnamomi* is only a moderate pathogen of holm

and cork oak seedlings, especially concerning cork oaks (Robin et al. 1998; Moreira et al. 2000; Robin et al. 2001; Sánchez et al. 2005). It was suggested that tree mortality was occasioned after an interaction of pathogen attack with abiotic factors, with special relevance to drought events (e.g., Brasier et al. 1993; Robin et al. 1998; Gallego et al. 1999).

The aim of this review is to analyze the strength of the association between *P. cinnamomi* occurrence and cork and holm oak decline. To achieve this main objective, we examined all field surveys and pathogenicity tests that were published, as well as studies about the relation between cork and holm oak decline and other factors than *P. cinnamomi*. The specific goals were:

1) To detect which factors are more associated with cork and holm oak decline,

2) To analyze possible interactions between the pathogen and abiotic factors and

3) To classify the role of *P. cinnamomi* in cork and holm oak mortality in South Europe.

3.3 ANALYSIS OF THE BIBLIOGRAPHY

3.3.1- Relationship between *P. cinnamomi* distribution and health status of cork and holm trees

Several field surveys were carried out in order to study the relationship between *P. cinnamomi* and cork and holm oak decline (Cobos et al. 1992; Brasier et al. 1993;

Robin et al. 1998; Gallego et al. 1999; Moreira 2001; Sánchez et al. 2002), however, these prospections only focused on declining sites where tree mortality was occurring. Plant resistance to attack by *Phytophthora spp.* may depend on the physiologic status of the host (Duniway 1983); therefore, it is possible that in declining stands some other conditions were significantly predisposing trees to attack by pathogens. Tree death often represents an arbitrary point on a continuum process with multiple contributors where the proximate causes of death (e.g., an insect or disease) may be a secondary factor, whereas the primary one (e.g., starvation) may not be obvious (Franklin et al. 1987). For this reason, the presence of a pathogen in declining trees is not sufficient to indicate causality since it may be a consequence of alterations in host resistance due to other stress factors. For example, *Biscogniauxia mediterranea*, the causal agent of the charcoal disease, is closely associated with cork oak declining stands; however, it was recurrently recovered in both declining and asymptomatic cork oak trees in north Sardinia (Franceschini et al. 2002). These fungal populations are endophytic and remain latent in healthy tissues, developing upon decrease in host defenses caused by unfavorable conditions (Franceschini et al. 2002; Santos 2003). To analyze an association between a pathogen distribution and a disease incidence, surveys should be carried out in both declining and healthy sites. Few *P. cinnamomi* prospections on healthy *montado*s have been published. In Portugal, an extensive survey covering 56 healthy and declining *montado*s, distributed throughout the country, showed a positive relation between the tree crown defoliation and the occurrence of *P. cinnamomi* in Algarve region, whereas no relationship was found in the other regions (Moreira 2001; Moreira and Martins 2005). This significant result was only possible after analyzing separately not only regions, but also the source of *P. cinnamomi* isolations: plant roots or soil rhizosphere of each selected tree. Thus, in Algarve region, trees with low

defoliation level showed a lower frequency of *P. cinnamomi* in roots and higher frequency of the pathogen in the rhizosphere than trees with high defoliation level. A similar trend was found in another study carried out in Cáceres region (Spain), where Vivas et al. (2009) found a positive relation between *P. cinnamomi* isolation from roots and crown decline symptoms of holm oak trees. However, this pattern is not consistently observed in cork oaks. In a survey carried out in Alentejo region (Portugal), the pathogen was recovered more often from root tissues of trees found in stands with average crown defoliation level lower than 25 % (Moreira et al. 2005); in this study, the pathogen was detected in almost all the *montado*s surveyed, however, positive isolation from roots was infrequent in *montado*s with higher crown defoliation. Possible explanations for this trend are described below. The study of relationship between *P. cinnamomi* detection and oak canopy status raises several questions concerning:

(A) Pathogen isolation.

(B) Time delay between infection and manifestation of above-ground symptoms.

(C) Quantification of disease symptoms.

(D) Use of different units in statistical analysis.

(E) Host species (fig. 3.1).

A) Pathogen isolation:

Although negative results are usually attributed to low soil moisture at the time of the sample collection, Robin et al. (1998) and Sánchez et al. (2003a) observed that isolation success was not significantly correlated with soil moisture or rainfall, and positive isolations were obtained in soils with relative soil water content as low as 6 %. The environmental factor associated with isolations success was the minimum

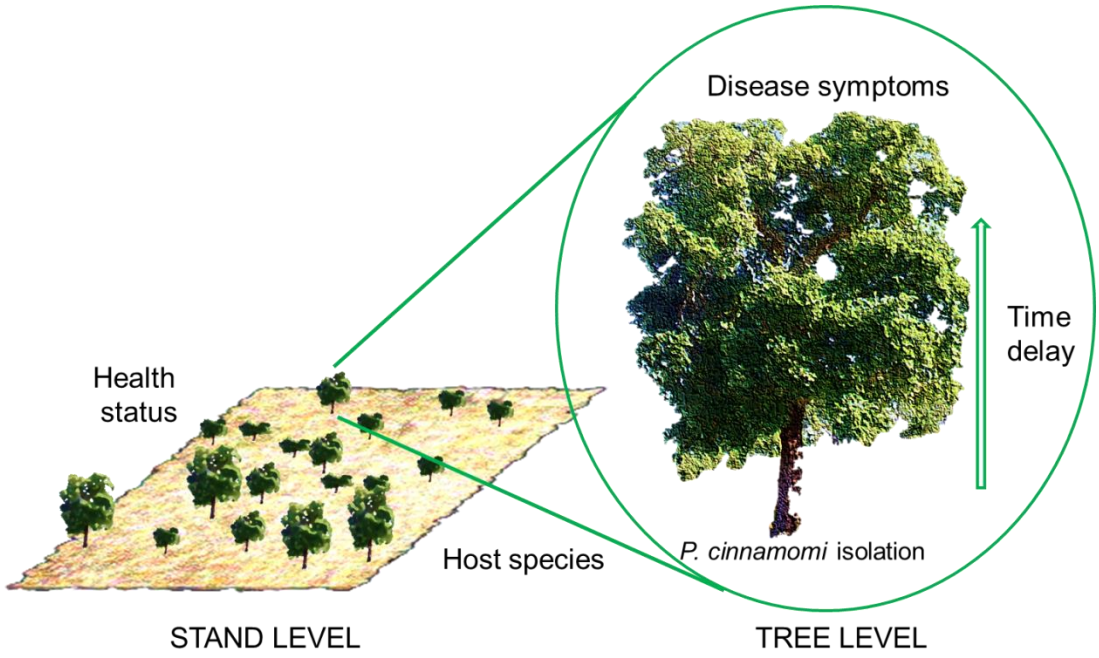


Figure 3.1 Scheme of the critical steps on the studies concerning the relationship between *Phytophthora cinnamomi* distribution and health status of cork and holm trees

temperature recorded in the 5-week preceding isolation attempts (Sánchez et al. 2003a). *P. cinnamomi* is a moderate temperature species and minimum temperature for growth is approximately 10ºC, with a few isolates being able to grow at 5ºC; free water is required for *P. cinnamomi* release of zoospores from sporangia and subsequent dispersal through the soil, however, water is not essential for production of chlamydospores and oospores, and for direct germination of the sporangia (Zentmyer 1980). *P. cinnamomi* is able to grow and reproduce in slightly drier conditions than other *Phytophthora spp.* (Weste 1983). Negative results in pathogen isolation may be instead due to sub-optimized isolation methods. Usually a combination of baiting and selective medium is preferred; however, this procedure has several critical steps that should be adjusted, otherwise, isolation is just a matter of luck. For example, the

amount of soil analyzed for *P. cinnamomi* detection mentioned in almost all the studies referred in Table 3.1 is about 10 g per sample, whereas it should be taken about 3–5 soil monoliths (20 x 30 x 30 cm) for analysis to increase the probability of obtaining sufficient inoculum for bait infection (Jung et al. 2000; Jung 2011). Several other precautions are required in order to avoid *Pythium spp.* contamination, a faster growing oomycete that inhibits *Phytophthora spp.* isolations. On the other hand, isolation success is also related to the material source. Authors usually use soil samples from the rhizosphere and fine feeder roots for *P. cinnamomi* isolation attempts and, to a lesser extent, bark tissue. Positive isolations are more frequent when using soil samples instead of root samples, since roots infected primarily by *Phytophthora spp.* are latter invaded by other opportunistic pathogens (Jung 2011). However, the presence of the pathogen in the rhizosphere confirms that the pathogen is active but it does not present an unequivocal evidence of root infection. Some authors developed other methods than the combination of baiting and selective medium for *P. cinnamomi* detection from the soil or infected plants. ELISA based kits for *Phytophthora spp.* are not species specific and may show cross reactivity with some species of Pythium (O'brien et al. 2009), but new developed molecular techniques appear to be sensitive and species specific, thought it requires specialized equipment (Cooke et al. 2007; O'Brien et al. 2009; Williams et al. 2009; Langrell et al. 2011).

Table 3.1: Occurrence of *Phytophthora cinnamomi* in *Quercus suber* and *Q. rotundifolia* recovered by the baiting method and selective medium

A) *Q. suber*, *Q. rotundifolia*

Unit	Date	Region	Health status criteria	Sample type	Total stands	Total trees	Healthy Units		Declining Units		Ref.
							positive for Pc	negative for Pc	positive for Pc	Negative for Pc	
Tree	Jun, Nov 95 Jun 96	SE France	ACD of 4 trees	Roots, soil, bark	24	96			28 (29%)	68 (71%)	G
	Sept. Au 95-98	C Portugal	Crown defoliation	Roots	20	65	0 (0%)[c]	4 (100%)[c]	7 (11%)[d]	54 (89%)[d]	H
	Sept. Au. 95-98	C Portugal	Crown defoliation	Soil	20	65	0 (0%)[c]	4 (100%)[c]	3 (5%)[d]	58 (95%)[d]	H
	Sept. Au. 95-98	S Portugal	Crown defoliation	Roots	30	192	3 (14%)[c]	19 (86%)[c]	29 (17%)[d]	141 (83%)[d]	H
	Sept. Au. 95-98	S Portugal	Crown defoliation	Soil	30	192	4 (18%)[c]	18 (82%)[c]	21 (12%)[d]	149 (88%)[d]	H
Stand	May, Nov 91 March 92	SW Spain, S Portugal	Decline symptoms [a]	Roots, soil	9	22			6 (67%)	3 (33%)	B
	Jun, Nov 95 Jun 96	SE France	ACD of 4 trees	Roots, soil, bark	24	96			7 (29%)	17 (71%)	G
	Spring 00	SW Spain	ACD in 4 ha	Soil	8	196			6 (75%)	2 (25%)	I
	autumn 91 to spring 92	W Spain	Stands with mortality	Roots, soil	9	21			3 (33%)	6 (67%)	D
	spring 99 to spring 00	W Spain	Stands with mortality	Roots, soil	6	3 -5 per stand			4 (67%)	2 (33%)	D

B) ***Q. rotundifolia***

Unit	Date	Region	Health status criteria	Sample type	Total stands	Total trees	Healthy Units		Declining Units		Ref.
							positive for Pc	negative for Pc	positive for Pc	Negative for Pc	
Tree	Sept to Dec 91	SW Spain	Decline symptoms [a]	Roots, soil	53	162			10 (6%)	152 (94%)	A
	May, Nov 91,March 92	SW Spain, S Portugal	Decline symptoms [a]	Roots, soil	5	9			3 (33%)	6 (67%)	B
	Jan 98	SW Spain	Crown defoliation	Soil	3	28			20 (71%)	8 (29%)	E
	Jan 98	SW Spain	Crown defoliation	Roots	3	28			27 (96%)	1 (4%)	E
	Dec 99	W Spain	Crown defoliation	Soil	1	25			19 (76%)	6 (24%)	F
Stand	2003-2005	SW Iberia	Declining stands[b]	Roots, soil	70	140			30 (43%)	40 (57%)	C
	Sept to Dec 91	SW Spain	Stands with mortality	Roots, soil	53	162			9 (17%)	44 (83%)	A
	autumn 91 to spring 92	W Spain	Stands with mortality	Roots, soil	21	63			9 (43%)	12 (57%)	D
	spring 99 to spring 00	W Spain	Stands with mortality	Roots, soil	17	3 -5 per stand			3 (18%)	14 (82%)	D

A) ***Q. suber***

Unit	Date	Region	Health status criteria	Sample type	Total stands	Total trees	Healthy Units		Declining Units		Ref.
							positive for Pc	negative for Pc	positive for Pc	Negative for Pc	
Tree	Sept to Dec 91	SW Spain	Decline symptoms [a]	Roots, soil	30	78			20 (26%)	58 (74%)	A
	May, Nov 91 March 92	SW Spain, S Portugal	Decline symptoms [a]	Roots, soil	5	13			5 (38%)	8 (62%)	B
Stand	2003-2005	SW Iberia	Declining stands[b]	Roots, soil	31	62			19 (61%)	12 (39%)	C
	Sept to Dec 91	SW Spain	Stands with mortality	Roots, soil	30	78			14 (47%)	16 (53%)	A
	spring 99 to spring 00	W Spain	Stands with mortality	Roots, soil	4	3 -5 per stand			0 (0%)	4 (100%)	D

Pc: *P. cinnamomi*, ACD: Average crown defoliation, Sp: Spring, Au: Autumn; A: Cobos et al. 1992; B: Brasier et al. 1993; C: Romero et al. 2007; D: Molina et al. 2003; E: Sánchez et al. 2002; F: Molina et al. 2005; G: Robin et al. 1998; H: Moreira & Martins 2005; I: Sánchez et al. 2003a.

[a] Crown defoliation, exudations, branch dieback

[b] Non-specified

[c] trees with defoliation inferior to 10%

[d] trees with defoliation superior to 10%

B) Time delay between infection and above-ground symptoms:

Root pruning precedes crown dieback since a tree can tolerate a great loss of its rootlets or feeder roots without showing visible above-ground symptoms (Tsao 1990). Tests showed that sweet chestnut seedlings, a highly susceptible species to *P. cinnamomi,* tolerate a loss of 90 % of the rootlets before exhibiting alterations in water status as measured through plant hydraulic conductance and leaf water potential (Maurel et al. 2001a). This indicates that expression of above-ground symptoms might be a quantitative rather than a qualitative problem affecting the root system (Jung et al. 1996). On the contrary, failure in detection of *P. cinnamomi* in the rhizosphere of declining plants is not unusual, because of the decrease in the fungal population due to antagonism and interference of fastgrowing- associated secondary microflora (Tsao 1983).

C) Quantification of disease symptoms:

In studies concerning oak decline, some authors evaluate aboveground symptoms as a visual and subjective measure of the percentage of crown defoliation (e.g., Jung et al. 2000; Sánchez et al. 2002; Vettraino et al. 2002; Jönsson et al. 2003; Sánchez et al. 2003a; Moreira & Martins 2005). Although cork oaks are considered evergreen trees, they have short-lived foliage and a late flushing pattern; average leaf longevity is about 12 months whereas holm oak leaves last 1–3 years, and both leaf shedding and leaf birth occur during spring (Escudero et al. 1992; Sá et al. 2005; Caritat et al. 2006). Overlapping between different leaf cohorts is very low, and leaves should

be classified as overwinter, rather than true perennial (Mediavilla & Escudero 2008). Therefore, caution should be exercised while taking measurements of cork oak crown defoliation during spring. Moreover, cork oak has been described as an extremely polymorphous species with many overlapping morphological attributes, mainly distinguishable by certain traits of the leaves, fruits, and cupules (Natividade 1950; Coelho et al. 2006b). Thus, density of the canopy may be influenced by factors other than health status, like phenological variability, effect of tree competition (fig. 3.2) or artificial pruning. Some authors considered additional criteria to infer on the tree health status, like dieback of the tip of branches (e.g., Hansen & Delatour 1999; Balcì & Halmschlager 2003). Oak trees undergo self-pruning of lower branches under the shade, but dieback of high branches is a reliable symptom of stress. However, after the collapse of dead branches, trees may present enough vigor to be considered asymptomatic, rendering unreliable evaluation of their health status (Ribeiro 2006). Nevertheless, dieback or lower leaf density in the upper part of the canopy can be related with water stress.

D) Use of different units in statistical analysis:

In studies of the association between *P. cinnamomi* distribution and oak decline, researchers analyzed data at tree level or at stand level. Usually, at tree level, the independent factor is the presence of the pathogen in the tree rhizosphere and the dependent factor is the degree of the tree crown defoliation, whereas at stand level, a set of trees are analyzed; the stand is positive for the pathogen if at least one soil or root sample yields the pathogen and decline symptom is calculated as average tree crown defoliation of part or of all the trees from the set (Table 3.1). Analyses at tree level may

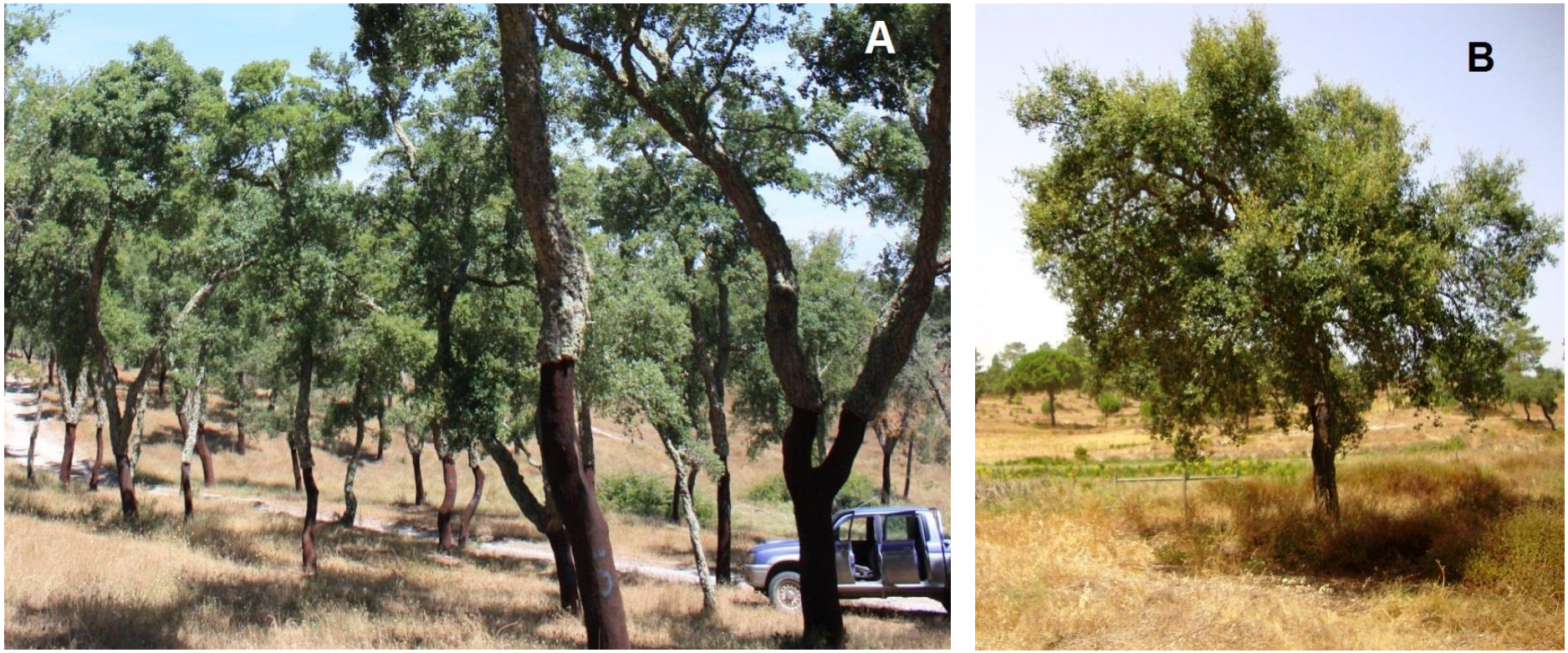


Figure 3.2: Asymptomatic trees with different canopy shape associated to intra-competition for light
A) with intra-competition; B) no competition

be hampered by eventual difficulties when isolating the pathogen or when evaluating disease symptoms, and by time delay between infection and aboveground symptoms, but at stand level one can use average values, thus avoiding great variation of data. Moreover, studies based on data obtained from nearby trees often display spatial autocorrelation, in that locations close to each other exhibit more similar values of independent factors than those further apart, increasing the chance of a type I error (incorrect rejection of a null hypothesis, Legendre 1993). On the other hand, analyses at stand level pose some subjectivity in relation to the methodology applied to select the area of the stand units and to calculate its health status, where different authors use their own criteria (Table 3.1). Tomé (2007) demonstrated that different criteria to infer the health status of the stands lead to different results and highlighted the importance in the implementation of standard and systematic methodology. In addition, stand units should be as homogeneous as possible, at least in relation to topographic characteristics that may influence *P. cinnamomi* distribution, like slope and orientation (Moreira and Martins 2005). Additionally, the absence of the pathogen in a stand should be based on more than two samples analyzed for *P. cinnamomi* detection; otherwise, the number of negative locations would be overestimated (Pryce et al. 2002).

E) Host species:

Several studies encompassed cork and holm oaks and both species are usually analyzed together (Brasier et al. 1993; Molina et al. 2003; Sánchez et al. 2003a; Moreira and Martins 2005). However, pathogenicity tests reveal that they exhibit differential susceptibility to *P. cinnamomi*, holm oak being more susceptible than cork oak (Maurel

et al. 2001b; Moreira 2001). Thus, pooling both species in the same analysis may lead to inaccurate results.

Different methods for classifying the health status of the trees, the use of different units and problems in *P. cinnamomi* detection pose difficulties in evaluation studies. Declining stands positive for *P. cinnamomi* varied between 0 % (cork oak *montado*s) and 61 % (cork oak *montado*s; Table 3.1). At tree level, symptomatic trees positive for *P. cinnamomi* also showed great variation, from 6 to 96 % (both extreme values observed in holm oak *montado*s). In two studies where both asymptomatic and symptomatic trees were surveyed, there was no strong relation between the presence of the pathogen and decline symptoms (Table 3.1), contrarily to other susceptible hosts like sweet chestnut (Vettraino et al. 2005) and Fraser fir (*Abies fraseri*; Griffin et al. 2009) as well as with susceptible hosts to other *Phytophthora spp.* (Hansen 1999; Jung et al. 2000; Balcì and Halmschlager 2003; Jönsson et al. 2005) where the pathogens were frequently isolated from declining stands and/or trees and less frequently from healthy ones. For example, *P. cinnamomi* was isolated in 96 % of declining sweet chestnut stands and only in 21 % of asymptomatic ones (Vettraino et al. 2005). *P. quercina* was isolated in 63 % of declining pedunculate oak (*Q. robur*) and sessile oak (*Q. petraea*) trees and only 23 % in asymptomatic trees (Jung et al. 2000). This result was consistent, irrespective of the unity level, evaluation methods for health status estimation and inherent difficulties in *Phytophthora* isolations. In USA, little leaf disease in shortleaf pine (*Pinus echinata*) affects 1/3 of the stands; *P. cinnamomi* seemed to be associated with both healthy as well as affected stands; however, careful, quantitative surveys showed that not only declining stands were infected in higher number than healthy ones, but also declining trees were, on average, more infected than

nearby healthy trees in affected stands (Hansen 1999). Although *P. cinnamomi* has been isolated from declining cork and holm oak stands, its prospection in healthy stands using an adequate method is essential to evaluate the status of the stands or of the trees. At stand level, units should be uniform concerning topographic characteristics and an index of mortality relating dead (or highly damaged) trees with the total number of trees should be used to evaluate health status. At tree level, preference should be given not only to crown defoliation, but also to dieback of branch tips and spatial autocorrelation should be considered.

3.3.2- Pathogenicity of *P. cinnamomi* in cork and holm oak seedlings

Along with surveys in declining stands, experimental host inoculations with *P. cinnamomi* were carried out in nurseries under controlled conditions using 6 months up to 2 years old seedlings (Tuset et al. 1996; Robin et al. 1998, 2001; Gallego et al. 1999; Moreira et al. 2000; Maurel et al. 2001b; Sánchez et al. 2002; Tapias et al. 2008a). The most evident result from these studies is the finding in differential susceptibility between holm and cork oak seedlings to infection. In the studies where both cork and holm oak seedlings were tested, the latter always showed more symptoms and mortality rates than cork oak seedlings (Table 3.2). Although both species showed necrosis in tap roots and a reduction in root and in foliar biomass, symptoms were much more severe in holm oak seedlings except in the study conducted by Sánchez et al. (2002). Cork oak mortality was barely observed whereas holm oak mortality occurred in half of the studies and varied between 1 and 67 %. This result is in agreement with histological,

Table 3.2: Morphologic and physiologic differences between *Quercus suber* and *Q. rotundifolia* seedlings inoculated with *Phytophthora cinnamomi* in relation to non-inoculated controls. Experiments were subjected to three different watering regimes. Each line refers to the effect of inoculation in relation to non-inoculated seedlings, according to the respective water regime

Treatment	Duration (months)	Dead plants	Taproot necroses	Foliar sympt	Root BM	Foliar BM	Length	g_s	Ψ	F_v/F_m	N,P K	Reference
Q. suber												
Flooding	1		>	=								Sánchez et al.
	9	0	>		<							Moreira et al.
	3	0 / 10	>	>								Romero et al.
	4	2.5%										Tuset et al. 1996
	5	3/74	>		<			<	<			Robin et al. 2001
Watered[a]	9	0	>		<							Moreira et al.
	6		>	>								Sánchez et al.
	5		>	=								Sánchez et al.
	5							=	=			Robin et al. 2001
	9	0	= (0)		>							Moreira et al.
	5	0	>		<			<	<			Robin et al. 2001
	4	0/135						=	< >			Tapias et al.
	2,5	0/25	✓ [b]						=			Robin et al. 1998
	2	1/ 3					<[c]	<		<		Luque et al. 1999
Drought	5	1/?						=	=			Robin et al. 2001
	9	0	>		=							Moreira et al.
Q. rotundifolia												
Flooding	1		>	=								Sánchez et al.
	9	25%	>		<							Moreira et al.
	4	64.3%										Tuset et al. 1996
	5	67%	>		<			<	<			Robin et al. 2001
Watered[a]	9	0	>		<							Moreira et al.
	6		>	=								Sánchez et al.
	5		>	>								Sánchez et al.
	5							=	=			Robin et al. 2001
	10	1	>		<	<	<	<	=		<N,<P,=K	Maurel et al.
	9	16.7%	>		<							Moreira et al.
	5	20%	>		<			<	<			Robin et al. 2001
	2,5	25%	✓ [b]						<			Robin et al. 1998
Drought	9	0	>		=							Moreira et al.
	10	1	>		<	=	=	=	>		<N,<P,=K	Maurel et al.
	5	6/32						=	=			Robin et al. 2001
F/D	8	8/18	>	>			<					Gallego et al.

Seedlings were 6 months to 2 years old. All *P. cinnamomi* inoculations were applied in the substrate, except in Luque et al (1999) were the pathogen was applied in a wound made on the host.

>: Significantly more symptoms than controls,

< : Significantly less symptoms than controls,

= No significant differences between control and inoculated plants;

Sympt: symptoms (wilting, yellowing, necrosis); BM: biomass; Length: Seedling length; g_s: Stomatal conductance; Ψ: Predawn stem water potential; F_v/F_m: Photochemical efficiency; LHC: Hydraulic conductance; N P K: Leaf nitrogen, phosphorus and potassium; F/D: Alternating flooding and drought

[a] According to soil type and plant needs

[b] non-specified symptoms

[c] Diameter of the stem

[d] There was no effect on Ψ until 92 days after inoculation

studies showing that *P. cinnamomi* is able to invade vascular cylinder in newly emerged plants of both species, however, progress is more rapid and severe in the holm root cortical parenchyma than in cork oak (Moreira 2001; Pires et al. 2008). However, comparatively to other susceptible species, like the sweet chestnut, holm oaks exhibit more tolerance; in a comparative study, all the sweet chestnut seedlings died compared with 10 % of the holm oak seedlings (Maurel et al. 2001b). In most of the experiments with seedlings in appropriate watering conditions (usually field capacity), there was only slight or even no root or leaf symptoms (Moreira et al. 2000; Maurel et al. 2001b; Sánchez et al. 2002) and no physiological alterations related to transpiration and photosynthesis (Tapias et al. 2008a). Furthermore, inoculated seedlings even presented better performances than the controls in some experiments. For example, inoculated holm oaks plants had better water use efficiency (Maurel et al. 2001b) and cork oak plants had better hydraulic conductance and photochemical efficiency (Tapias et al. 2008a) and showed higher root biomass than controls as a response to infection by *P. cinnamomi* (Moreira 2001). In relation to leaf water potential and stomatal conductance, results were contradictory; Tapias et al. (2008a) observed that the decrease in cork oak leaf water potential was not accompanied by changes in stomatal conductance, whereas Robin et al. (2001) and Maurel et al. (2001a, b) observed marked decrease in stomatal conductance of cork and holm oaks even at high values of water potential. Causality observed in these physiologic parameters may have different implications in the mechanism of infection. Decrease in stomatal conductance associated with leaf water potential is probably related to hydraulic signals acting in the stomata but decrease in stomatal conductance independent of leaf water potential may be related to non-hydraulic signals like an increase in abscisic acid concentrations, associated with root pruning caused by *P. cinnamomi* infection (or drought), as it was found for the

susceptible sweet chestnut (Maurel et al. 2004). In relation to interaction between water regime and *P. cinnamomi* infection, it was observed that both waterlogging and water shortage altered the host symptomatology to infection. Waterlogging treatment resulted in more mortality, more necrosis and less root and foliar biomass for both cork and holm oaks compared to controls. A global data analysis shows a synergistic effect between excess water and infection by *P. cinnamomi* on the severity of the disease (Moreira et al. 2000; Robin et al. 2001; Sánchez et al. 2002), since waterlogging by itself did not cause major symptoms in the seedlings. Waterlogging caused some root necrosis (Moreira et al. 2000) and root weight losses (Robin et al. 2001), but waterlogging combined with *P. cinnamomi* increased disease symptoms exponentially and was related to major mortality. It is considered that waterlogging increases the severity of diseases caused by root pathogens, primarily by adversely affecting host physiology while increasing the mobility of the pathogen through the soil (Schoeneweiss 1975; Zentmyer 1980). The observed synergistic effect could be attributed to a strong increase in the pathogen population causing multiple infections on the host (Moreira et al. 2000; Robin et al. 2001; Sánchez et al. 2002) acting together with higher host susceptibility after root hypoxia caused by excess water (Jacobs et al. 1996). This author observed that levels of defense barrier compounds (e.g., polymerized phenols) in cork oak roots changed at near-anoxic oxygen conditions. Contrasting to excess water, the effect of the pathogen was reduced in plants subjected to drought. In cork oaks subjected to water shortage, the pathogen did not affect root biomass (Moreira et al. 2000) and though holm oak root biomass decreased, infection did not alter root collar diameter and aerial biomass (Moreira et al. 2000; Maurel et al. 2001b). Inoculation of both cork and holm oaks subjected to water stress had no effect on stomatal conductance (Maurel et al. 2001b; Robin et al. 2001) and in leaf water

potential (Robin et al. 2001), except in one study where inoculated holm oaks had leaf water potential values as well as plants in good watering conditions (Maurel et al. 2001b). This may happen if stomatal closure following root infection reduces water losses. In relation to midday stem water potential, decreases were only related to water shortage and not to inoculation (Turco et al. 2004). Water stress also limited necrosis length caused by the pathogen when comparing to necrosis length in cork oaks plants subjected to good watering conditions (Luque et al. 2000). When subjected to water shortage, inoculated plants may not suffer from water stress since they already reduced water absorption and water losses as a consequence of *P. cinnamomi* infection (Maurel et al. 2001b). Physiological responses to infection like stomatal closure, better water use and photochemical efficiency, observed in plants infected by *P. cinnamomi* in good watering regimes, may enable trees to tolerate some water stress, at least temporarily. However, when irrigation is reduced long enough to significantly decrease soil moisture, there is an indication that the combination of water stress and infection increases severity symptoms (Moreira et al. 2006). Long-term experiments under water shortage are necessary to understand the relationship between drought and infection. Other studies, concerning germination and survival of newly emerged plants, showed high damping-off in artificially inoculated soils, with holm oaks being more affected; damping-off in naturally inoculated soils was very low and eventually attributed to low inoculum values or to the presence of antagonistic factors (Tapias et al. 2006, 2008b; Moreira 2001). On the contrary, other experiment showed high holm oak damping-off in naturally inoculated soils; however, part of the samples were subjected to alternation of flooding and drought conditions (Gallego et al. 1999) which may affect plant tolerance and pathogen aggressiveness. In relation to open field experiments in soils naturally infested with *P. cinnamomi*, damping-off occurred in 12.3 % of the

germinated cork oak seedlings (Moreira et al. 2007) and in 19.6 % of the planted holm oak seedlings after the first year of experiment (Molina et al. 2005); however, authors considered that not all mortalities could be ascribed to the pathogen. Finally, studies regarding selection of more resistant seedlings detected some differential resistance/tolerance to *P. cinnamomi* infection among new emerging cork and holm oak seedlings from different origins (Moreira et al. 2007; Tapias et al. 2008b). The possibility of using plants more tolerant or resistant to *P. cinnamomi* infection can be an important tool to the reforestation of highly infested areas (Moreira et al. 2007); however, older seedlings from diverse origins had similar physiologic responses to infection (Tapias et al. 2008a). In general, authors considered reactions shown by inoculated oaks very similar to the response usually observed in trees subjected to drought. Both pathogen infection and water stress may reduce root biomass and leaf water potential. Although in some circumstances, major roots and the lower stem may be infected (Shea et al. 1982; Dawson and Weste 1984), it is considered that the main effect of *P. cinnamomi* is the destruction of fine roots; therefore, reducing water absorption capacity and causing water stress symptoms. Exceptions were found on silvertop ash (*Eucalyptus sieberi*), a susceptible host that suffers from water stress when only about 1/6 of the roots are infected; thus failure in water transport cannot be due directly to decay of the root system (Dawson & Weste 1984). Likewise, in jarrah (*E. marginata*), there was a reduction in cytokinins before significant reduction in root tips (Cahill et al. 1986). The authors suggested that changes in the balance between this phyto-hormone and abscisic acid could cause water transport failure and symptoms of drought. In holm and cork oak trees, there are no studies concerning hormonal changes after infection, but there are indications that the water absorption deficit is related to root pruning (Robin et al. 2001). *P. cinnamomi* invades holm oaks roots more rapidly

than cork oak ones, but penetration and intra- and intercellular progression of the pathogen through the cortical parenchyma and vascular cylinder are similar in both species (Pires et al. 2008). Both species respond with accumulation of phenolic compounds close to the hypha, which are not able to prevent root invasion (Pires et al. 2008). However, histological examinations of other resistant species showed that their root tissues were also invaded by the pathogen; nevertheless, those species were able to restrict colonization and necrosis (Cahill et al. 1989; Jang & Tainter 1990). The authors observed deposition of phenolic compounds in infected roots, as well as granulation of the cytoplasm, shrinkage of the protoplast and cell-wall distortion and disruption, regardless the species was considered resistant or susceptible. No specific change has been consistently associated with resistance, though deposition of phenolic compounds, lignification of cell walls and formation of papillae are observed more often in resistant ones (Cahill et al. 1989; Cahill & Weste 1983; Cahill et al. 1993). For example, resistant sweet chestnut hybrids increase production of leaf phenolic compounds after infection, whereas in the susceptible sweet chestnut no difference in leaf phenol content was observed (Dinis et al. 2011). Numerous plant species considered resistant to *P. cinnamomi* exhibit horizontal resistance, opposed to vertical resistance where disease does not occur (Erwin and Ribeiro 1996; Irwin et al. 1995). There are no reports of species being able to block pathogen ingress. It is thought that field-resistant plants are able to restrict colonization, sealing the lesions off by the periderm and shedding them (Tippett et al. 1985; Irwin et al. 1995; Cahill et al. 2008). When an infected plant can prevent further spread of the pathogen determines the severity of infection (Cahill et al. 2008). In conclusion, the pathogenicity tests indicate that holm and cork oak seedlings present some susceptibility to *P. cinnamomi* infection, particularly in conditions of excess water, with holm oaks being more susceptible. Both cork and holm oaks have

limited capacity in preventing *P. cinnamomi* progression, particularly in new root tissues, but in appropriate watering and nutritional conditions, infected cork oak seedlings may replace necrotic roots (Moreira 2001), thus avoiding water stress caused by the reduction in water absorption following root destruction.

3.3.3- Relationship between cork and holm oak mortality and site characteristics

Cork oak mortality events have been usually empirically ascribed to complexes involving abiotic stress factors related to soil properties, particularly hydromorphic and shallow soils, and drought, inadequate silvicultural practices and secondary attacks by insects and fungi (Natividade 1958; Cabral et al. 1992; Diniz 1994). Studies attempting to statistically relate abiotic factors and mortality are shown in Table 3.3. Results varied from region to region. Since trees are subjected to several local abiotic factors that interact between them, the relative effect of each one depends on that of the others. Thus, a negative effect in one region may be positive or neutral in another. For example, the presence of understory is associated with increase in mortality of cork oak trees in SW Portugal (Costa et al. 2010), but in Sardinia, unshrubed stands do not affect trees vitality (Ruiu et al. 2005a). Diniz (1994) and Cabral et al. (1992) observed that the shrub gum rockrose (*Cistus ladanifer*), present in some severely affected areas, may compete for limiting water sources in shallow and sun-exposed soils. In areas with no water limitations or with other shrub species, competition may be absent. Moreover, shrub clearing may alter soil properties, exposing them to sunlight, temperature oscillations, erosion and lixiviation, which may increase tree mortality (Macara 1975).

Table 3.3: Abiotic factors associated with cork oaks (*Quercus suber*) and holm oaks (*Q. rotundifolia*) decline.

			Abiotic factors														
			Stand					Soil									
Species	**Scale**	**Region**	**Health status criteria**	**Tree age**	**Cultural practices**	**Struture**	**Orien tation**	**Texture or type**	**Depth**	**Topo graphy**	**OM**	**P**	**N**	**K_2O**	**Perm**	**SC**	**Reference**
Q. suber																	
	Tree	N Portugal	Crown defoliation	-						Declive (-)	+	-				+	Martins et al 2006
		S Portugal	Crown defoliation								+	-		-		+	Martins et al 2006
		C Portugal	Dead/ alive	+[b]		NS[d]	NS		-	Slope: NS							Ribeiro 2006
	Stand	SW Portugal	% defoliation					Clay (+) Silt (-)	-			-	-	-			Bernardo et al 1992
		Sardinia Italy	Average defoliation	NS	Pasture > Shrub clearing	NS[d]	NS	NS[i]							NS		Ruiu et al 2005a
		Portugal	% of dead Trees					✓	-	Usually< in plains							Ribeiro & Surový 2007
		SW Portugal	5 dead trees / ha			Shrub.> Agrosil.> Wood.[e]	South (+)	Leptosols > Luvisols > Arenosol	-								Costa et al 2010
Q. rotundifolia																	
	Tree	W Spain	Crown defoliation					Gravel > Sand =Clay > Silt							+	-	Solla et al 2009
		W Spain	30% wilted					Clay NS Sand NS					-			+	Vivas et al 2009[g]
		W Spain	Symptomatic: 30% wilted					Clay >Sand					=			+	Vivas et al 2009[h]
	Stand	Sardinia Italy	Average defoliation	NS	NS[c]	NS[d]	NS	NS[f]							NS		Ruiu et al 2005b
Q.suber Q. rotundifolia																	
	Stand	W Spain	Symptomatic trees[a]	-	Cork harvest > Pruning > No activity = Harrowing= Shrub clearing			Schist > Rock > Clay= Sand		Valleys> Slopes > Plains							Perez 1993

OM: Organic matter; K_2O: Potassium oxide; P: Phosphorus; N: Nitrogen; Perm: Permeability; CEC: Cation exchange capacity; SC: Soil compaction; C Port: Center Portugal; (+) Positively related with mortality; (-) Negatively related with mortality; NS: Non-significant; >: Category more associated to decline than the next one; =: Both categories equally associated to decline;

[a] Symptoms non specified

[b] Related to tree size

[c] Pasture, shrub clearing

[d] Density

[e] Shrubland, agro-silvo-pastoral system, woodland

[f] Substrates: basalt, trachyte, granite, schist, sedimentary

[g] Analysis made in trees located in slopes

[h] Analysis made in trees located in streams

As a consequence, the effect of the understory in tree vitality depends on plant species that are involved and on water availability, which, in turn, may depend on other stand characteristics like orientation or topography. Additionally the effect of shrub clearing in tree vitality depends also on the method applied. Shrub removal with soil mobilization causes disturbance in the root system of the trees and may increase tree vulnerability to adverse conditions. Concerning orientation, it was reported higher mortality values in south facing slopes (Costa et al. 2010; Moreira & Martins 2005; Brasier 1996; Cabral et al. 1992) but in some studies this pattern was not observed (Table 3.3). It is expected that plants growing in south facing slopes are more subjected to drought conditions, though in some regions the absolute humidity values may not be low enough to be reflected in tree vitality and no significant pattern is observed.

Although the relationship between site characteristics and tree decline varied among studies (Table 3.3), we estimated the relative significance of each independent factor in tree decline as the proportion of the number of studies where the factor was significant in relation to all the studies where it was analyzed; we also included the relative significance of *P. cinnamomi* in tree decline, calculated as number of declining stands positive for the pathogen in relation to the total of declining stands (fig. 3.3). Since there are mixed cork and holm oak stands and little information concerning separate species, we analyzed both species together. Cork and holm oaks are moderate susceptible to *P. cinnamomi* and are also affected by the same abiotic factors. Although the strength of association between each factor and the health status of the trees may vary between species, for a general screening, we opt to group both species. It is possible to observe in fig. 3.3 that soil compaction and depth were the characteristics most associated with decline, whereas *P. cinnamomi* was detected in 40 % of declining stand; however, the presence of the pathogen is probably underestimated, since false

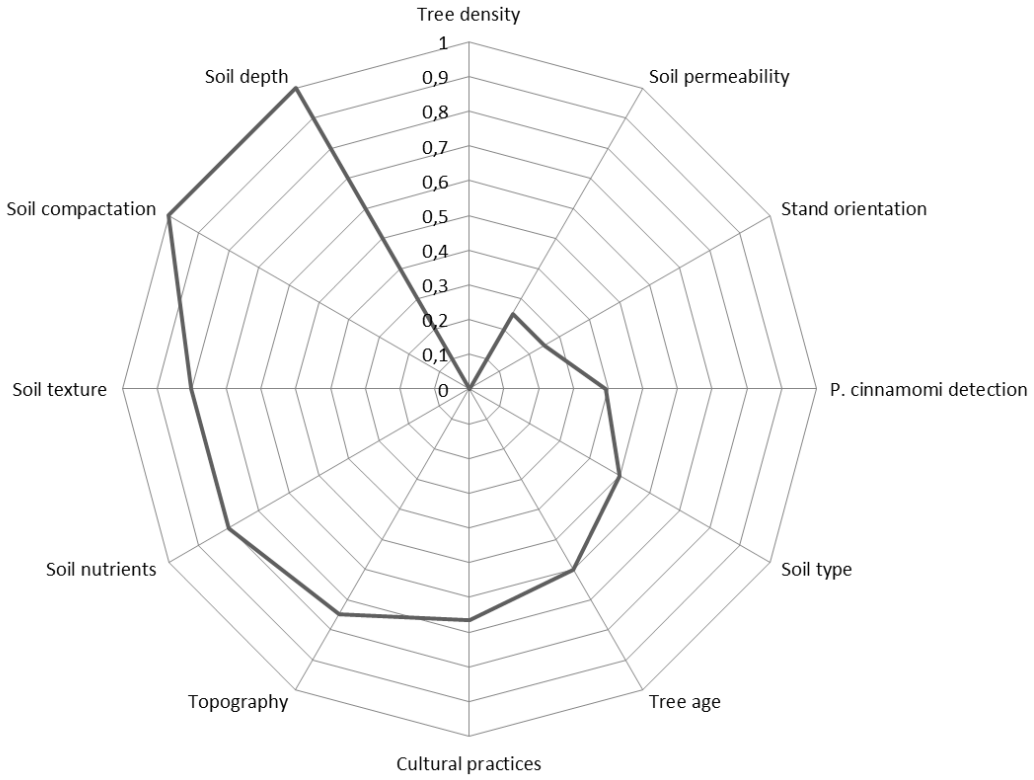


Figure 3.3 Relative significance of each independent factor associated with cork (*Quercus suber*) and holm oak(*Q. rotundifolia*) decline in the studies referred in Tables 3.1 and 3.3

negatives are common when using the baiting method for *Phytophthora spp.* detection (O'Brien et al. 2009). Factors limiting vertical root expansion such as compact or shallow soils may limit root access to deep groundwater tables during the dry season (Otieno et al. 2006) and, on the other hand, expose the roots to disturbances caused by soil management, waterlogging events and root pathogens. As a consequence, weakened and predisposed trees may not be able to regenerate the reduced fine-root capacities and will suffer extreme drought stress during the dry season and/or after drought episodes, as it was observed in beech and in silvertop ash decline (Cahill et al. 2008; Jung 2009).

Reduced soil compaction associated with high percentage of gravel increases soil infiltration capacities favoring holm oaks decline caused by water stress (Solla et al. 2009). Other factors like orientation, topography, soil texture or understory are usually associated with mortality and all affect water availability, either limiting or in excess. Shrub competition, south orientation or soils with high gravel content may reduce water availability to values below the appropriate range, thus imposing drought conditions to the trees. On the other hand, topographic depressions, soils with high clay content and shallow soils have poor drainage, contributing with excess water to root hypoxia, toxicity and tree decline (Bernardo et al. 1992; Natividade 1958; Cabral et al. 1992; Diniz 1994). Nutrient availability was also related to tree decline, though cork and holm oaks are adapted to nutrient-poor soils. Bernardo et al. (1992) observed that soils with deficient internal drainage and with low effective thickness for root expansion have less accessible nutrients. Cork oak trees growing on these stands showed less vitality and their leaves exhibit nutrient imbalanced concentrations. Tests carried out in sandy and schistose soils during four sequential years showed an overall cork oak vitality recovery after fertilization treatments, however, the response was only observed during the first year after the first fertilization (Sousa et al. 2005). Site characteristics may act directly in the health status of the trees but also they affect *P. cinnamomi* survival acting in synergy with the pathogen. In particular, factors influencing soil moisture levels and microbial populations are factors governing the growth, reproduction and inoculum potential of the pathogen (Weste and Marks 1987). Soil compaction was reported to yield more *P. cinnamomi* inoculum (Vivas et al. 2009), though this relation may interact with water condition (Rhoades et al. 2003). Nevertheless, mortality associated with *P. cinnamomi* is usually much more severe in compact or shallow soils (Weste & Marks 1987; Fonseca et al. 2004). South-oriented stands and soils with high percentage of clay

are both favorable to *P. cinnamomi* survival (Moreira & Martins 2005; Vivas et al. 2009) and unfavorable to cork oak vitality.

Pathogenicity test (Table 3.2) indicated a synergism between waterlogging conditions and infection by *P. cinnamomi* and these results should be considered in the studies concerning mortality in the open field. Soils retaining high levels of moisture provide conditions to the increase in *P. cinnamomi* inoculum causing multiple fine root infections and, in addition, negatively affect cork and holm oak roots and the overall health status of the trees. Although pathogen preferences for high levels of moisture, it was preferentially recovered in the driest side of the hills (Moreira & Martins 2005). This pattern was also found in Australia, and it was suggested that those soils may also have a low level of microbial antagonism (Newhook & Podger 1972). Soil dryness inactivates most of the suppressive microorganisms before affecting *P. cinnamomi* (Weste & Marks 1987), which may explain the preferential occurrence of the pathogen in south facing slopes. Furthermore, the occurrence of susceptible species like the shrub *Cistus ladanifer* in sunlight exposed slopes can provide an important basis for inoculum production and survival, thus acting as reservoirs for *P. cinnamomi* (Moreira 2001). Regardless of sites with south orientation yielding high amount of *P. cinnamomi*, the relation between drought and infection by root pathogens in tree decline in those sites is unclear, given that pathogenicity tests showed that the symptoms of the infection are limited when plants are also subjected to moderate water stress (Table 3.2). Supporting this assumption is the result obtained in a *montado* at Extremadura, Spain, where trunk injections with potassium phosphonate, which have been used successfully to control *P. cinnamomi,* had no effects on holm oaks shoot growth and acorn production (Solla et al. 2009). In this study, water stress was more likely to contribute to decline than *P. cinnamomi*. Drought has been considered a factor associated with mortality (Macara

1975; Cabral et al. 1992). In Spain, there was synchronism between exceptionally dry years and holm oak mortality (LLoret a&nd Siscart 1995; Peñuelas et al. 2001; Sánchez & Garcia 2007) and recovery occurred after long periods of rain (Tuset & Sánchez 2004). Cabral & Lopes (1992) also refer to a synchrony between atypical dry years (1943–1945, 1975–1976, 1980–1993) in Portugal and cork oak mortality events referred in bibliography; however, this pattern was not found in the study of Pereira (2007), which was also carried out in Portugal, though with no information concerning the period analyzed. On the contrary, in the southern regions of Portugal, there was a positive relationship between higher mortality and average annual precipitation (Ribeiro & Surový 2007). The exception was found in the driest region, where lower values of precipitation presented more mortality values. These patterns might suggest that usually holm oaks are more susceptible to drought events and cork oaks to excess water.

3.3.4 Approaches to prevent decline

Development of infection is usually explained with a disease triangle, a general concept in plant pathology. The three main factors that must operate in concert to produce the disease are the presence of the pathogen, a susceptible plant host and environmental conditions favoring infection. Methods of disease control can be thought of as modifying the disease triangle by reducing or eliminating one of the three factors. Researchers have been trying to reduce host susceptibility through selection of resistant varieties to *P. cinnamomi* with promising results (Moreira et al. 2006, 2007) and studies about the mechanism of pathogenesis of *P. cinnamomi* on cork oak have been carried

out with potential implications for disease control via resistance breeding (Coelho et al. 2006a; Horta et al. 2008; Maia et al. 2008). The initial approach to reduce the presence of the pathogen in the field was through application of the fungicide Metalaxyl (Coffey et al. 1984), however, some resistance has been found in some *Phytophthora* spp. (Cohen & Coffey 1986) and prolonged use of Metalaxyl reduces its efficacy (Darvas & Becker 1984). The fungicide may slow *P. cinnamomi* tissue infection but it does not eliminate the pathogen from infected plants (Marks & Smith 1992). Potassium phosphonate is other fungicide believed to have fungistatic activity and to stimulate the defense mechanisms of the fine roots (Guest & Grant 1991). Its application successfully improved vegetative growth of cork and holm oak seedlings in controlled situations (Navarro et al. 2004) as well as in adult holm oak trees in open field (Fernández-Escobar et al. 1999); however, other studies on treated trees have reported a lack of effectiveness of the fungicide (Porras et al. 2007; Solla et al. 2009). In order to suppress the pathogen, greenhouse experiments have been successfully realized with extracts from native plants (Neves et al. 2007), vegetable composts (Moreira et al. 2010) and calcium fertilizers (Serrano et al. 2011). The latter are not indicated to cork oak due to its preference for soils free of calcium carbonate. These experiments were conducted with seedlings in controlled situations and for the moment there are no curative treatments that can be carried out in adult trees, despite potassium phosphonate applications. Finally, restriction of human access to undisturbed sites is recommended to prevent further dispersal of the pathogen (Dawson & Weste 1985); however, these guidelines are not feasible to these human-made agro-silvo-pastoral systems. Other approach to reduce pathogen dispersal is through the control of nursery stocks used to reforestation, since there is strong evidence that *Phytophthora* dispersal and infested nursery stock are linked (Brasier & Jung 2003). Approaches described above present

some limitations: selection of resistant hosts is a long-term run since it will take time to replace susceptible with resistant varieties or to improve resistance through genetic manipulation. *P. cinnamomi* suppression through products application would be demanding since the species is widespread in the Mediterranean region and will probably widen its distributions with the expected climatic alterations (Brasier & Scott 1994; Brasier 1996). Data from Tables 1, 2 and 3 indicated that cork and holm oaks appear to be moderately susceptible to *P. cinnamomi* infection. For this reason, and considering disease triangle, the occurrence of infection is strongly dependent on environmental characteristics that not only favors pathogen survival, but also reduce host resistance. Figure 3.3 shows that cultural practices were one of the factors associated with decline. Moreover, management practices affect soil properties in its chemical, biotic and physical characteristics (Vacca 2000; Soru et al. 2006; Moreno and Obrador 2007; Moreno et al. 2007; Azul et al. 2011; Schnabel et al. 2011), including alterations in soil compaction and effective depth. A recovery from decline after long periods of rain was referred in some holm oak stands when management practices that cause root damage, soil degradation, and lack of natural regeneration were minimized (Tuset & Sánchez in 2004; Solla et al. 2009). Diniz (1994) also point out that soil management could increase decline in stands sub-optimal for cork oaks vitality, thus cultural practices should be adapted to site characteristics. Stand management offers several possibilities in the control and prevention of cork and holm oak decline, since it interferes with several other site characteristics associated with host vitality and pathogen survival and is one of the factors associated with decline that we effectively control.

3.4 CONCLUSION

Forest declines are considered a complex multifactorial phenomenon involving the combination of several factors. It is challenging to identify a cause that overcomes others, either because it may be related to other factors or because the proximate cause of death may mask the primary one. Following Manion's (1981) disease spiral concept, *P. cinnamomi* appears to act as a predisposing stress factor that, combined with other predisposing factors such as soil compaction, shallow soils, reduces cork and holm oak trees resilience, thus increasing their susceptibility to inciting and contributing stress factors, like drought or excess water events and other diseases (fig. 3.4). The effect of *P. cinnamomi* appears to be a chronic root pruning, more severe in holm than in cork oaks, forcing the trees to expend more energy in the production of more fine roots. To succeed, trees should be located in soils favoring root expansion and with adequate nutrient and hydric conditions. Otherwise, trees may not be able to replace necrotic roots and, moreover, the use of limited resources for the defense system and for root reposition may limit their response to other adverse situations. The main difference between cork and holm oaks and highly susceptible species is probably a higher dependence of other unfavorable conditions to occur decline. Despite this, the role of *P. cinnamomi* in oak decline should not be ignored.

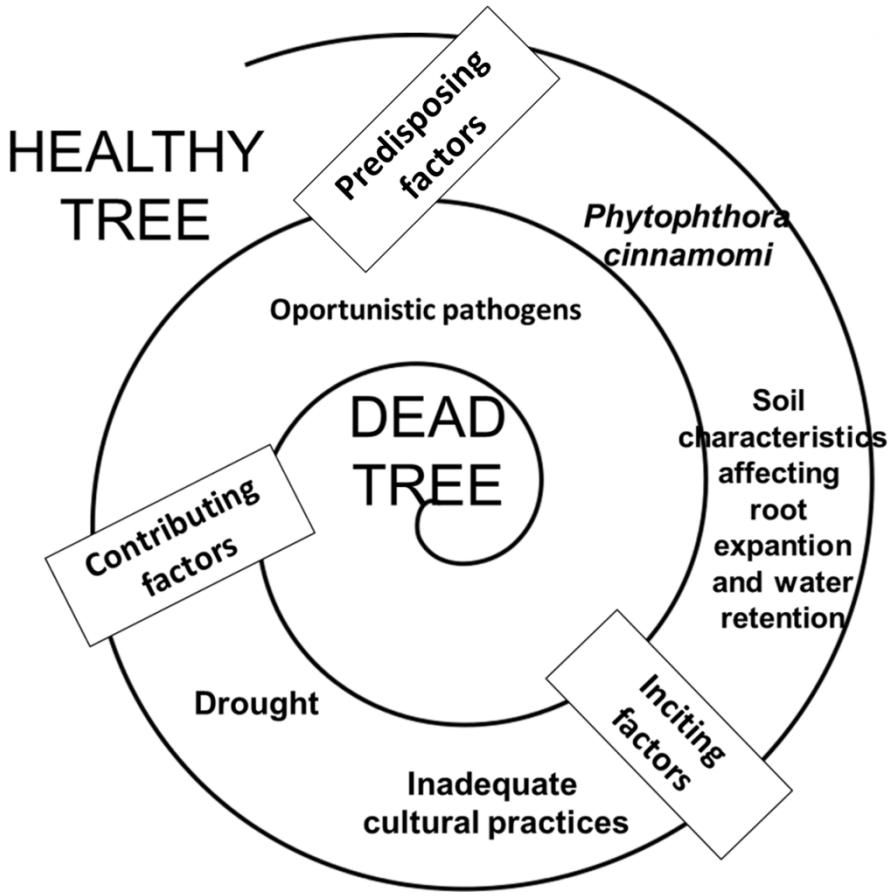


Figure 3.4 Adaptation of Manion's (1981) disease spiral with main interacting factors associated with cork and holm oak decline classified according to their role in decline

CHAPTER 4

Testing methods

for field diagnosis of *Phytophthora cinnamomi*:

Baiting selective-medium vs. Nested PCR

4.1- ABSTRACT

Baiting selective-medium methods are routinely used for *P. cinnamomi* diagnosis from soil samples, but they present some limitations resulting in the production of false negatives. On the other hand, DNA-based molecular protocols were recently developed to diagnose *P. cinnamomi* from soil samples. In this study, it was tested three variations of the baiting selective-medium method and a nested-PCR protocol for *P. cinnamomi* detection from soil samples collected in the rhizosphere of up to 60 declining and non-declining cork trees distributed in 30 stands. The objective was to analyze *P. cinnamomi* distribution in the study area and its association with tree health status using a reliable *P. cinnamomi* diagnosis. The main obstacle in the recovery of the pathogen using baiting selective-medium methods was pronounced contamination by *Pythium spp.* However, there are several improvements that were be applied to reduce contamination, namely the use of intact leaves for baiting, maintaining them floating at a distance of 3-4cm from the soil surface, the use of small parts of the infected tissue when plating in selective-medium and frequent observation for posterior sub-culturing of the growing hyphae. Overall success rate was 10.1%. Nested-PCR methods failed in detecting *P. cinnamomi* on 56 of the 60 soil samples (6.7%). 3 of the positive results were obtained from soils already positive for *P. cinnamomi* through baiting selective-medium method. Several attempts to optimize the reaction were tested, but reactions using soil DNA with aliquots of *P. cinnamomi* DNA showed amplification of the target DNA, indicating that PCR conditions were optimized and no inhibitors were present. It was applied other method for DNA extraction that allowed the use of 4-fold more soil sample. Purified DNA extracted with CTAB method resulted in similar DNA concentration despite the use of more soil sample. These results suggested that

failure in detecting the pathogen was due to small amount of soils samples, reducing the probability of acquiring sufficient *P. cinnamomi* inoculum. An alternative would be the use of combined techniques, applying selective-medium for pathogen isolation and molecular methods for identification. *P. cinnamomi* was detected in stands with high mortality rate, but also in stands with no mortality and with tree regeneration.

4.2- INTRODUCTION

Routine methods for detection of *P. cinnamomi* usually consist on baiting selective-medium procedures and involve two stages, isolation and identification: generally the isolation stage is also separated in two procedures: the first is the baiting stage where the goal is to attract zoospores to a trap; in the second stage the infected trap is placed on a selective medium for mycelium growth. Although the use of traps increases the probability of positive isolation, some researchers opt to apply directly the soil sample (diluted in water) onto the selective medium in order to quantify the active inoculum, or to plate directly infected roots with the aim to assure that the inoculum is active on the host tissues. There are several selective and susceptible traps used to "fish" *P. cinnamomi*, which can be part of plants (leaves, fruits) or whole seedlings and are chosen according to their availability. An extensive description of the vegetal traps and their efficacy was reported by Zentmeyer (1980). After verification of infection, isolation of the pathogen should be performed by plating out diseased tissues on selective media with antibiotics and fungicides to prevent growth of contaminant bacteria or fungi. The use of selective media prevents the masking or inhibition of the

relatively slow growing *Phytophthora* by the tremendous range of other microorganisms (Cooke et al. 2007). The mycelium developing in the selective medium can be finally identified. In the identification stage, plating out *P. cinnamomi* onto PDA (potato dextrose agar) medium should result in a characteristic gross morphology, or phenotype, with a camellioid or rosaceous growth pattern (Zentmeyer 1980). However, there are some variants in the shapes that are formed by the colony and, moreover, other *Phytophthora spp.* may form rosaceous colonies (Erwin & Ribeiro 1996), therefore, posterior identification of the species based on their structures like hyphae, sporangia and chlamydospores should be attempted. Although plating out in PDA medium the mycelium growing in the selective medium is useful to select *Phytophthora* instead of *Pythium spp.* or fungi mycelium, other procedures should be realized to identify *P. cinnamomi* since it does not form spores neither their characteristics hyphal swellings in this medium. Sporangium production can be stimulated by non-sterile soil extract (Jeffers & Aldwinckle 1987) and chlamydospores are abundantly produced in carrot or V8 agar medium. Oospores formation can be, by turn, stimulated by the presence of other *Phytophthora spp.* (Zentmeyer 1980). Microscopic observations of the spores are a good procedure for species identification, but it is time consuming, requires expertize and are not above misidentifications. Thus, molecular analyses are probably the most accurate method to identify the species the mycelium formed in PDA medium belong to, despite being more cost consuming than morphologic identification. Polymerase Chain Reaction (PCR) -based approaches have recently been developed and their application in the study of *Phytophthoras* in natural ecosystems is opening an incredible number of research opportunities (Cooke et al. 2007). PCR assays developed for *P. cinnamomi* identification usually target on pure cultures, soilless medium, or plant tissues (ex: Dobrowolski & O'Brien 1993, Coelho et al. 1997, Kong et al. 2003), but

two for them were specifically designed for *P. cinnamomi* detection from soil samples (William et al. 2009; Langrell at al. 2011). William et al. (2009) developed a protocol to detect *P. cinnamomi* inoculum from soil samples through a nested-PCR where both pair of primers were designed specifically for *P. cinnamomi* based on Internal Transcribed Spacer (ITS) region. Langrell at al. (2011) developed a touchdown nested multiplex PCR protocol for the simultaneous detection of *P. cinnamomi* and *P. cambivora* direct from soil with pre-existing and novel primers, based on ITS region as well.

An accurate detection of the root pathogen is crucial in studies concerning tree decline. Visible symptoms of oak decline observed in the field are unspecific and refers to defoliation and upper branch dieback or, occasionally, fast drying of the leaves (see chapter 2 for an exhaustive description of decline symptoms). These symptoms were associated to different tree physiologic response to water stress, as it was studied and explained in detail in chapter 5. However, only after accessing the origin of tree water impairment it will be possible to develop approaches for an eventual prevention of the problem. Infection of fine roots by root pathogens, destruction of shallow roots during management practices with soil tillage in the rizosphere, flooded soils enhancing root hypoxia and rot, or limited access to deep water resources may cause the same symptoms. In cork and holm oak *montados, P. cinnamomi* diagnosis was only realized through traditional baiting-selective medium methods. Its success rate varied considerably among studies (see chapter 2) and unsuccessful attempts may or not be real negatives isolations. Therefore, studies about cork and holm oak decline, including studies on the interaction between the pathogen distribution and abiotic factors, or studies about the physiologic status of host trees with *P. cinnamomi* in the rhizosphere, the effect of ectomycorrhizae in preventing *P. cinnamomi* infection in the field, or

approaches to prevent host mortality, all need a reliable method for *P. cinnamomi* diagnosis.

4.3- OBJECTIVES

In this study we tested the efficacy of traditional methods *vs* PCR-based methods for *P. cinnamomi* diagnosis from soils samples obtained in a cork oak *montado*. The goal of this study was to analyze the distribution of *P. cinnamomi* in *montado* woodland showing variable mortality indexes with a reliable methodology.

4.4- MATERIAL AND METHODS

4.4.1- Study site

Since 1995 a set of 64 permanent plots with were installed in *montados* at Machuqueira do Grou (39°6'N 8°22'W, 130–150 m a.s.l.), near Coruche, Portugal (16 °C mean annual air temperature, 640 mm mean annual precipitation) in the Alentejo region, on undulating terrain, with the assistance of forest producers associations. In the permanent plots cork oaks have been monitored in regards to tree growth and density (mortality and tree replacement) and cork production (Ribeiro et al. 2003) which was

linked with information regarding site characteristics, stand structure, human management and meteorology. This approach has allowed long term studies with a detail and complexity that otherwise would be difficult to achieve. With information obtained in the permanent plots it was produced special tree growth models and mortality models (CORKFITS, Ribeiro et al. 2004, 2006) and a decision support system (ECCORK, Pinheiro et al. 2008), both being useful tools to help forest producers in their management practices decisions. Mortality of each stands was calculated as a percentage of tree mortality in relation to total trees (Ribeiro 2006).

4.4.2 - Soil sample collection

Soil samples were collected during four field surveys, 19 stands in spring 2010, 16 stands in autumn 2010, 31 stands in spring 2011, and 4 stands in autumn 2011, after seasonal rains when soil inoculum of *P. cinnamomi* is supposed to increase due to water availability and, additionally, occurs the sprout of young cork oak leaves, necessary for baiting. In the 4th survey, efforts were concentrated in a study-case for ecophysiologic measurements and only 6 trees were needed for sampling (chapter 5). In the surveys, two widely separated trees per stand with some decline symptoms, namely the presence of upper branch dieback, were selected for soil sampling (fig. 4.1). Careful was taken not to select much damaged trees (Jung 2011). In the case of no dieback symptoms, trees were selected randomly. In the 4th survey it was selected 12 trees in the 4 stands with half presenting decline symptoms, as explained in chapter 5. After removing the

Figure 4.1: Cork oak (*Quercus suber*) with decline symptoms like upper branch dieback

first layer of soil litter, soil samples were collected in four locations distant 1m from the trunk base of each tree, and about 10 cm deep, making a total of 0.5 L. Utensils used to excavate and collect soil samples were disinfected with alcohol 70% before use in another tree.

Soil samples were homogenized and stored at 10ºC for up to four weeks until utilization. Storage at low temperatures stimulates zoospores release from sporangium (Zentmeyer 1980). A 50 ml sample of each soil was freeze dried for two days and stored at -20ºC for posterior molecular analyses.

4.4.3 - Baiting and selective-medium methodology

It was tested three different baiting methodologies, where the proportion of infected baiting leaves and the proportion of contamination in selective-medium was measured. Success rate was calculated as the proportion of soils (1 composite sample by tree) positive for *P. cinnamomi* in relation to total soils analyzed in each methodology.

1st baiting selective-medium method

The samples obtained in spring and autumn 2010 were analyzed according to methodology adopted by Moreira (2001). 15 g soil was mixed with 20 ml of distilled water in a petri dish with 90 mm diameter. It was performed 4 replicates by soil sample. After soil sedimentation traps concerning of 15–20 pieces of young cork oak leaves

were placed floating under laboratory conditions (20ºC and diffuse daylight, fig. 4.2).

Up to 3 days of incubation, baits with necrosis were removed, washed with tap water and half of them surface sterilized (60 s in 1% aqueous sodium hypochlorite) and blotted dry. All baits were then transferred to the MA+PARBHY medium

Figure 4.2: Baiting method: 15 g soil submersed in 20 ml of distilled water with floating cork oak discs to bait *Phytophthora cinnamomi* zoospores.

Figure 4.3: Direct application of roots onto selective MA+PARBHY medium for *Phytophthora cinnamomi* isolation.

(Jeffers & Martin 1986, Robin 1998) which is partially selective for several *Phytophthora*, including *P. cinnamomi* (table 4.1). Additionally, some samples of cork oak roots were also applied directly onto MA+PARBHY medium (fig. 4.3).

Table 4.1: MA+PARBHY composition for 1 L solution

Components	Quantity
Malt extract	15 g
Agar	20 g
Pimaricin	10 mg
Rifampicin	10 mg
Ampicillin	250 mg
Benomyl	15 mg
Hymexazol	50 mg
Distilled water	1 L

For 1 L solution, malt extract, agar and 990 ml of distilled water were mixed and autoclaved at 121ºC for 20 min. Before this, antibiotics and fungicides were stirred in 10 ml of autoclaved distilled water for complete dissolution. Particularly, benomyl is not easily dissolved in water. When the sterilized solution cooled to about 45ºC antibiotics and fungicide were added and final solution distributed onto petri dishes with 60 mm diameter (about 10 ml in each).

If there was mycelium growing in the selective-medium, a small piece was removed and placed on the center of petri dishes containing PDA medium (39 g Potato Dextrose Agar and 100 ml distilled water, autoclaved at 121ºC for 20 min). If the colony developed in the typical rosaceous or camellioid pattern, petri dishes were stored at ambient temperature and eventually replicated until molecular analyses for its identification.

2nd baiting selective-medium method

The samples obtained in spring 2011 were baited with a different methodology: Soils inoculum per replica was increased to 25 g and distilled water to 100 ml (fig. 4.4). By this means it was expected to enhance *P. cinnamomi* recovery using more inoculum source and increasing soil dilution which is favorable to sporangium formation (Tsao 1983). Baits placed onto MA-PARBHY medium were not surface sterilized but washed with sterilized water and dried on.

Figure 4.4: Baiting method: 25 g of soil submersed in 100 ml of water, with floating cork oak discs to bait *Phytophthora cinnamomi* zoospores.

3rd baiting selective-medium method

Samples obtained in autumn 2011 (4th survey) were baited according to Jung (2011). About 1 L of soil sample was flooded with autoclaved water in a plastic

container until the distance between the soil surface and waterline was about 3-4 cm. Litter and debris were removed from the waterline. Young and intact cork oak leaves were placed floating on the water. Right after signs of infection leaves were observed under light microscopic for the presence of sporangia and infected tissues were cleaned and cut into small pieces and placed onto PARBHY medium.

4.4.4 – Nested PCR-based methodology

DNA extraction was performed using three methodologies:

1) PowerSoil DNA Isolation Kit (Mo Bio laboratories, Inc.) was used for DNA extraction according to manufactures instructions. A total 60 samples were analyzed, each with 250 mg of soil prepared for molecular analyses. Cell lysis occurs by mechanical and chemical methods. The mechanical shaking was performed with a vortex mixer, after securing tubes horizontally with tape, at maximum speed for 10 minutes.

2) Alternatively, other 30 subsamples obtained from the same soil used in the first method were macerated with liquid nitrogen instead of shaken in the vortex mixer. About 40g of the freeze dried soil were macerated with a pestle on a mortar with liquid nitrogen. Right after maceration, samples were maintained on ice until stored at -20ºC before use. Ceramic mortar and pestles were washed in 1% aqueous sodium

hypochlorite and autoclaved at 121ºC for 20 min before reuse. 250 mg of the macerated soil was added to the extraction buffer of the PowerSoil DNA Isolation Kit and DNA extraction followed the subsequent steps of the kit.

3) Total soil DNA was also extracted using Graham et al. (1994) methodology for fungal genomic DNA. Briefly, 30 subsamples with 1 g obtained from the macerated soil (prepared as explained above) were added to a 1.5 ml microcentrifuge tube with 1ml extraction buffer (2% wt/vol CTAB, 100 mM tris-HCl, 1.4 M NaCl 20 mM EDTA). The blend was mixed by gentle inversion and incubated at 55ºC for 20 min, following 5min centrifugation at 15,000 x *g*. The supernatant was collected in a new microcentrifuge tube and 1 volume of chloroform: isoamyl alcohol (24:1) was added and mixed by gentle inversion for 2 min. After centrifugation at 15,000 x *g* for 20 s, 640 µl of the upper aqueous phase was collected carefully to a 2 ml microcentrifuge tube and 1/10 volumes of ammonium acetate and 2 volumes of ice-cold absolute ethanol were added and mixed by gentle inversion. Samples were stored at -20ºC for at least 60 min to precipitate genomic DNA. Supernatant was discarded after centrifugation at 15,000 x *g* for 1 min and the pellet was washed twice with 1 ml of 70% ethanol and air dried for about 1 hour. DNA was eluted in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Additionally, 6 samples of eluted soil DNA were cleaned with PowerSoil DNA Isolation Kit: Addition of a high concentrated salt solution to the eluted soil DNA allows binding of DNA to the silica present in the spin filters, but not non-DNA organic and inorganic material that pass through the filter membrane. After centrifugation at 10,000 x g for 1 minute, the flow-through was discarded and the silica membrane of the spin filters washed with an ethanol based solution. 2 more centrifugation at 10,000 x g

for 1 minute were performed in order to remove the ethanol based solution and to dry ethanol residues. DNA was eluted with 100 µl of a elution buffer.

DNA quantification

After elution, DNA samples were analyzed in a NanoDrop 2000c/2000 UV-Vis Spectrophotometers (Thermo scientific) for nucleic acid concentration and purity measurements. 5 samples of total DNA extracted with CTAB protocol was diluted 100 fold since it showed a yellow-brown color which may interfere with wavelength measurements.

PCR amplification protocol

Unless indicated otherwise, PCR amplification conditions were following William et al. (2009) nested PCR protocol, developed specifically for *P. cinnamomi* diagnosis from soil samples. The authors designed two sets of primers (table 4.2) developed from the rDNA ITS sequences of *P. cinnamomi* that can detect as little as 1 pg DNA and used a thermophilic DNA polymerase (Tth^+, Promega, table 4.3 A) that showed to be less sensitive to soil inhibitors. Additionally, it was also tested the Dream Taq Polymerase (Fermentas, table 4.3 B) and Supreme NZTaq 2x Green Master Mix (Nzytech, table 4.3 C).

Table 4.2: Specific primers for amplification of the rDNA ITS region of *Phytophthora cinnamomi* used for first and nested rounds of PCR (William et al. 2009). Tm: melting temperature

Primer	Sequence	Tm (ºC)	PCR Reaction	DNA Fragment Size (bp)
CIN3A	CATTAGTTGGGGGCCTGCT	57.7	1st round	783
CINITS4	TGCCACCACAAGCACACA	57.9	1st round	
CIN3B	ATTAGTTGGGGGCCTGCT	56.6	Nested	396
CIN2R	CACCTCCATCCACCGACTAC	57.1	Nested	

PCR cycling consisted of 5 min denaturation at 94ºC, followed by 30 cycles of 94ºC for 1 min, 60ºC for 1 min, 74ºC for 1 min; and a final extension of 74ºC for 5 min. For Dream Taq and Supreme NZTaq denaturation was adjusted to 95ºC.

Nested PCR: For the second round of amplification, products of the first round were diluted by 1 ⁄ 100 and 1 µl added to the reaction. The same amplification conditions of the 1st round were used for the nested PCR.

The thermal cycling program was run in a programmable heat block. A negative and a positive control were added in each set of 8 reactions. The positive control was extracted with the powersoil kit from a *P. cinnamomi* pure culture obtained with the baiting selective-medium method and posteriorly sequenced. For the negative control, template DNA was replaced by 1 µl of nuclease-free water in order to test contamination of reagents and reaction mixtures. Nested PCR had two negative controls, one with no DNA template and a second with a dilution of the negative control product of the first round.

Table 4.3: Components combined in the reaction mixture to amplify *Phytophthora cinnamomi* DNA by Nested PCR, for 25 µl total volume

A) Using Tth^{+}DNA polymerase:

Reagent	Volume (µl)	Final concentration
10X reaction buffer	2.5	10 mM Tris-HCl (PH 9), 50 mM KCl, 0.1% Triton X-100
25 mM MgCl	2	2 mM
10 mM DNTP's	0.5	0.2 mM
2 Primers	5 x 2	1 µM each
DNA template	1	2.5 ng
Tth^{+} DNA polymerase	0.5	-
Nuclease-free water	8.5	-

B) Using Dream Taq polymerase:

Reagent	Volume (µl)	Final concentration
10X reaction buffer	2.5	20 mM Tris-HCl (PH 8), 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, 0.5% (v/v) Nonidet P40, 0.5% (v/v) Tween 20, 50% (v/v) Glycerol, 2mM MgCl
10 mM DNTP's	0.5	0.2 mM
2 Primers	1 x 2	0.5 µM each
DNA template	1	10 ng
Dream Taq polymerase	0.5	2.5 U
Nuclease-free water	14	-

C) Using supreme NZTaq 2x Green Master Mix:

Reagent	Volume (µl)	Final concentration
2x Green Master Mix	12.5	n.a
2 Primers	1 x 2	0.5 µM each
DNA template	1	10 ng
Nuclease-free water	9.5	-

- Several alterations to amplification conditions were performed:

- 3 DNA template dilutions were tested: 0.05, 1 and 20 ng in a 25 µl reaction.
- Annealing temperature in the first round was reduced to 58ºC and in the second round was increased to 62ºC and 65ºC.

- The first PCR product was diluted ½, 1/50 or added with no dilution to the Nested PCR reaction.
- Reduction in primers concentration to 0.2 µM each
- To test for potential inhibitors in the DNA template, it was performed several different dilutions with total DNA from soil (a positive sample obtained with the baiting selective-medium technique) and DNA from a *P. cinnamomi* pure culture (table 4.4)
- To avoid inhibitory effect of soil extract it was added 400 ng ⁄ µl bovine serum albumin and 4% formamide in the PCR reaction (William et al. 2009)

Table 4.4: DNA dilutions tested in Nested PCR reactions for *Phytophthora cinnamomi* rDNA fragment amplification.

Reaction	**template DNA (ng / µl)**	***P. cinnamomi* DNA (ng / µl)**
1	0.32	-
2	0.08	-
3	0.008	-
4	0.32	0.052
5	0.64	0.052
6	0.64	0.026
7	-	0.002

Agarose gel electrophoresis of PCR products

20 µl of the nested PCR reaction was loaded into a 1% (wt/vol) agarose gel in 1x Tris-acetate-EDTA (TAE) buffer. Gels were stained by immersion in an ethidium bromide solution for 10 min and gel images digitally recorded under UV light. Fragment sizes were determined by comparison with a 1 kb Plus molecular weight standard (Invitrogen).

DNA Sequencing of the amplified products

Fragments with 396 bp from 7 samples were excised from the 1% agarose gel and purified using QIAquick Gel PCR purification kit (QIAGEN) according to the manufacturer's instructions and were sequenced using the CIN3B primer in Macrogen Standard Sequencing Service (Amsterdam, The Netherlands). DNA sequences were compared with the sequences in the National Center of Biotechnology Information nucleotide databases (Genbank; Zhang et al. 2000, Morgulis et al. 2008; using the BLASTN search: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.4.5- Statistical analyses

Statistical analyses were made using Systat v.13.1 software package. One-way ANOVA was used to test significance of differences observed between DNA concentration and impurities ratio between the 3 DNA extraction methodologies. Significant differences between treatments means were evaluated with Tukey's HSD tests with $p < 0.05$. Before applying ANOVA tests, data were tested for normality by using Kolmogorov-Smirnov test at the significance level of 0.05. A one-sample z-test was performed to compare the mean of the total DNA concentration and impurities of the 5 diluted CTAB samples with the mean of the not diluted CTAB samples and to compare the 6 purified CTAB samples with non- purified CTAB samples .

4.4.6 - *Phytophthora cinnamomi* occurrence in the study site and its relation with tree mortality

In each stand, health index was previously calculated based on variation in tree density since plot installation and latest cork oak inventory (Ribeiro, unpub. data), considering not only dead trees but also tree regeneration. Stand health index = (number of dead trees – number of new trees) / number of initial trees. *P. cinnamomi* occurrence in each stand, obtained will all the diagnosis methods, was compared with stand health index.

4.5 - RESULTS

4.5.1- Baiting and selective-medium methodology

In the 1st survey only one stand (in a total of 19) was positive for *P. cinnamomi* and in the 2nd survey there was 3 positive stands (in a total of 17), all adjacent to each other. In the 3rd survey 3 stands from a total of 31 prospected were positive for the pathogen. Finally, the 4th survey was on the study-case comprising 4 adjacent stands with 12 trees where physiologic analyses were performed for studies described in chapter 5. In 6 of the 12 trees *P. cinnamomi* was already detected in the 2nd survey, thus only the remaining 6 trees were prospected again. 1 tree yielded the pathogen in the rhizosphere, belonging to a stand were *P. cinnamomi* was not recovered before.

Considering a total of 48 different stands, 16 of them being surveyed twice, *P. cinnamomi* was recovered from only 8 stands, 4 of them during the 2nd survey. Regarding sampled trees, the pathogen was recovered in the rhizosphere of 14 trees from a total of 138 trees (10.1%, fig. 4.5). According to methodology, success rate varied between 4.8% and 16.7% (table 4.5). The roots applied directly in PARBHY medium did not yield *P. cinnamomi*. In samples were *P. cinnamomi* was recovered no other contaminant mycelium had grown.

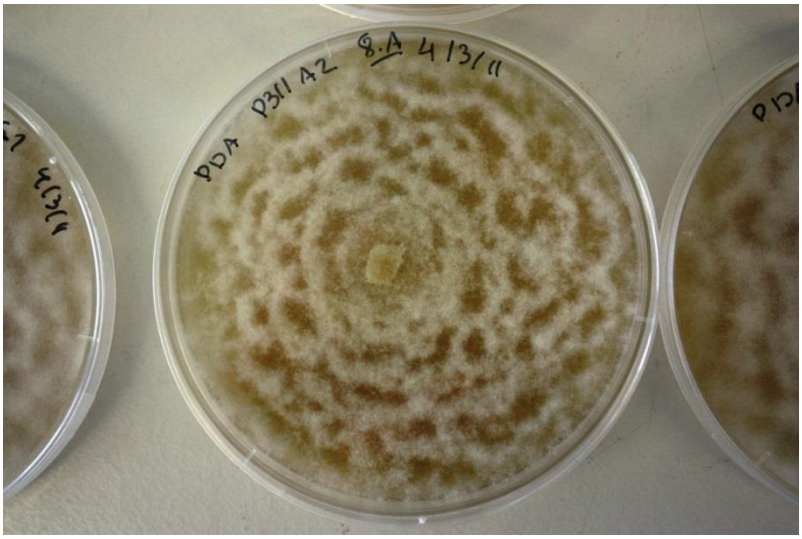


Figure 4.5: *Phytophthora cinnamomi* recovered from cork oak (*Quercus suber*) rhizosphere and growing on PDA medium forming rosaceous colonies

Table 4.5: Proportion of soil samples, obtained in the rhizosphere of cork oaks (*Quercus suber*), positive for *Phytophthora cinnamomi* (pc) according to isolation method,

Baiting methodology	**Sampled soils (n. trees)**	**Positive for pc**	**Success rate (%)**
15 g soil/ 20 ml water, baiting leaf sterilization	70*	8	11.4
15 g soil/ 20 ml water, no baiting leaf sterilization		6	8.6
25 g soil/ 100 ml water	62	3	4.8
1 L soil submerged 3 to 4 cm	6	1	16.7

*half of the baiting leaves from each sample were sterilized

Baiting selective-medium method faced some difficulties, in particular regarding *Pythium spp.* contamination. In table 4.6 is resumed the difficulties, resolutions performed to overcome them and obtained results.

Table 4.6: Difficulties encountered when using baiting selective-medium method for diagnose of *Phytophthora cinnamomi*, troubleshooting and results obtained

Difficulties	**Troubleshooting**	**Results**
Overall baiting leaves contamination	Use of intact leaves instead of leaf pieces	Reduced contamination of the floating leaves (from 70% to 14%)
	Distance of 4 cm from the soil surface to waterline	
Growth of other agents in MA-PARBHY medium, particularly *Pythium spp.* mycelium in 58% of the petri dishes with MA-PARBHY medium	Baits surface sterilized	Reduced contamination by 62% and yielded *P. cinnamomi*
	Very small fragments of infected tissues were placed onto MA-PARBHY medium	Strong reduction in contamination (76%)
	Frequent observation of growing mycelium for posterior subculturing	Success may be limited to low *Pythium spp.* inoculum
Low success rate	Increase in soil amount sampled	Apparently with no differences

4.5.2- Nested PCR methodology

60 soil samples were analyzed using different methodologies for DNA extraction and PCR reaction.

DNA extraction

Total DNA from each soil sample was extracted using three methodologies. DNA concentration obtained with PowerSoil DNA Isolation Kit was similar despite the

use of different cells lysis methods (14.4 ± 9.1 ng/µl, $p = 0.146$). Total DNA obtained with CTAB apparently was more concentrated (293.7 ± 157.9 ng/µl) however, excess of impurities may result in an overestimation of the nucleic acid concentration. Indeed, DNA extracted with CTAB protocol showed lower 260/280 ratio that that with the extraction kit (1.73 ± 0.18; Anova: $F_{(2,87)} = 10.2$; $p < 0.001$). Dilutions by 1/100 of 6 samples reduced total DNA concentration (113.94 ± 77.0 ng/µl; $Z = -2.5$; $p = 0.011$), but not in the proportion of dilution, and 260/280 ratio remained similar ($Z = -1.32$; $p = 0.188$). 6 samples of total DNA extracted with CTAB and posteriorly cleaned with the PowerSoil DNA isolation kit reagents showed similar DNA concentration and impurities to DNA extracted with the same Kit ([DNA]: $Z = -0.9$; $p = 0.361$. 260/280 = 1.99 ± 0.33; $Z = -0.034$; $p = 0.973$). In regard to 260/230 ratio, there was no differences between protocols (Anova: $F_{(2,87)} = 1.97$; $p < 0.145$), dilutions ($Z = -0.31$; $p = 0.975$) and posterior DNA cleaning ($Z = -0.17$; $p = 0.867$).

PCR amplification protocol

The PCR reaction with Tth^{+} DNA polymerase and DNA template extracted with PowerSoil DNA Isolation Kit yielded no positive results, not even the positive control. Alterations to PCR conditions, namely DNA and primers dilutions, different annealing temperatures and several dilutions of the product from the 1st round to the nested PCR did not result in amplification of the positive control as well.

Both Dream Taq polymerase and Supreme NZTaq polymerase amplify the target DNA fragment of the control sample; however, there were only 4 template DNA samples that yielded positive reactions (6.7%, fig. 4.6). DNA from the positive results

was extracted with PowerSoil DNA Isolation kit after maceration with liquid nitrogen and standard amplification conditions with Dream Taq Polymerase, 4.5 to 5 ng template DNA and no dilution of the first product to be added to Nested PCR reaction. All the 4 positive samples were obtained from soils tested with baiting selective-medium methodology, and 3 of them were positive for the pathogen using the convencional methods .

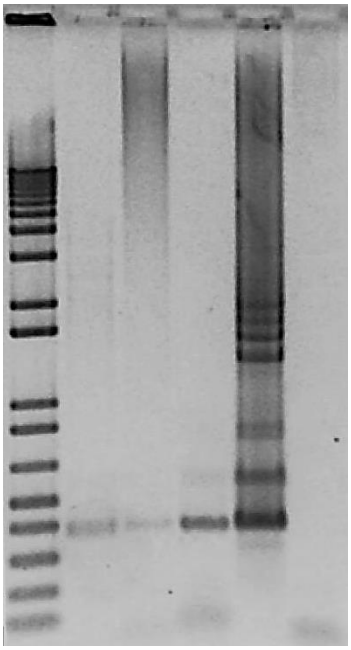


Figure 4.6: Amplification of the 396 bp fragment in nested PCR reaction with specific primers for *Phytophthora cinnamomi*
rDNA ITS amplification fragment: CIN3A and CINITS4 in the 1st round, CIN3B and CIN2R in the 2nd round. Fragments were separated on a 1 % agarose TAE gel.
Lane 1: 1 kb plus ladder as marker.
Lane 2, 4, 5: template DNA from soils positive for *P. cinnamomi* with baiting method.
Lane 3: template DNA from soil not positive for *P. cinnamomi* with baiting method.
Lane 6: Negative control

No other extracted DNA yielded positive results, thought several attempts to optimize the reaction were performed. The addiction of *P. cinnamomi* DNA to soil

DNA resulted in positive amplifications, meaning that there was no PCR inhibitors in the template DNA solutions (fig. 4.7). As little as 0.02 ng of *P. cinnamomi* DNA was amplified and, in contrast, addiction of as large as 16 ng of template DNA did no prevent amplification of the *P. cinnamomi* DNA.

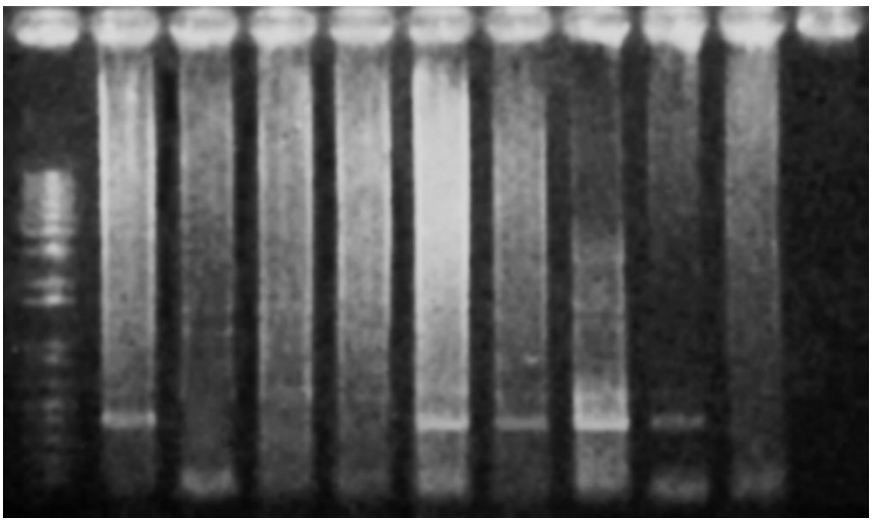


Figure 4.7: Optimization of template DNA concentrations and test for potential inhibitor with addition of *Phytophthora cinnamomi* DNA.
Bands correspond to amplification of the 396 bp fragment in nested PCR reaction with specific primers CIN3A and CINITS4 in the 1st round, CIN3B and CIN2R in the 2nd round. Fragments were separated on a 1 % agarose TAE gel.
Lane 1: 1 kb plus ladder as marker
Lane 2: positive control: 13 ng of *P. cinnamomi* DNA
Lane 3: 8 ng of template DNA
Lane 4: 2 ng of template DNA
Lane 5: 0.2 ng of template DNA
Lane 6: 8 ng of template DNA and 1.3 ng of *P. cinnamomi* DNA
Lane 7: 16 ng of template DNA and 1.3 ng of *P. cinnamomi* DNA
Lane 8: 16 ng of template DNA and 0.65 ng of *P. cinnamomi* DNA
Lane 9: 0.054 ng of *P. cinnamomi* DNA
Lane 10, 11: Negative controls

Total DNA extracted with CTAB methodology did not yield positive results, not even after addiction of bovine serum albumin and formamide or after purification with appropriate solutions from PowerSoil DNA isolation kit.

4.5.3- *Phytophthora cinnamomi* occurrence and mortality index of the stands

P. cinnamomi was detected in 16.7% of the 44 different prospected stands, being more frequent in stands with high mortality though it was also recovered from stands with no mortality and from a stand with regeneration (table 4.7).

Table 4.7: Positive *Phytophthora cinnamomi* recovery from the rhizosphere of cork oaks (*Quercus suber*), in relation to health index of the stands

Surveyed stands	***P. cinnamomi* recovery**	**Success rate (%)**	**stand health index**
5	1	20	Positive tree regeneration
11	1	9	No tree variation
14	1	7	Tree mortality up to 49%
14	5	35.7	Tree mortality above 50%

Health index: (n. dead trees – n. new trees) / initial n. trees in the stand

4.6 - DISCUSSION

4.6.1 – Baiting selective-medium methodology

The principal difficulty with the baiting selective-medium was to prevent the infection of the baiting leaves by the faster-growing mycelium of *Pythium spp.* Hymexazol, used in the selective-medium, avoids the growth of several *Pythium spp.* (Jeffers & Martin 1986), however, it is not efficient for a some common strains. Sterilization of baiting leaves reduced contamination; however, *P. cinnamomi* recovery

was slightly higher when using sterilized leaves: in 8 positive samples, 6 were recovered from both sterilized and unsterilized leaves. The other 2 positive samples were only recovered from sterilized leaves. This suggests that surface sterilization of baiting leaves is a better procedure than no sterilization at all. However, positive results were still very low (8 in 70) and it is possible that surface sterilization also reduced *P. cinnamomi* inoculum along with reduction in contaminants.

Increasing total soil sample from 10 g to 25 g was not more effective, probably because contamination by other agents was still very high (about 70%) and could limit *Phytophthora* slow-growing mycelium.

Placing intact baits in the selective-medium also resulted in contamination by several fungi and *Pythium*. Although they may not be able to growth in the selective medium, infection of the leaves by contaminants would inhibit the desired pathogen. Several procedures, applied in the baiting method with 1 L soil, were efficient in reducing undesired contaminations. Using intact leaves floating about 4 cm distant from the soil surface and then plating out small fragments of the infected tissues, all reduced contaminations. Intact leaves are less susceptible to opportunistic pathogens and if maintained distant from the soil surface they are less exposed to *Pythium* sporangia (Jung 2011). The use of small pieces of necrotic tissue also reduces contamination since they contain less inoculum. However, to increase successful isolations baiting leaves should be observed under light microscopic for the presence of *Phytophthora* sporangia, which is time consuming and requires expertise. Although success rate was low with this last methodology (1 sample in 6), it was probably due to reduced replicates. The method was applied to scrutinize *P. cinnamomi* in the rhizosphere for the case study of tree eco-physiologic measurements (chapter 5) and only 6 samples were needed for analyses. Those 6 trees were negative for *P. cinnamomi* after baiting 15 g soil in the

previous surveys. After baiting 1L soil sample and using the above referred procedures to reduce contaminations, it was detected 1 positive result. The other 5 negative results should be reliable since leaves used to trap *Phytophthora spp.* were considerably not infected by other pathogens, indicating that *P. cinnamomi* isolation was not severely affected by the presence of other contaminants, and failure in detecting the pathogen could be attributed to inoculum shortage.

4.6.2- Nested PCR methodology

Positive results obtained with PCR diagnosis were inferior to those obtained with baiting selective-medium methodology. Several attempts were made to optimize the PCR reactions, with no success in detecting *P. cinnamomi* DNA from the total soil DNA extract. All the steps of the procedure were repeated and adjusted to overcome possible impediments in DNA amplification, from alterations in cell lysis to the use of more soil sample, adjustment of the components in the reaction and alteration of some amplification conditions, like temperature annealing. The enzyme recommended in the protocol for *P. cinnamomi* detection from soil DNA through molecular methods (Williams et al. 2009) was not effective in amplifying *P. cinnamomi* DNA from a pure culture (positive control), neither after optimization of the annealing temperatures and template DNA dilutions. Taq Polymerases (both Dream Taq and Taq polymeraze from the Master Mix) always amplified the positive control and were selected in posterior amplification attempts. After recurrent negative amplifications of soil DNA extracted with PowerSoil DNA Isolation Kit, it was tested if cell lysis was not being performed

properly. Following kit protocol, micro-centrifuge tubes were fixed horizontally with tape on a vortex mixer to be shaken as recommended; however, they eventually could not be shaken evenly or efficiently, leading to inconsistent results or lower yields caused by incomplete homogenization and cell lysis (PowerSoil DNA isolation Kit instruction manual). Maceration with liquid nitrogen was performed to overcome this question, however, total DNA concentration was not superior than that obtained using the vortex mixer for homogenization and cell lysis. Nevertheless, the only 4 positive results were obtained after maceration with liquid nitrogen previously to DNA extraction with PowerSoil DNA isolation kit, using Dream Taq polymerase with the conditions described in the methods. Since no more positive results were obtained using the same conditions, it was tested the possible existence of PCR inhibitors in the template DNA. Addiction of any amount of *P. cinnamomi* DNA along with template DNA did not prevent DNA amplification. Moreover, *P. cinnamomi* DNA amounts as small as 0.002 ng/µl (0.05 ng in a 25 µl reaction) were amplified. This showed that soil extracted DNA had no relevant PCR inhibitors and reaction conditions were appropriate for DNA amplification. It was then decided to test more soil amounts to increase the probability in obtaining *P. cinnamomi* DNA. CTAB methodology is a cost-friendly approach to extract DNA that imposes no limitation in the amount of substrate to be used. However, it has the disadvantage of possible contamination by PCR inhibitors co-extracted along with total DNA from soil samples. Measurements of the DNA purity showed high contamination

William et al. (2009) incorporated bovine serum albumin and formamide to PCR reaction and synergistically increased PCR sensitivity and specificity to target DNA. In this study, addition of those additives did not yield positive results. These negative results could be due to inefficiency of the additives, but the same extracted soil DNA

samples were posteriorly cleaned with appropriate reagents and filters resulting in purified DNA and results were negative as well. Additionally, DNA concentration was similar to that obtained with 250 mg soil samples, though it was used 1 mg soil sample for DNA extraction. Therefore, all the realized experiments and adjustments suggested that PCR methodology failed in amplifying *P. cinnamomi* DNA due to lack of target DNA. Although William et al. (2009) successfully recovered *P. cinnamomi* from 1 mg soil samples, this study suggests that soil samples with up to 1 mg are very low for *P. cinnamomi* diagnosis through PCR methods. DNA extracted with Powersoil kit was not significantly contaminated by impurities like proteins but required only 250 mg of soil sample for DNA extract. However, DNA extraction from 1 mg of soil sample through CTAB protocol, following purification, did not yield more total DNA.

Former attempts in detecting *P. cinnamomi* from 1 g soil samples using a highly specific, sensitive and reliable PCR-method based for *P. cinnamomi* identification resulted in no amplification success when soil samples were naturally infested with the pathogen, even after purification steps; however, artificial infested soil samples, independently of their composition, yielded DNA amplification in a proportion of 100 ng and more of mycelium to 1 g of soil (Moreira 2001, Moreira et al. 2007). The authors considered that failure in detection of the fungus in naturally infested forest soil reflected the difficulty of eliminating polymerase inhibitors from this soil. In other experiment with naturally infested soil samples, Langrell et al. (2011) used 10 g of soil samples for *P. cinnamomi* and *P. cambivora* diagnosis and were able to detect the pathogens in naturally infested soils; they detected the target DNA but success rate was equal to the baiting method. In relation to soils artificially amended with *P. cinnamomi* inoculum, PCR-based methods consistently detect the target DNA, detecting as little as 1 pg DNA (William et al. 2009, Langrell et al. 2011,). As well, in this study low

amounts of *P. cinnamomi* DNA added to the PCR reaction were amplified, reinforcing the assumption that negative amplifications resulted from absence of target DNA and not from possible presence of PCR inhibitors. *P. cinnamomi* is a weak competitor and its saprophytic ability improved only in saturated soils. Thus, many naturally infested soils have low levels of *Phytophthora* propagules and their populations can fluctuate from non-detectable to a high inoculum density in a very short period of time (Eden et al. 2000, Cooke et al. 2007) and that soil sampling represents an important factor with respect to detection success (Langrell et al. 2011). Furthermore, *P. cinnamomi* distribution at spatial scales of 1-m intervals was found to be random, independently of the health status of the surveyed stands (Pryce et al. 2002). Thus, detection of *P. cinnamomi* in such low amount of soils appears to be a matter of luck and failure in detection cannot be considered true negatives.

Conjoining techniques, like using the baiting selective-medium to trap *P. cinnamomi* propagules and then applying a molecular method in bait tissues (Moreira 2001), may be an alternative to increase the probability in obtaining the desired DNA. Other approaches to attract *P. cinnamomi* zoospores involve the use of dipsticks coated with chemo-attractants, which can be an easy way to concentrate the inoculum on a small surface before running a molecular assay (Martin et al. 2000). Nevertheless, efficient and reliable methods should allow direct detection in small samples of roots or soil without the need for isolation intermediate steps such as the use of baits (Moreira et al. 2007). Development of methodologies to increase sensitivity, like Nested or Real-time PCR instead of conventional PCR, helps amplifying pathogen DNA extracted from plant tissue (Martin et al. 2012). Infected tissues, either from baits or host roots, probably have an adequate *Phytophthora* inoculum allowing the use molecular techniques for pathogen diagnosis (Moreira et al. 2007).

4.6.3- *Phytophthora cinnamomi* occurrence and stands health index

It is referred that *P. cinnamomi* detection is reduced in areas with high mortality since declining trees are posteriorly affected by opportunistic pathogens, reducing the probability of detecting this weak competitor (Tsao 1983, Jung 2011). In the study *P. cinnamomi* was surveyed in the rhizosphere of trees with moderate to low declining symptoms -in order to avoid possible antagonism and interference of fast-growing associated secondary microflora- regardless of health index of the stands, thus it was expected a similar likelihood in detecting *P. cinnamomi* in any of the stands. Higher occurrence of *P. cinnamomi* in stands with high tree mortality suggests an association between the pathogen and tree mortality. However, *P. cinnamomi* was also present in stands with significant regeneration where apparently it as no injurious effect. The role of *P. cinnamomi* in cork oak mortality is considered to be toughly associated with other abiotic factors like soil characteristics and climate conditions (Brasier et al. 1993 Moreira & Martins 2005) and it may be possible to detect the pathogen in apparently healthy stands if no other factors are affecting the trees. In declining stands, a synergism between *P. cinnamomi* and unfavorable conditions may cause tree mortality.

Finally, in the study site management practices like soil tillage with harrowing are performed by machines that may spread the pathogen through the stands, along with dispersal by water flow. It should be expected that a true negative in pathogen detection should be due to the existence of suppressive soils. A reliable method *P. cinnamomi* diagnosis would be an important tool to study the association between tree decline and the pathogen, as well identification of suppressive soils for posterior studies.

4.7 –Conclusions

Baiting selective-medium technique is cost-saving though time consuming and requires expertise for species identification. Moreover, it requires a permanent stock of vegetal traps, otherwise diagnosis could only be realized in the appropriate seasons, in this case during sprouting of cork oak leaves. On the other hand, molecular methods are more expensive and require special equipment, though less time consuming and efficient in species identification. However, success obtained with diagnostic PCR was negligible probably due to reduced likelihood in obtaining the target DNA from small amount of soil samples. Baiting selective-medium methods efficiency was reduced due to contamination by opportunistic pathogens, however, it is possible to overcome these obstacles through specific approaches, making this method more appropriate for *P. cinnamomi* diagnosis. When detection of *P. cinnamomi* is not overlaid by contaminants, negative results can be interpreted as lack of inoculum in the soil sample, and limitations to *P. cinnamomi* detection can be reported to amount of soil samples. On the other hand, using PCR methods to diagnose the pathogen from infected tissue – either from host or bait, combining the two methodologies- may be a feasible alternative, with emphasis in the detection of the pathogen from naturally infected tissues, avoiding the use of several techniques.

CHAPTER 5

Ecophysiologic studies on cork oaks under decline

This chapter was submitted to Tree Physiology with the reference:

Camilo-Alves CSP, Vaz M, Clara MIE, Ribeiro NMCA. Effect of prolonged stress in cork oak water relations – loss of resilience

5.1 ABSTRACT

Two main types of syndromes associated to cork oaks (*Quercus suber*) decline have been observed: a sudden death of the tree or a progressive and chronic decline. Decline symptoms are unspecific and usually attributed to water stress. In this study it was compared leaf water status of 22 declining and 22 asymptomatic cork oak trees in spring and summer using a pressure chamber. Reduced cork thickness previously observed in declining trees indicated that those trees had been under chronic decline for several years. Regardless of health status, all trees were in good watering conditions during spring, showing predawn leaf water potential (Ψ_{pd}) ≈ -0.4 MPa and midday leaf water potential (Ψ_{md}) ≈ -2.8 MPa, but in the summer symptomatic trees showed lower Ψ_{pd} (-1.99 ± 0.60 MPa) compared to asymptomatic trees (Ψ_{pd}= -0.80 ± 0.42 MPa). However, contrarily to expected, Ψ_{md} was higher in declining than in asymptomatic trees (-2.81 ± 0.42 and -3.28 ± 0.51 MPa respectively) and, consequently, sapflow driving force was found to be 1.66 MPa less in trees suffering chronic decline. Drop of Ψ_{pd} is usually related to short-term water stress but this study shows that high values of Ψ_{md} are related to chronic stress. This may occur when stomatal conductance and, consequently, transpiration, are significantly reduced. It was measured leaf gas exchange and chlorophyll fluorescence in a sub-sample of 12 trees with an infrared gas analyzer and leaf chlorophyll content with a portable steady-state photosynthetic system and observed that transpiration, stomatal conductance, efficiency and quantum yield of photosystem II, as well chlorophyll content were lower in declining trees in comparison to asymptomatic ones during the dry season. Lower summer sapflow driving force and down-regulation of photosynthesis suggests that chronic dieback is associated to reduction in nutrient root uptake and/or leaf carbon assimilation, eventually leading to

plant death of starvation. On the other hand, one tree underwent sudden death after this study. This tree presented the lowest predawn leaf water potential (Ψ_{pd}= -3.62; Ψ_{md}= -3.75 MPa) well below the cavitation threshold (-3 MPa) suggesting mortality caused by dehydration. Contrarily to chronic decline, where dying trees close stomata to avoid hydraulic failure, limiting CO2 and nutrients uptake, "sudden death" appears to be a result of stomatal control failure in preventing xylem cavitation and runaway embolism – in accordance with the hydraulic-failure hypothesis. Finally, analyses to relative amount of shallow fine roots showed no relation with tree health status, but a significant association with *P. cinnamomi* occurrence. Tree water status was related to fine-root ratio, thought one could not infer causality and further studies are needed to infer the role of shallow fine roots in tree water status. *P. cinnamomi* effects on tree physiology were linked to symptoms of water stress, even when pathogen presence was not associated to decrease in tree water status. Starch content in coarse roots was vestigial and in leaves was only 7.24 ± 0. 91 mg g^{-1}, though a slightly more was measured in leaves from trees under *P. cinnamomi* effect (8.94 ± 1.09 mg g^{-1}) which could be due to sink-limitations of the trees. More studies are needed to understand the role of starch mobilization in cork oaks affected by the pathogen.

5.2- INTRODUCTION

Cork oaks (*Quercus suber*) are of high conservation and socioeconomic value within their areas of geographic distribution around the Mediterranean basin (DGRF et al. 2007). Cork is the outer layer that covers the tree trunk and branches and is removed every 9 years for commercial purposes. It is the main product obtained from this

silvopastoral system with its global production reaching 201.428 ton in 2010 and cork stoppers correspond to 70% of cork market (APCOR 2011). However, cork oaks are facing disturbances that are reducing their resilience, affecting the sustainability of the system. Two main types of syndromes associated to decline have been observed (Cobos et al. 1992; Tuset et al. 1996; Gallego et al. 1999; CAMA 2001; Moreira 2001; Ruiu 2006; Sousa et al. 2007): (1) a sudden death of the tree, characterized by the fast drying of the crown followed by tree death in one or two seasons, particularly in early summer after the winter rains and in early autumn following the dry season; yellow or brown leaves may remain attached to the tree for some time. (2) A progressive decline and gradual loss of foliage, where the first symptoms are drying of the tree top and sprouting of epicormic shoots, an intense leaf drop which may affect the whole crown or only some branches. Decline symptoms are unspecific and are usually attributed to water stress. Factors promoting impaired water absorption have been studied and were mainly attributed to drought (Macara 1975; Cabral et al. 1992; LLoret & Siscart 1995; Peñuelas et al. 2001), to soil characteristics that may limit root expansion (Bernardo et al 1992; Ribeiro and Surový 2007; Costa et al 2010) or root rot caused by the soilborne root pathogen *Phytophthora cinnamomi* associated to unfavorable abiotic conditions (Cobos et al 1992; Brasier et al 1993, Robin et al 1998; Sánchez et al 2002; Moreira and Martins 2005).

Mediterranean-climate regions are characterized by recurrent droughts, with 90% of annual precipitation falling in the six cool season months, and frequent periods of extended summer drought (Rundel et al. 1995) which may be aggravated in the future. According to most climate change scenarios for the region, the severity of the summer drought may increase as well as the frequency of severe droughts (Miranda et al. 2002; Giorgi & Lionello 2008). Several studies on physiologic responses of adult

cork oak trees to environmental stress, specially short-term droughts, have been performed (e.g., Faria et al. 1996; Garcia-Plazaola et al. 1997; Faria et al. 1998; Otieno et al 2006; Passarinho et al 2006; David et al. 2007; Otieno et al 2007; Grant et al. 2010; Vaz et al 2011; Pinto et al 2012). However, to our knowledge there are no published studies under field conditions on the physiologic status of cork oak trees showing chronic decline, nor on the physiologic differences between each type of declining syndrome. Studies on trees pushed beyond their optimal conditions for a long term will provide information about their physiological limitations to overcome stress events and may help us to minimize or reverse injury.

5.3- OBJECTIVES

Since water stress, irrespective of its origin, is considered the main cause of cork oak decline, this study focused on seasonal water relations of 43 *Q. suber* showing decline symptoms and asymptomatic ones under natural conditions. The study was complemented with exploratory measurements realized in a case-study of 12 trees located in 4 adjacent stands. In the case study several ecophysiologic measurements were realized in order to analyze tree physiologic status and in relation to occurrence of *P. cinnamomi* in the rhizosphere, and to fine-root ratio of the trees. The goal of the study was to analyze if: 1) trees suffering chronic decline present different water status than healthy trees under short-term water stress and 2) There was variation in photosynthetic parameters according to tree health status. 3) Tree water status was related to fine roots amount. 4) There could be physiologic differences between trees under water stress and trees with *P. cinnamomi* in the rhizosphere.

5.4- MATERIAL AND METHODS

5.4.1- Study site

The present study was carried out in 2010, between June and September, at Herdade da Machoqueira do Grou, Coruche, Portugal (see chapter 4 for the description). In winter and early spring precipitation was higher than long-term mean in Portugal (1971 to 2000, fig. 5.1) and cumulative precipitation between October 2009 and March 2010 was 40% higher than average for the region (IPMA 2010, 2011).

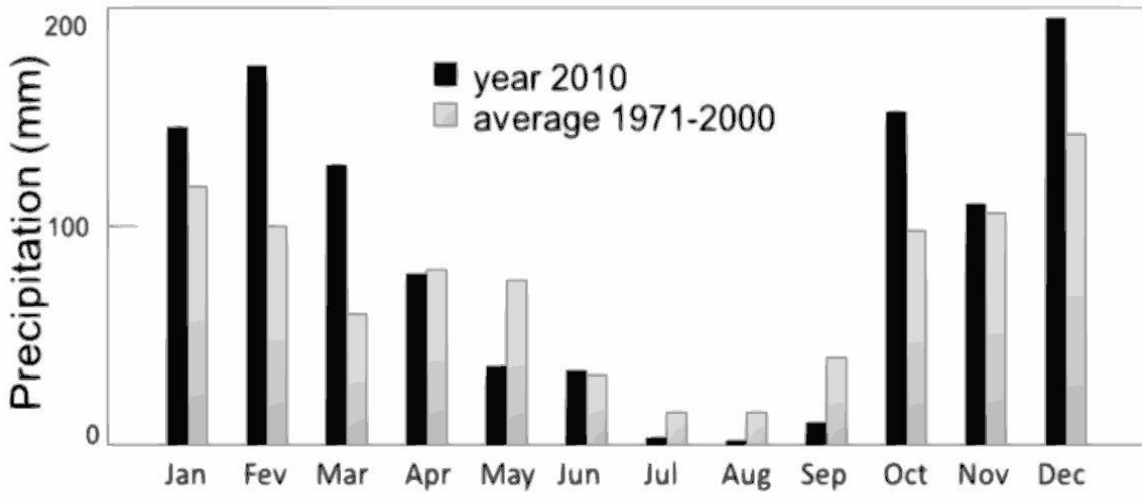


Figure 5.1: Monthly long-term average precipitation (1971-2000) and monthly precipitation in the year 2010 for Coruche region.

Eight managed cork oak stands were selected for the study, differing in soil conditions and mortality rate. Five stands located on Cambisols soils have no relevant limitations to cork oak growth and the remain three stands, located on gleyc lixisols and fluvisols soils, show deficient water drainage and present excess water holding capacity

for cork oak growth. Four to eight trees per stand, in a total of 43 trees, were selected in order to measure water status during two seasons. Perimeter at breast height (PBH) was about 33.76 ± 12.4 cm and trees were 9.18 ± 1.54 m high. About half of them presented decline symptoms like upper dead branches and epicormic shoots. The remaining trees were asymptomatic. For some statistical analyses, trees were divided into three classes: 0 – no declining symptoms; 1 – declining trees alive in the following year (2011); 2 - declining trees dead in the following year (2011); 3 – trees with sudden death symptom by the end of the summer (2010). Additionally, a sub-set of 12 trees, 7 of them presenting decline symptoms, were selected in 4 adjacent stands. This case study was performed in 3 stands with pronounced mortality were *P. cinnamomi* is active (chapter 4) and probably plays an important role in tree decline. From the 7 selected trees, all presented *P. cinnamomi* in the rhizosphere and 5 of them declining symptoms. In the 4th stand mortality rate was null, though 2 of the selected 5 trees showed upper branch dieback. Only 1 of the 2 declining trees had *P. cinnamomi* in the rhizosphere, according to previous surveys (chapter 4).

5.4.2- Dendrometric measurements

In 2007 and 2008 perimeter at breast waist was measured before (PBHb) and after (PBHa) cork extraction. Cork thickness was determined using the formula:(PBHb – PBHa) / 2π. Trunk diameter increment was calculated as follows: (PBHa – PBH) / 2π, where PBH is the initial perimeter at breast waist measured during plots installation, in 1995.

5.4.3- Water potential measurements

Predawn leaf water potential (Ψ_{pd}) and midday leaf water potential (Ψ_{md}) were measured using a Scholander pressure chamber (PMS 1000, PMS Instruments, Corvallis, Ore., Scholander et al. 1965). Three to four leaves from the south-facing side of the crown were sampled just prior to sunrise and at midday (12–13 h, local time). Samples were taken at similar height above ground to avoid variability due to hydrostatic water potential and leaf water potential was measured immediately after cutting. Measurements where realized during spring (June) and summer (August/ September) 2010.

5.4.4- Root measurements

The subsample of 12 trees was selected in order to avoid great proximity between trees, in order to reduce the probability of acquiring roots from other trees. Trees were distant from neighboring by at least 8 m. Shrubs are removed periodically through harrowing, and cattle is allowed to pasture in the stands, thus, no significant shrubs, whose roots may be mistaken with cork oak roots, grow in the study area. Grass roots can be easily identified and removed from the soil samples. Soil monoliths with 30 x 30 x30 cm were collected in four locations distant 1m from the trunk base of each tree. When cutting soil monoliths, it was observed if coarse roots were attached to main roots from the trees. Soil monoliths were transported to laboratory, where roots were sieved from the soil and carefully washed with tap water. Root samples were oven-dried at 80ºC during three days after what they were separate into fine roots (non-lignified roots, about up to 0.5 mm diameter) and coarse roots, measured and weighted.

5.4.5- Instantaneous gas exchange and chlorophyll fluorescence measurements

In the same sub-sample of 12 trees used for root analyzes, leaf gas exchange was determined simultaneously with measurements of chlorophyll fluorescence using the open gas exchange system Li-6400 (LI-COR Inc., Lincoln, NE, USA) with an integrated fluorescence chamber head (Li-6400-40; LI-COR Inc.). All measurements were made on young, fully expanded leaves, with photon flux density (PFD) at 1500 µmol m^2 s^1, with a CO_2 concentration in the leaf cuvette of 400 µlmol CO_2 per mol air. Block temperature was kept at 30 C during all measurements.Measurements were realized in summer in the morning period (10.00–11.00 h, local time) on four sun exposed leaves per tree.

The actual photochemical efficiency of photosystem II (/PSII) was determined by measuring steady-state fluorescence (Fs) and maximum fluorescence (F´m) during a light-saturating pulse of 8000 µmol photon m-2 s-1 following the procedures of Genty et al. (1989):

$$\phi PSII = \frac{F´m - Fs}{F´m}$$

The electron transport rate (Jflu) was then calculated as:

$$Jflu = \phi PSII \times PPFD \times \alpha \times \beta$$

where PPFD is the photosynthetically active photon flux density, α is leaf absorptance and β reflects the partitioning of absorbed quanta between photosystems II and I. The product $\alpha \times \beta$ was determined, following Valentini et al. (1995), from the relationship between /PSII and /CO_2 obtained by varying either light intensity under non-photorespiratory conditions in an atmosphere containing less than 1% O_2.

5.4.6- Starch measurements

In the sub-sample of 12 trees it was measured starch reserves from coarse roots with about 5 mm root and leaves collected in August. Bark and heartwood from roots were discarded. The remaining sapwood tissues and leaves were oven dried for 48h, being grinded and analyzed in duplicates for starch reserves using the amyloglucosidade α-amylase method (total starch kit, megazyme®). Initially, all glucose and maltodextrins were first removed from the samples with an 80% ethanol solution at 85ºC for 5 min and resistant starch was pre-dissolved by stirring the samples with dimethyl sulphoxide at 100ºC. Thereafter, starch was hydrolyzed into maltodextrins and then into glucose, following the manufacturer protocol. Glucose was quantitatively measured in a colorimetric reaction and the absorbance for each sample was read at510 nm in a spectrophotometer. Starch was estimated according to the following equation:

$\text{Starch (g /100 g)} = \Delta_A * F * 1.8$

Δ_A = Absorbance (reaction) read against the reagent blank.

$$\text{F (conversion from absorbance to µg)} = \frac{100\mu g \text{ of D} - \text{glucose}}{\text{absorbance for 100 µg of glucose}}$$

The same procedure was applied in leaves collected during summer.

5.4.7- Chlorophyll measurements

In the sub-sample of 12 trees, relative chlorophyll content was measured with a portable chlorophyll analyzer (Hansatech chlorophyll meter CL-01) and, in addition, chlorophyll concentration was also determined using an extraction method: Six circular disks, each 6.25 mm in diameter, were punched from the leaves where optical properties were measured. The disks were placed immediately into 8 mL of 100% methanol, and

pigments were allowed to extract in the dark at 30ºC for 24 h. Absorbances (*A*) of the clear extract at 652.0, 665.2, and 750 nm were read with a spectrophotometer and concentrations of chlorophylls *a, b,* were computed after Porra et al. (1989). Measurements at wavelength of 750 nm are used to correct turbidity and contaminating colored compounds but in these samples they were virtually zero.

Equations for chlorophyll concentration extracted with methanol, in nmol ml^{-1}

Chlorophyll a = $18.22 * A^{665.2} - 9.55 * A^{652.0}$

Chlorophyll b = $33.78 * A^{652.0} - 14.96 * A^{665.2}$

Chlorophyll a+ b = $24.23 * A^{652.0} - 3.26 * A^{665.2}$

Chlorophyll concentration of the extract (8 ml) was related to total disk surface area of 1.84 cm^2 [6 leaves each with area = 3.14 * (6.25mm / 2) ^{2}] were used to compute leaf chlorophyll concentrations per unit projected area.

5.4.8- Specific leaf area determination

To determine Specific leaf area (SLA, cm^2 g^{-1}), 20 leaves collected during summer from the sub-sample of 12 trees transported to the laboratory in refrigerated bags to avoid weight loss by respiration, oven-dried at 80ºC for 48 h, weighted after petiole removal and digitalized to calculate surface area with Imaje J 1.45s software.

5.4.9- *Phytophthora cinnamomi* occurrence

Occurrence of *P. cinnamomi* in the rhizosphere of each tree from the subsample was tested using the baiting selective-medium referred in chapter 4.

5.4.10- Statistical analyses

Statistical analyses were made using Systat v.13.1 software package. One-way ANOVA was used to test significance of differences observed between tree health status and cork thickness, trunk diameter and tree water status along seasons. Significant differences between treatments means were evaluated with Tukey's HSD tests with $p<0.05$. Before applying ANOVA tests, data were tested for normality by using Kolmogorov-Smirnov D test at the significance level of 0.05. When variance across groups was unequal, i.e. the usual ANOVA assumptions were not satisfied, the Welch-ANOVA test was applied. Two-sample T-Test with separate variance was used to compare significant differences between the means of tree water status according to soil limitations, and between the means of photosynthetic parameters according to tree health status and *P. cinnamomi* occurrence. Linear regression was applied to assess relationship between fine root ratio and Ψ_{pd}. Principal components analysis was applied to correlate ecophysiologic parameters with fine root ratio, *P. cinnamomi* occurrence and tree health status in the subsample of 12 trees.

All measurements shown are the mean ± standard error of the mean.

5.5- RESULTS

5.5.1- Cork thickness

Thickness of the 9 years old cork that was extracted two to three years before the study was on average 26.25 ± 8.34 mm and was thicker in healthy trees. Trees that died in the year following this study presented the narrowest cork ($p < 0.001$, fig. 5.2). On the other hand, there was no relation between tree health status and trunk diameter increment since plots installation ($p = 0.456$).

5.5.2- Trees water status

Variations in Ψ related to tree health status were only observed during summer (table 5.1). In spring, all trees showed high Ψ_{pd} and water status decrease equally at midday, resulting in similar sap flow driving force ($\Psi_{pd} - \Psi_{md}$) regardless of tree health status. In summer, trees were not able to maintain the same Ψ_{pd} observed in spring, but reduction was stronger in declining trees ($p < 0.001$, fig. 5.3).

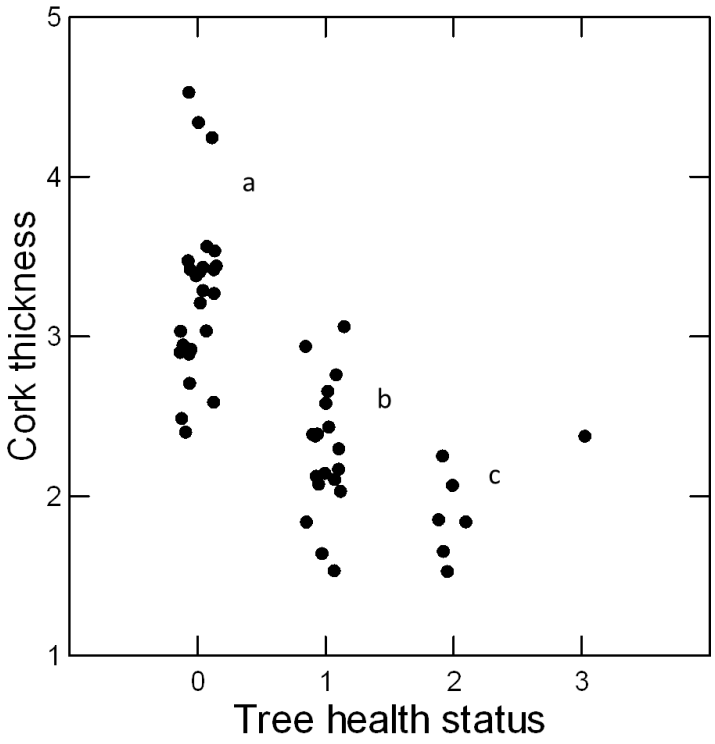


Figure 5.2: Relationship between thickness of the 9 years old cork extracted in 2007 and 2008 and tree health status (n= 37) classified in 2010 according to decline symptoms: 0- asymptomatic, 1- trees with upper branch dieback and epicormic shoots, 2- symptomatic trees considered dead in 2011, 3- tree that suffers from sudden death in the end of the summer 2010. Different letters denote statistically significant differences at the 5% level

On the other hand, significant variation in Ψ_{md} along seasons was only observed in healthy trees, showing reducedΨ_{md} in summer and maintaining the same sapflow driving force along seasons (fig. 5.4). Therefore, contrary to what was expected trees showing symptoms of water stress were better hydrated at midday summer than asymptomatic trees (p = 0.002, fig. 5.3) and, consequently, sap flow driving force was strongly reduced (table 5.1, fig. 5.4). In the year following this study, six of the declining trees were considered dead and cut down. Yet, during the study those trees had the same summer water status than the remaining declining trees (Ψ_{pd}: p = 0.13; Ψ_{md}: p = 0.39; sap flow driving force: p = 0.09; fig. 5.3 and 5.4).

Table 5.1: Average water status and physiologic parameters on asymptomatic and declining *Quercus suber* trees (n= 43).

	Spring		Summer	
Parameter	**Asymptomatic trees**	**Declining trees**	**Asymptomatic trees**	**Declining Trees***
Ψ_{pd} (MPa)	-0.44 ± 0.227[a]	-0,41 ± 0,16[a]	-0.80 ± 0.42[b]	-1.99 ± 0.60[c]
Ψ_{md} (MPa)	-2.92 ± 0.48[ab]	-2,78 ± 0,43[a]	-3.28 ± 0.51[b]	-2.81± 0.42[a]
Ψ_{pd} - Ψ_{md} (MPa)	2,48 ± 0,50[a]	2.37 ± 0.51[a]	2.48 ± 0.71[a]	0.82 ± 0.72[b]
gs** (mol m^{-2} s^{-1})			0.24 ± 0.09[a]	0.10± 0.02[b]
*A*** (µmol CO_2 m^{-2} s^{-1})			7.82 ± 5.39[a]	1.93 ± 1.81[b]
WUE (A/gs) ** (µmol CO_2 mol^{-1} H_2O)			31.54 ± 3,78[a]	20.01 ± 8.18[b]
*E*** (mmol m^{-2} s^{-1})			5.74 ± 3.79[a]	2.02 ± 0.18[b]
*Ci*** (ppm)			294.0 ± 28.7[a]	322.9 ± 27.9[b]
F'_V/F'_M**			0.63 ± 0.55[a]	0.43 ± 0.05[b]
ϕ_{PSII}**			0.27 ± 0.20[a]	0.14 ± 0.01[b]
QP**			0.45 ± 0.37[a]	0.32 ± 0.05[a]
QN**			2.75 ± 2.30[a]	1.79 ± 0.16[b]
Leaf chlorophyll µmol m^{-2}**			401.6 ± 41.0[a]	300.9 ± 30.5[b]
Fine roots (g) / coarse roots (m)**			21.80 ± 14.12[a]	7.61 ± 7.08[a]

Declining trees presented upper branch dieback and epicormic shoots. Different letters denote statistically significant differences at the 5% level. Ψ_{pd}: Predawn leaf water potential; Ψ_{md}: midday leaf water potential; Ψ_{pd} - Ψ_{md}: sapflow driving force; gs: stomatal conductance; *A*: photosynthetic rate; *WUE*: intrinsic water-use efficiency; *E*: transpiration rate; *Ci*: intercellular CO_2 concentration; F'_V/F'_M: maximum efficiency of photosystem II; ϕ_{PSII}: quantum yield of photosystem II; QP: photochemical quenching; QN: non-photochemical quenching.
* Excluded values from the tree that showed sudden death
** n = 12
**n = 12

Another declining tree suffered from sudden death by the end of the summer in the year of the study. Contrarily to others that became gradually defoliated, this one showed a different dieback symptomatology, where leaves suffered chlorosis in a few weeks and remained attached to branches. This tree presented the lowest predawn leaf water potential (Ψ_{pd} = -3.62; Ψ_{md} = -3.75 MPa, fig. 5.3).

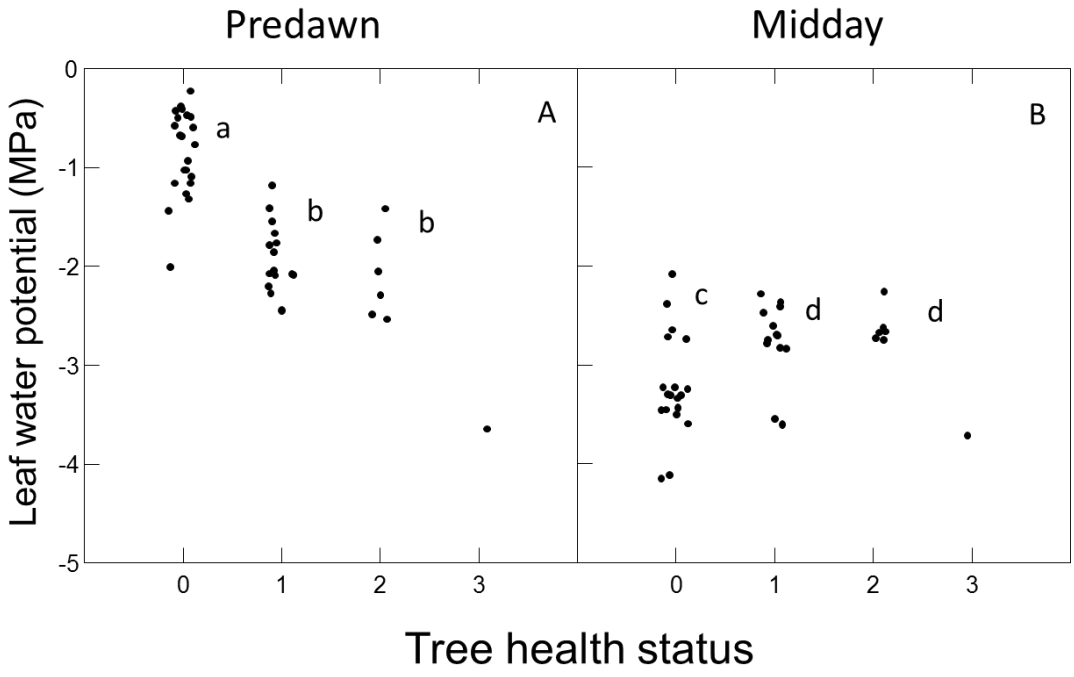


Figure 5.3: Water status of *Quercus suber* trees (n = 43) at (A) predawn and (B) midday measured in summer 2010, according to tree health status. Decline symptoms: 0- asymptomatic, 1- trees with upper branch dieback and epicormic shoots, 2- symptomatic trees considered dead in 2011, 3- tree that suffers from sudden death in the end of the summer 2010. Different letters denote statistically significant differences at the 5% level

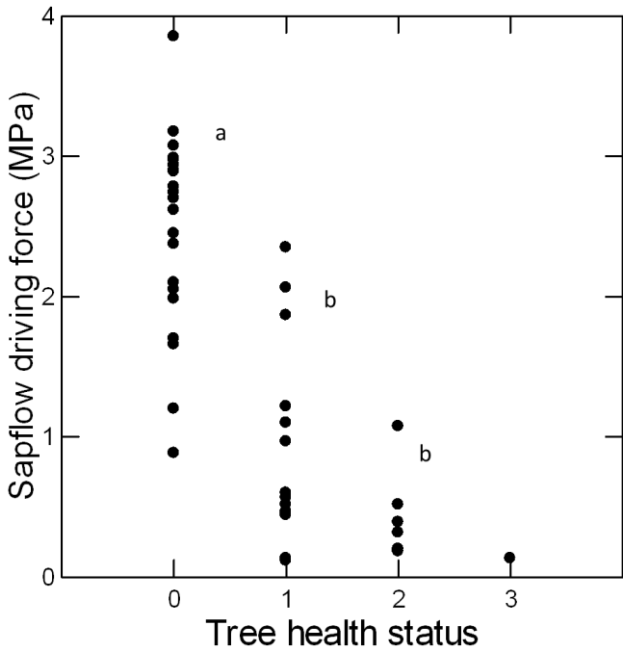


Figure 5.4: Sapflow driving force of *Quercus suber* trees (n= 43) measured in summer 2010 according to decline symptoms: 0- asymptomatic, 1- trees with upper branch dieback and epicormic shoots, 2- symptomatic trees considered dead in 2011, 3- tree that suffers from sudden death in the end of the summer 2010. Different letters denote statistically significant differences at the 5% level.

5.5.3-Gas exchange and chlorophyll fluorescence measurements

Physiologic analysis realized in summer on the subsample showed that declining trees had reduced leaf gas exchange, but reduction in net CO_2 assimilation was not accompanied by reduction in intercellular CO_2 concentration in the mesophyll, though it was accompanied by decline in efficiency, quantum yield of photosystem II and leaf chlorophyll content (table 5.1). Declining trees also showed reduction in water used efficiency and non-photochemical quenching.

5.5.4-Fine root ratio

There was not enough independent data to apply statistical analyses to infer a relation between soil limitation and root ratio, but it was observed that all trees located in soils with tendency to accumulate water had reduced fine-roots ratio (fig. 5.5) and, moreover, trees in soils with deficient water drainage were the poorest hydrated at predawn in summer (p = 0.001; fig. 5.6). Accordingly, in the sub-sample it was observed a gradual decrease in Ψ_{pd} associated to reduction in fine root ratio (p = 0.04, fig. 5.7).

Reduction in fine-root ratio was strongly related with *P. cinnamomi* occurrence (fig.5.7). Trees with the pathogen in the rhizosphere had less than 11g of unlignified fine roots by 1m of lignified roots. In contrast, trees where the pathogen had not been recovered showed more than 15 gm^{-1}.

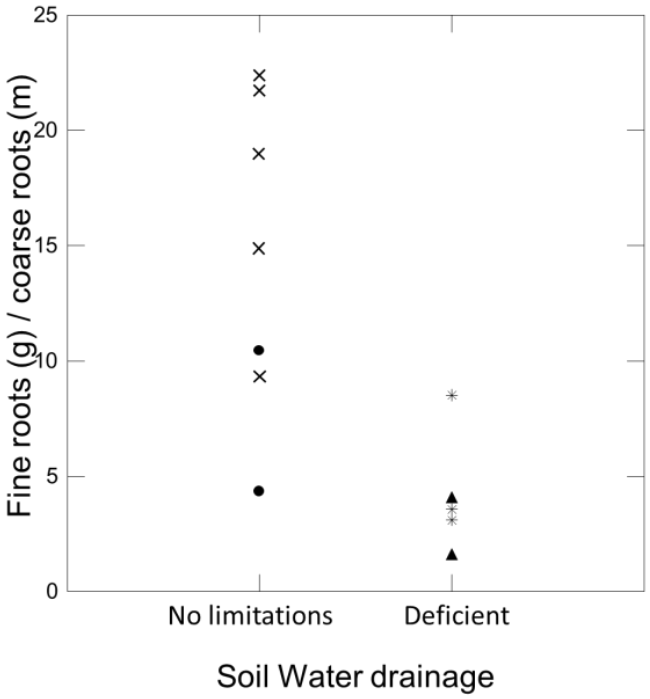


Figure 5.5: Shallow fine root ratio of *Quercus suber* trees (n= 12) in relation to stand soil limitations regarding water drainage. Similar symbols denote trees in the same stand

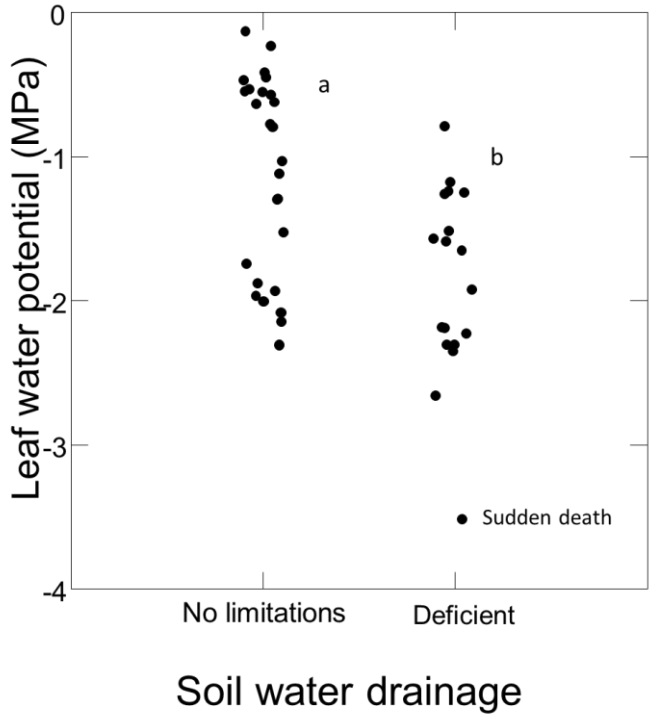


Figure 5.6: Predawn water status of *Quercus suber* trees (n= 43) measured in summer 2010 in relation to stand soil limitations regarding water drainage. Different letters denote statistically significant differences at the 5% level. Tree that suffers from sudden death was not considered in statistical analyze

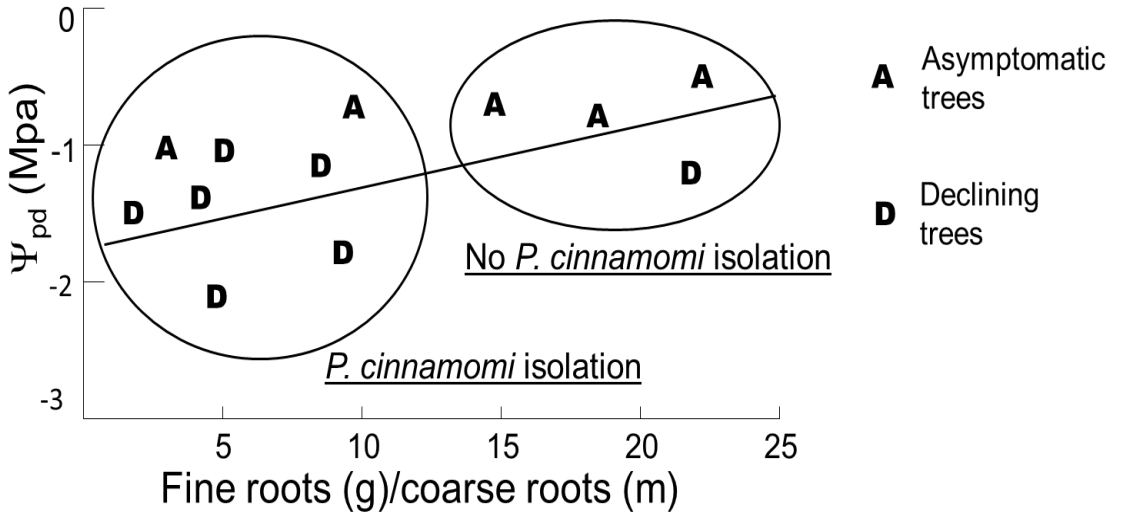


Figure 5.7: Association between predawn leaf water potential and shallow fine root ratio of *Quercus suber* trees (n= 12) in relation to *Phytophthora cinnamomi* occurrence and tree health status

5.5.5- Relationship between *Phytophthora cinnamomi* occurrence and trees parameters

With principal component analyzes, parameters were not clearly separated since there were somewhat correlated, but it was possible to observe a tendency to separate parameter related to Ψ_{pd} with parameters related to *P. cinnamomi* occurrence. Accordingly, tree health status was strongly related with Ψ_{pd} and efficiency of photosystem II. On the other hand, fine-root ratio and leaf starch content were associated with *P. cinnamomi* occurrence (fig. 5.8, table 5.2). Other parameters like stomatal conductance, photosynthesis and chlorophyll content were partially associated to both groups. In fact, those parameters are significantly related to either Ψ_{pd} and *P. cinnamomi* occurrence (T-tests: $p< 0.05$)

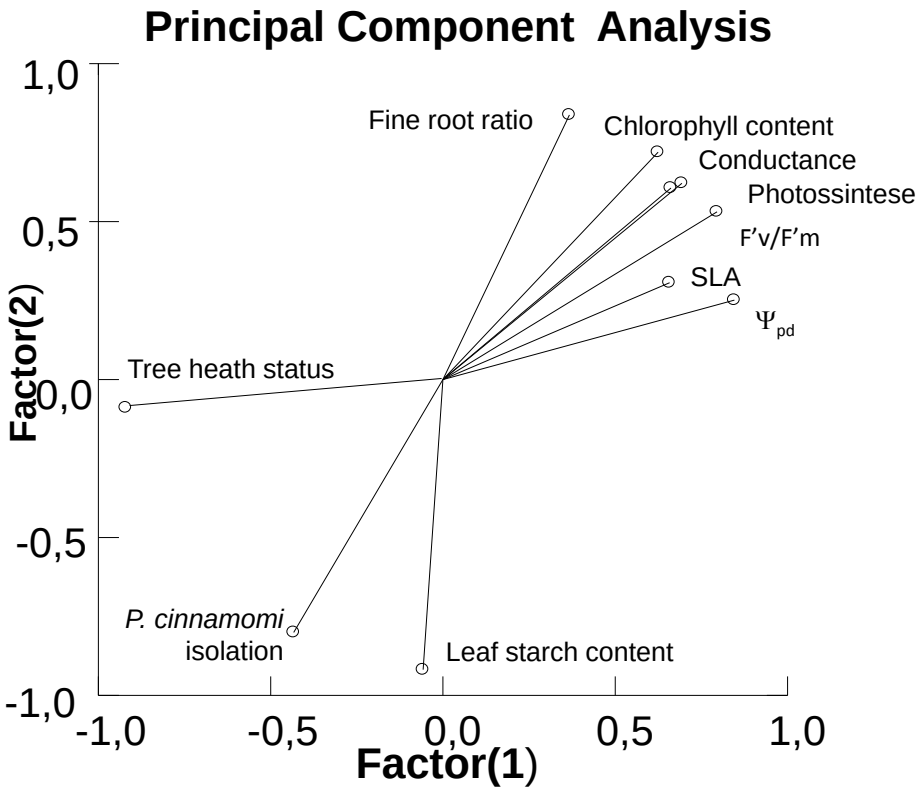


Figure 5.8: Principal component analysis of the ecophysiologic and structural parameters measured in healthy and declining *Quercus suber* affected or not by *Phytophthora cinnamomi*

Table 5.2: Correlation between the parameters and the factors extracted with principal component analyses.

Parameter	Factor 1	Factor 2
Tree health status	**-0.923**	-0.098
Ψ_{pd}	**0.846**	0.251
F'_V / F'_M	**0.796**	0.531
Photosynthetic rate	0.693	0.622
Stomatal conductance	0.662	0.606
Specific leaf area	0.658	0.306
Chlorophyll content	0.624	0.720
Leaf starch content	-0.057	**-0.919**
Fine-root ratio	0.367	**0.838**
P. cinnamomi occurrence	-0.433	**-0.801**

Extraction method: Maximum likelihood. Rotation method: Varimax with Kaiser normalization. In bold the 3 parameters that explain better each factor

Leaf starch content was the only parameter related to the presence of *P. cinnamomi* (T-test: p = 0.024) and not with tree health status or tree water status (T-tests: p> 0.05) being significantly higher in trees affected by the pathogen (8.94 ± 1.09 mg g^{-1} *vs*. 7.24 ± 0. 91 mg g^{-1}; fig. 5.9).

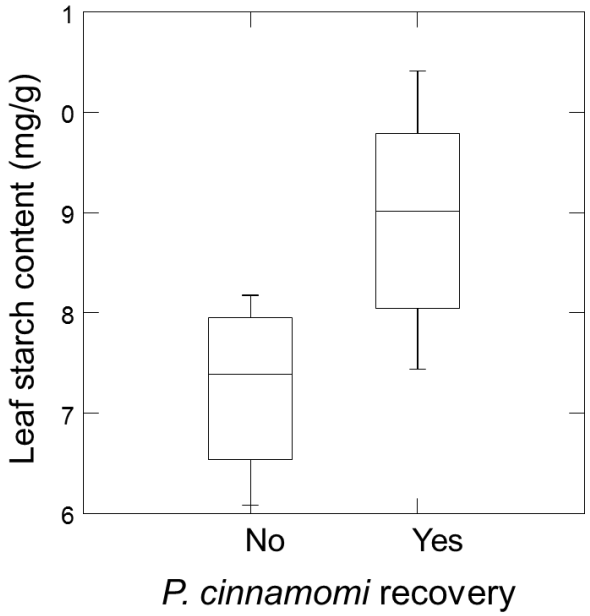


Figure 5.9: Box plot of *Quercus suber* leaf starch content in relation to *Phytophthora cinnamomi* recovery in the rhizosphere

5.6- DISCUSSION

5.6.1- Effect of prolonged stress in cork oak water relations: loss of resilience

Dieback of branches and epicormic shoots are unspecific symptoms and can be associated with changes in soil moisture or virulent pathogens (Ciesla & Donaubauer 1994**).** Epicormic shoots, common in oaks, are often stimulated by sudden exposure to

light (Kerr & Harmer 2001; Kozlowski & Pallardy 1997) which, in turn, may be a consequence of defoliation after decline in water supply (Larcher 1995). Dieback of the most upper twigs suggests impairment in tree water balance. In fact, those symptoms were strongly associated to low tree water status (fig. 5.8, table 5.2). In general the upper part of the canopy is subjected to the lowest tree water potential because of the greater effect of the gravity (Larcher 1995) and hydraulic architecture of the tree (Tyree & Sperry 1989). As decline in water supply intensifies, embolism will preferentially occur in minor branches where xylem tensions are greatest (Tyree & Sperry 1989; Rust & Rolof 2002). After branch shedding trees shrink in height (Ribeiro 2006) but eventually improve water balance in surviving shoots (Tyree & Sperry 1989), indicating that branch sacrifice may provide adaptation to drought events (Rood et al. 2000). However, though branch dieback and epicormic leaves are an indication that trees are under stressful events, it is difficult to infer whether it refers to a temporary situation or that the trees are under chronic stress. In this study it was analyzed cork thickness since it may be a good indicator of the long term tree water status. Several studies reported an association of cork thickness with water availability, as cork growth was limited in trees subjected to drought (Caritat et al. 2000) and trees presenting symptoms of water stress, like upper branches dryness and defoliation, tend to have narrower cork width (Costa et al. 2003; Ben Jamâa et al. 2005). Furthermore, cork thickness is related to variables that interfere with tree water availability, like tree size (Ben Jamâa et al. 2005; Sánchez-Gonzaléz et al. 2007) and intra-competition (Ribeiro 2006; Sánchez-Gonzaléz et al. 2007). Usually, plants under water stress show reduction in photoassimilates and increase their allocation to root growth in order to increase root/shoot ratio (Dickson and Tomlinson 96). Particularly in healthy cork oaks, trunk increment is related to climatic variation but cork increment show less inter-annual variations (Costa et al.

2002), however, trees in poor sanitary conditions have narrower cork (Costa et al. 2003). Our results are in accordance since only cork thickness is related to tree health (fig. 5.2). In order to occur significant reduction in cork development, trees should be under intense environmental stress and reduced cork thickness indicates that the studied symptomatic trees where under chronic decline.

Although summer precipitation was insignificant during the study year (fig. 5.1), cumulative precipitation in previous seasons was considerably higher than long-term mean and deep water resources should be still available for tap root trees uptake in the dry season. Thus, asymptomatic trees were in good hydric conditions at predawn by the end of the dry season (table 5.1, fig. 5.3): average summer Ψ_{pd} slightly decline and was higher than -1 MPa. Other authors found markedly reduced values during the same season, usually lower than -2 MPa (e.g. Chaves et al. 2002; Otieno et al. 2007; David et al. 2007; Vaz et al 2010; Pinto et al. 2012) which were associated to years with lower precipitation than average and/or limitations in root access to groundwater. The role of deep water sources in maintaining a good tree water status during summer is an important strategy for sclerophyllous oaks survival in the hot and dry conditions of the south Iberian Peninsula (David et al. 2004; Otieno et al. 2006). The authors observed that during summer water is extracted progressively from shallow to deeper soil layers and differences in summer Ψ_{pd} among trees is usually related to differences in access to soil water resources at progressively deeper soil layers. Thus, plant potentials reflect the wettest soil water potential accessed by roots (Otieno et al 2006, David et al. 2007).
Moreover, tap roots access of deep water resources also facilitates shallow root growth and persistence by hydrating them during the summer night through hydraulic lift, i.e. a passive mechanism driven by a water potential gradient that transports water through the root system from deep moist soil layers up to shallower and drier soil layers

(Richards and Caldwell 1987, Caldwell et al. 1998, Kurz-Besson et al. 2006, Nadezhdina et al 2008). Therefore, differences in water status found between asymptomatic and declining trees in this study, as well differences in fine root survival, may reflect different access to deep soil water resources by the trees.

Reduction in fine roots may also be caused by excess water, since waterlogging causes cork oak root hypoxia (Jacobs et al.1996). There was no trees in soils with deficient water drainage with fine root ratio as high as values found in soils with appropriate water drainage (fig. 5.5) and, additionally, there was a significant tendency to find less hydrated trees in soils with excess water retention (fig. 5.6).However, Ribeiro (2006) found no relationship between deficient soil drainage and mortality for the same study area; instead, mortality was related to soil depth and was higher in soils limiting access to deep water sources, suggesting the importance of the deep root system in cork oak survival. Other studies also reinforce the role of deep roots in maintaining tree water status during the dry season (Otieno et al 2006, David et al 2007), but the association between tree water status and shallow fine roots in adult trees remains to be established.

In our study, cork oaks showing chronic stress symptoms were able to maintain high Ψ_{pd} during the growing season (spring, table 5.1) and the significant decrease inΨ_{md} indicates they are transpiring as healthy ones. This pattern was also observed in holm oaks affected trees, with Ψ_{pd} measured during the wet seasons was as high as in healthy trees (Sala & Tenhunen 1994). However, plants with favorable water status in the rainy season may already have some physiological functions, like photosynthesis, affected by chronic stress. This was observed in holm oak seedlings subjected to repeated drought, where seedlings presented appropriate water status during the re-watering cycles, but photosynthesis recovered only to 80% of control values due to

persistently low stomatal and mesophyll conductances to CO_2 (Galle et al 2011). Drought associated with high temperatures and excess of light may result in a chronic photoinhibition or down-regulation of photosynthesis (Osmond 1994; Ripullone et al. 2009).In fact, our results show that summer reduction in the photosynthetic apparatus was stronger in declining trees. In studies concerning healthy trees suffering seasonal drought events (Faria et al. 1998, Chaves et al. 2002, Grant et al. 2010, Vaz et al. 2011) it was also observed a decrease in the pool and efficiency of the photosystem II open centers, driven by low chlorophyll content. However, plant water use efficiency increases with water stress, as well plant ability to dissipate excitation energy by other mechanisms than photosynthetic C-metabolism (non-photochemical quenching). In this study, reduction in water use efficiency observed in trees under chronic decline, along with high intercellular CO_2, shows the role of non-stomatal limitation of photosynthesis under increasing drought conditions (Shardendu et al. 2011). Although this study shows that trees under chronic stress are less protected against the potential for photo-oxidative damage since they show inferior values of non-photochemical quenching (Müller et al. 2001), higher values of initial fluorescence may suggest some protection against heat stress (Chaves et al. 2002).

Differences between healthy and declining tree water status were visible only during the drought period: reduced sapflow driving force as well reduced stomatal conductance found in declining trees in the dry season (table 5.1, fig. 5.4) suggest a strong stomatal control in order to avoid runaway embolism caused by water loss through transpiration until below the xylem cavitation threshold (Vilagrosa et al. 2003). These results are consistent with carbon-starvation hypothesis that associates stomatal closure to prevent hydraulic failure during long lasting drought conditions and reduction of photosynthetic uptake of carbon, as well mineral absorption by the roots, until the

plant starves (McDowell et al. 2008). In other studies (David et al. 2004; Otieno et al. 2006; Pinto et al. 2012), non-symptomatic cork oaks subjected to summer water stress (with Ψ_{pd} lower than -2MPa) presented higher sap flow driving force due to lower Ψ_{md}, indicating that occasional drought did not force trees to markedly reduce transpiration: healthy trees allow the drop of Ψ to about -3 MPa, operating near the critical value (-2.9 MPa; Pinto et al. 2012) when occurs 50% loss in hydraulic conductivity. Although predawn water status indicates that trees are under temporary water shortage, in this study it was found that midday water status was related to chronic water stress and, contrary to expected, was higher in symptomatic trees (fig. 5.3). To our knowledge this was not yet reported and it is significant since it shows that the main cause of chronic decline is not associated to reduction in tree water status to below safety margins. This assumption is reinforced by similar water status and sap flow driving force in trees at different stages of decline (fig. 5.3 and 5.4), indicating that mortality was not a consequence of tree dehydration. Instead, our results showed that chronic decline is associated to reduction in transpiration caused by stomata closure and down-regulation of photosynthesis. Limitation in water flow through the soil-plant-atmosphere continuum may reduce mineral root uptake, down-regulation of photosynthesis reduces leaf carbon assimilation and plants will eventually die of starvation (Kramer and Boyer 1995). Healthy trees subjected to recurrent droughts or dryer conditions acclimatize their metabolic and structural capabilities in order to improve their functioning under stress (Chaves et al. 2002; Limousin et al. 2010). Mortality processes operate on long timescales, with recurrent water stress likely weakening trees and pushing them to their physiologic limits, until they lose resilience.

Contrarily to chronic decline, where dying trees close stomata to avoid hydraulic failure and down-regulate photosynthesis, thus limiting CO_2 and mineral uptake,

"sudden death" appears to be a result of stomatal control failure in preventing xylem cavitation and runaway embolism – in accordance with the hydraulic-failure hypothesis (McDowell et al. 2008). The tree that died suddenly presented the lowest water potential at predawn (-3.62 MPa, fig. 5.3). It has already been established for *Q. suber* that leaf Ψ values of –3 MPa are equivalent to xylem water potentials of –2MPa at which cavitation commences, i.e., cavitation threshold (Tyree & Cochard 1996; Cruiziat et al. 2002; Otieno et al. 2007). It is possible that sudden death occurred after xylem embolism when leaf Ψ dropped below the critical threshold. This observation shows the difference in water status between chronic decline and sudden death (fig. 5.10). One suggest that this may be eventually caused by rapid root destruction, either caused by excess water during spring, root rot pathogens or a synergism between them. If abcisic acid (ABA) is produced in root tips or very close to it (Schachtman & Goodger 2008) their rapid destruction may prevent ABA root signaling to control stomatal aperture. Embolism disrupts the water flow through the soil-plant-atmosphere continuum and trees eventually dye from dehydration.

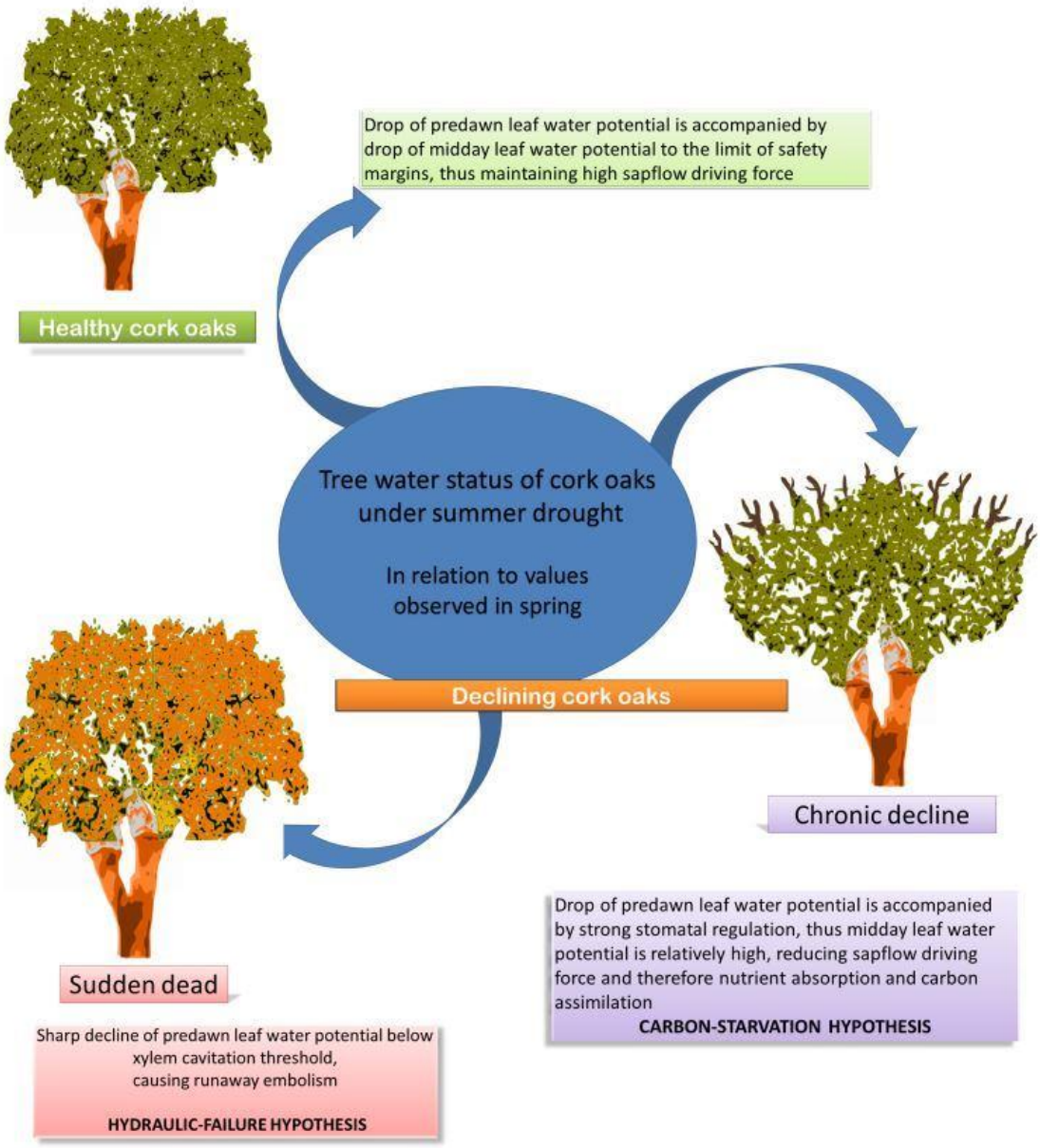


Figure 5.10: Variation in tree water status from spring to summer in relation to health status of the trees.

5.6.2- Preliminary data on physiologic conditions of cork oaks located in stands where *Phytophthora cinnamomi* is active

This study was an exploratory approach to analyze whether there would be any physiologic parameter associated to *P. cinnamomi* infection rather to waters stress. The sampling size is therefore reduced, joining information previously collected with the occurrence of *P. cinnamomi*, allowing a new approach for the physiologic measurements already gathered on this research.

It is thought that the main effect of *P. cinnamomi* attack in oak trees is impairment in water absorption caused by destruction of the root system, leading to tree water stress. For this reason, search for specific symptoms of *P. cinnamomi* infection is a challenging purpose. Studies concerning the effect of pathogen infection were only realized in seedling, which may not reflect the response of adult trees. Cork oak seedlings growing in containers do not possess sink root for water absorption, like adult ones. Thus, the effect of *P. cinnamomi* in the root system of adult trees should be rather different and, consequently, its effect on tree water relations. In this study, the strong relation between *P. cinnamomi* and fine-root ratio suggested their destruction by the pathogen; Although there was a tendency for decline in tree water status with reduction of fine-root ratio (fig. 5.7), tree water status was not significantly associated with *P. cinnamomi* occurrence, suggesting a more complex relation between these factors. Corcobado et al. (2013) also observed that fine roots were considerably lower in *P. cinnamomi*-infected than in *P. cinnamomi*-non-infected holm oaks and, moreover, declining holm oaks also showed reduced water status; however, there were no reports on the significance of *P. cinnamomi* infection in tree water status.

There was 1 asymptomatic tree with *P. cinnamomi* in the rhizosphere that showed reduced fine root ratio (10.43 g m^{-1}) but presented good water status (Ψ_{pd}= - 0.6 MPa). However, photosynthetic rate (= 2.81 µmol CO_2 m^{-2} s^{-1}) and stomatal conductance (= 0.094 mol m^{-2} s^{-1}) were lower to that expected for the observed water status (similar to values observed in declining trees, table 5.1). This may happen if destruction of fine roots causes ABA root signaling to control stomatal aperture despite favorable water status, and decrease in stomatal conductance reduces in turn photosynthetic rate, explaining these results. These results indicate that the effect of *P. cinnamomi* infection on cork oak trees in some cases may not be only related to enhancement of water stress by impairment in water absorption. Instead, the pathogen may induce reduction of photosynthetic rate caused by stomatal closure, triggering tree starvation. Studies with seedlings in controlled conditions, as well studies with other host and *Phytophthora* species, also report these 2 different reactions to infection (Sterne et al. 1978; Ploetz & Schaffer 1989; Robin et al. 2001; Maurel et al. 2001a, b; Fleischmann et al. 2002, 2005; Tapias et al. 2008a), which are reflected on the sequence of the symptoms: reduction in leaf Ψ followed by stomatal closure, or on the contrary, stomatal closure before variation in leaf Ψ. Nevertheless, the most observed response was stomatal closure after drop of leaf Ψ.

Other exception to the results was a tree with dieback symptoms, negative for *P. cinnamomi* and with high fine-root ratio (fig. 5.7). It is referred that trees suffering water stress increase their root/shoot ratio by leaf shed or allocating assimilated carbon to root growth (Larcher 1995; Dickson and Tomlinson 1996, Chaves et al. 2002). This tree showed the highest fine-root ratio, indicating that, as response to water stress, upper branches are being sacrificed and resources are being invested in root development. In the other studied trees, it is possible that strong infection by *P. cinnamomi* prevent trees

from reacting to water stress through fine root production. However, hosts under water stress – indicated by decline symptoms- are able to produce great amount of fine roots even in the presence of the pathogen (Blom et al. 2009). Highly susceptible sweet chestnuts with decline symptoms had significantly more fine roots than asymptomatic ones, indicating that under certain circumstances susceptible hosts suffering water stress can produce high root amounts in the pathogen presence.

Most of physiologic parameters were related to both *P. cinnamomi* occurrence and water stress (indicated by low Ψ_{pd}) however, leaf starch content was related only to the presence of *P. cinnamomi* and, peculiarly, was higher in trees affected by the pathogen. It was expected that partitioning of assimilated carbon would favor secondary metabolites, like phenolics, that play a role in the resistance mechanisms of plants against pathogens (Lattanzio et al. 2006). Instead, it was detected a slight increase in the starch reserves. This pattern was already observed in an experiment (Clemenz et al. 2008) were infection by *Phytophthora alni* in *Alnus glutinosa* saplings increased leaf starch, which was thought to be related with impaired phloem transport from leaves to roots after cortical tissue destruction. Higher concentrations of starch was also observed in the leaves of low watered Olives (*Olea europaea*), in spite of the lowest photosynthetic rates, suggesting again that carbon was not translocated out of the leaves because these plants were sink-limited (Bacelar et al. 2006). Nevertheless, overall leaf starch in this study was only residual when compared to values observed in other studies (leaf starch content in Olives: 60.5 to 70.4 mg g^{-1}, Bacelar et al. 2006; in *Alnus*: 35.8 to 87.7 mg g^{-1}, Clemenz et al. 2008) and with values obtained with leaves of cork oak seedlings (≈ 50 to 80 mg g^{-1}, Vaz et al. 2002). Low leaf starch content were detected in trees with photosynthetic rate higher as 15.82 µmol CO_2 m^{-2} s^{-1} and with good water status, indicating it was not a consequence of reduced carbon assimilation

nor a response to water stress. Starch is often considered as the mainform of carbon storage in plants. When facing water stress, there are usually is a shift in carbon flow from starch to sucrose and other low molecular weight compounds. Such shifts aid in the maintenance of turgor and increase transportable compounds, enabling osmotic adjustment and sustaining export during stress events (Morgan 1984; Chaves 1991, Dickson & Tomlinson 1996). It would be expected a reduction in starch, particularly an increase in the sucrose/starch ratio (Chaves & pereira 1992, Épron & Dreyer 1996). In this study it was not observed a relationship between starch and water stress, probably due to overall low content of leaf starch.

Starch stored in roots was virtually none. Additional tests in roots with similar diameter from olives were performed, resulting in much more starch measured, meaning that the enzymatic test was able to analyze starch content in roots. It is considered that starch reserves in oaks are mainly stored in roots, but it is possible that stems or lignotubers may account for preferential starch pools (Molina & Verdaguer 1993; Dickson & Tomlinson 1996) during summer, and/ or photosynthetic products transported to roots are been used for production of structural tissues or other compounds like phenolics, associated with cork oak defense response *P. cinnamomi* infection (Pires et al. 2008, Horta et al. 2010). After all, starch is often considered a storing or "overflow" carbohydrate pool for excess carbon fixed during periods of high photosynthetic rates (Dickson & Tomlinson 1996).

5.7- CONCLUSIONS

In conclusion, this study shows that, thought tree water status is similar during the wet season regardless of tree health status, in the dry season there are different physiologic responses to water stress, where trees under chronic decline have reduced sap flow driving force and stronger down-regulation of photosynthesis compared to asymptomatic trees subjected to seasonal water stress, suggesting loss of resilience. Moreover, chronic decline is associated to reduction in water flow and not to dehydration, since trees are well hydrated during the wet season and water potential does not drop below critical values. On the contrary, sudden death appears to be a consequence of tree dehydration after drop of water status to values below cavitation threshold, though more replicates are needed to confirm the assumption.

Physiologic symptoms of trees with *P. cinnamomi* in the rhizosphere are linked to symptoms of water stress, mainly because the pathogen destroy fine roots and may cause impairment in water absorption. However, it is possible that the effect of root destruction may be stomatal closure instead of decrease in water status, at least in some cases. Moreover, though overall starch content was much reduced, trees affected by *P. cinnamomi* showed a slightly more starch in leaves which may be related to sink-limitations. Further studies with more replicates and with seedlings in controlled conditions should be undergone to understand the dynamic of starch after *P. cinnamomi* infection

CHAPTER 6

Effectiveness of cork oaks artificial mycorrhization in the nursery and in the field

6.1-ABSTRACT

Application of ectomycorrhizal fungi (ECM) aim to provide conditions for establishment and growth of a variety of forest seedlings produced in nurseries for reforestation programs. Several studies in controlled conditions showed the role of ECM in enhancing nutrient acquisition, drought tolerance, soil aggregation in eroded soils and pathogen resistance of their hosts. The following question would be if ECM application in natural conditions is able to colonize and benefit the host. Artificial inoculation in sterilized conditions requires a large investment of resources and may not be feasible for large reforestation campaigns. On the other hand, application of ECM in the field could be a possibility for overall adult tree health improvement, decline reversion and *P. cinnamomi* prevention. In this study, it was tested artificial ECM inoculations in cork oak seedlings growing in a nursery with non-sterile soil as well artificial inoculations in a healthy young cork oak plantation. A commercial ECM product with 6 ECM strains was used in both experiments. In the nursery, seedlings showed high ECM root tips, but morphological and molecular analysis identified only *Scleroderma spp.* and *Thelephora terrestris*, strains not present in the commercial ECM product. Those strains are known for their capacity in colonizing forest nurseries, including recently fumigated ones. Artificial ECM may have reduced capacity in competing with well adapted strains. Other approaches, like cultural practices toward enhancement of natural ECM root colonization may be an interesting alternative. On the other hand, application of ECM in the field resulted in improvement of height growth in trees that showed reduced increment in the year before ECM application. Introduced ECM may be effective in colonizing some cork oak roots or, on the other hand; nutrients found in commercial product may have favored development of natural ECM.

The study of ECM colonization in the field is still undergoing, where the next step is to analyze root tips to access differences in ECM composition or abundance in relation to treatments. Nevertheless, these positive results are promising toward the use of ECM in established trees in the field.

6.2- INTRODUCTION

In the last decades, reforestation and afforestation programs have been carried out mainly by artificial field transplantation of container-grown seedlings produced from seeds in nurseries (Chirino et al 2008, Oliveira et al 2010, Pinto-Gomes et al 2001; Ribeiro & Teixeira 2001, Pera & Parladé 2005). Cork oaks are the second species produced in nurseries, corresponding to 25% of the total plants from nursery; however, cork oak seedling transplantation did not correspond to the expected success which could be attributed to inadequate techniques, seeds quality, diseases and ecological conditions (Pinto-Gomes et al 2001, Costa-e-Silva et al. 2001). Seedling establishment is a critical phase in the reforestation process and depends on the capacity of tree seedlings to capture resources quickly to resist pests and pathogens and to survive climatic stress (Perry et al., 1987; Duñabeitia et al. 2004). The lack of mycorrhizal fungi on root systems of nursery seedlings may cause poor plant establishment and growth in a variety of forest, restoration, agricultural, suburban and urban landscapes, particularly when indigenous fungal population is low (Miller et al. 1994; Ortega et al 2004).

Application of ectomycorrhizal (ECM) fungi aim to provide conditions for increase growth and vigor of seedlings under nursery conditions (Brundrett et al. 2005; Chen et al. 2006; Vosátka et al. 2008) and for improvement in quality, performance and survival

of outplanted seedlings (Oliveira et al. 2010; Domínguez et al. 2006; Quoreshi et al. 2008; Tarango-Rivero et al. 2009). There is considerable published research in the world literature proving the biological, physiological and ecological significance of ECM to the survival, growth, development and health of many species of agricultural and horticultural plants, and of forest trees (Smith & Read 1996; Quarles 1999a, b; Marx et al. 2002). Mycorrhizae are symbioses of fungi and fine roots of plants (Smith & Read 1996). Mycorrhizal associations are regulated by features of the host plant and mycorrhizal fungus, as well as by soil conditions and environmental factors (Harley & Smith 1983; Mosse & Hayman 1980).

ECM change root morphology by enhancing its bifurcation, ramification and enlargement, thus increasing root absorption surface; moreover, ECM mycelium may spread along the soil acting as extensions of root systems. These organisms form a linkage between plant roots and the soil by transferring inorganic nutrients to the plant in exchange for carbon; in this two-way movement of nutrients, up to 20% of plant assimilated carbon can be transferred to the fungus (Walbert 2005).

Several studies showed the role of ECM in enhancing nutrient acquisition, drought tolerance, soil aggregation in eroded soils (Caravaca et al. 2002), and pathogen resistance of their hosts (ex: Slankis 1974; Perez-Moreno & Read 2000; Högberg & Högberg 2002; Read & Perez-Moreno 2003; Leake et al. 2004; Selosse et al. 2006; Smith & Read 1996; Azul et al. 2011). ECM root tips may also live longer and be more tolerant to adverse conditions (Brundrett 2009). Additionally, ECM fungi have been postulated as a biological option to prevent *Phytophthora* infection in new plantations and nursery stock (Blom et al 2009). ECM fungi are potential biocontrol agents, as several of them have shown a positive effect on growth and survival of infected plants. Marx & Davey (1969a, b) were the first demonstrating the protective role of ECM in

Pinus spp. against *P. cinnamomi*. Mechanisms underlying these effects may result from biochemical antagonism or a physical barrier of the ECM fungus over the pathogen, promotion of antagonistic rhizosphere microorganisms, improving plant vigor or other active responses by the plant (Malajczuk 1982). Thus, even strains incapable to produce antagonistic chemicals may have protective effect on plants against root pathogens: Rodrigues & Martins (2005) observed that, though the ECM *Pisolithus tinctorius* showed no in vitro antagonistic effect against *P. cinnamomi*, *Castanea sativa* seedlings colonized with this species showed more survival rate after *P. cinnamomi* inoculation than seedlings with no ECM application. Some studies in controlled conditions showed the inhibitory capacity of certain ECM fungal species in the virulence of *Phytophthora spp.* in tree species like *Pinus echinata* and *Pinus taeda* (Marx 1973; Marx & Davey 1969a,b; Barham et al 1974), *Pinus patula* (Marais & Kotze 1979), *Eucalyptus marginata* (Malajczuk 1988) or *Castanea sativa* (Branzanti et al. 1999, Martins 2004).

After studies in controlled conditions indicating that ECM improve overall plant fitness, including protective effect against root pathogens, the following question is if ECM application in natural conditions are able to colonize and benefit the host. Each ECM species has its own nutritional requirements and their persistence in ecosystems is dependent on the interactions with host, soil and environmental conditions (Brundrett 1991). Factors like tree health status and management practices also affect significantly the composition of the ECM fungal community, including in cork oak woodlands (Hagerman et al. 1999; Jones et al. 2003; Kuikka et al. 2003; Saravesi et al. 2008; Azul et al. 2010; Blom et al. 2009; Barrico et al. 2010; Lancellotti & Franceschini 2013). Therefore, success in artificial ECM inoculation may be increased with a compost of several ECM species with different requirements, covering a wide range of edaphic conditions.

Ribeiro et al. (unpubl. data) realized a study with the intend to analyze the efficacy of several treatments in growth and survival of cork oak seedlings. Their methodology was toward plant manipulation for field reforestation. Plants, acquired in a nursery, were submitted to fertilization and commercial ECM application treatments and afterwards transplanted to the field. Their goal was to test the efficacy of each treatment in plant growth and survival after reforestation. In this study, we selected randomly a sample of cork oak seedlings to analyze ECM colonization in each treatment. The goal of this approach is to analyze the effectiveness in artificial ECM application on seedlings for posterior reforestation. Additionally, we also applied the same commercial ECM in an experimental cork oaks site. In this experimental site young adult trees are under competition between them and dendrometric measurements have been realized since seedlings plantation. In this approach, the objective is to evaluate the effect of mycorrhizal inoculation in the growth of young adult cork oaks under competition effect. Overall, we want to test if the use of ECM inoculum before or after reforestation is an asset for cork oak forest producers and a good management practice for a general improve in tree health status, eventually protecting them against *P. cinnamomi*.

6.3- MATERIAL AND METHODS

6.3.1- EXPERIMENT 1:

6.3.1.1- Plant Treatments

Two years old cork oak seedlings were acquired in a nursery and planted on containers with 10 L capacity and filled with non-sterilized soil. Substrate was obtained from a cork oak *montado* in a stand selected for further seedlings transplantation.

Four treatments where applied:

C- Control (no application of additives);

F: Fertilization treatment,

FM: Fertilization + ectomycorrhizal fungi,

FA: Fertilization + amino acids

FAM: Fertilization + amino acids + ectomycorrhizal fungi.

Fertilization:

In all treatments, except control (C), it was applied 8.3 mg N, 3.7 mg P_2O_5, 16.0 mg K_2O, 8.1 mg CaO, 4 mg MgO, 7.8 mg SO_3-, 0.005 mg B, 0.004mg Cu, 0.001 mg Fe, 0.02 mg Mn and 0.001 mg Zn in each application per plant.

Amino acids:

For FA and FAM each plant was subjected in each application to a supply of 0.19g of amino acids and 0.47g of vegetable organic matter.

Ectomycorrhizal fungi:

In FM and FAM it was applied the commercial mixture ECTOVIT (Symbiom Ltd.). The mixture is compound by 4 strains of mycorrhizal fungi on a liquid medium and 2 strains of mycorrhizal fungi on a peat-based carrier with ingredients supporting the development of mycorrhiza (humates, ground minerals, extracts from sea organisms), naturally degradable granules of a water-retaining gel. The ECM species are *Cenococcum geophilum*, *Hebeloma sinapizans*, *H. crustiliforme*, *Pisolithus tinctorius*, *Amanita rubescens* and *Tricholoma acerbum*.

Plants were distributed randomly in a greenhouse and watered every five days according to their needs. During the experiment 18 applications, dissolved in 0.5 L water, were made every 10 days.

6.3.1.2- Root analyses

After 18 months, a subsample of about 10g of fine roots was removed in 5 plants per treatment from four distal points of the containers, in a total of 25 plants. Roots were wrapped in wet newspaper and sealed in plastic bags to maintain humidity and

stored at 4°C for up to 2 weeks until being processed. Soil particles adhering to roots were removed with water and fine forceps under a stereomicroscope. Roots were digitalized and the degree of mycorrhizal formation was expressed as the percentage of colonized root tips. ECM roots samples were observed under a dissecting microscope using a black background and lamps of daylight quality and categorized into morphotypes according to macroscopic morphology (Goodman et al. 1996): color and texture of the mycorrhizal tips, shape of unrumified tips, form and abundance of emanating hyphae, and the presence and type of rhizomorphs. Microscopic observation of the mantle peels, rhizomorphs and emanating hyphae was realized in a few samples. Samples of each ECM morphotypes were stored at -20ºC for posterior molecular analyses.

6.3.1.3- Classification of the macroscopic ectomycorrhizal characters:

A. Branching type (ramification): Simple, pinnate, irregular, dichotomous, coralloid or tubercle-like

B. Mantle surface texture: Smooth, reticulate, grainy, spiny, cottony or woolly.

C. Mantle luster: matte, shiny, reflective, stringy, short spiny, long spiny

D. Shape of unramified ends: Straight, bent, tortuous or beaded

E. Emanating hyphae: Frequency: none, rare, common;

F. Rhizomorphs:

   a) Attachment to the tips: restricted point, restricted, angled or fanned;

   b) Shape: filamentous, smooth or hairy;

   c) Frequency: none, rare, common

6.3.1.4- Molecular identification of the ectomycorrhizal tips

Molecular characterization was carried out by sequencing fragments of the nuclear ribosomal DNA region of representative ECM root tips.

DNA was extracted following Graham et al. (1994) protocol for fungal genomic DNA extraction with slightly modifications adapted to our samples and laboratory conditions, described above:

Protocol for fungal genomic DNA extraction (adapted from Graham et al. 1994).

* Adjustments from the original protocol

A) Roots tips samples (stored at -20ºC) were crushed and grinded with a pestle for 1.5 ml microcentrifuge tubes where 1ml extraction buffer (2% wt/vol CTAB, 100 mM tris-HCl, 1.4 M NaCl 20 mM EDTA) was added.

* ECM root tips samples are very small (about 1 mm long) and lightweight (mg?) and maceration with liquid nitrogen was not possible. Samples were imediately crushed while still frozen and then completely grinded after addiction of the extraction buffer. Although samples were less than 1 g, 1 ml of extraction buffer was added for convenience in the following collection phases.

B) The blend was mixed by gentle inversion and incubated at 55ºC for 20 min.

C) After 5min centrifugation at 15,000 x *g* the supernatant was collected in a new microcentrifuge tube.

D) 1 ml of chloroform: isoamyl alcohol (24:1) was added and mixed by gentle inversion for 2 min

* Although the supernatant was always inferior to 1 ml (about 0.9 ml) the nucleic acid was purified with a standard 1 ml of the reagent (instead of 1 volume).

E) After centrifugation at 15,000 x *g* for 20 s, 640 µl of the upper aqueous phase was collected carefully to a 2 ml microcentrifuge tube.

* It was decided to collect only 640 µl in order to avoid possible contamination by the reagent when collecting the upper aqueous phase. Moreover, it is the maximum amount possible to use in one individual reaction according to equipment and material limitations.

F) 64 µl (1/10 vol) of ammonium acetate and 1280 µl (2 vol) of ice-cold absolute ethanol were added and mixed by gentle inversion. Samples were stored at -20ºC for 60 min to precipitate genomic DNA.

* The reaction had the volume of 1984 µl : [640 µl sample + (64 µl + 1280 µl) reagents], suitable for a 2 ml microcentrifuge tube.

G) Samples were centrifugated at 15,000 x *g* for 1 min and the supernatant discarded.

H) 1 ml of 70% ethanol was added to wash the pellet by inversion, centrifuged at 15,000 x *g* for 1 min and the supernatant discarded.

I) Subheading 8 was repeated.

J) The pellet was dried in a DNA desiccator for 15 min.

K) DNA was eluted in 30 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

* Elution in a volume inferior to 200 µl was used in order to yield more DNA concentration.

After elution, DNA samples were analyzed in a NanoDrop 2000c UV-Vis Spectrophotometer for nucleic acid concentration and purity measurements.

PCR amplification protocol

PCR amplification conditions were followed from Gardes & Bruns (1993). The authors designed two primers that in combination preferentially amplify the ITS (Internal Transcribed Spacer) region of the basidiomycetes rDNA. The basidiomycete primer ITS4-B, when paired with the fungal specific primer ITS1-F, efficiently amplified DNA from all basidiomycetes; although some plant DNA (including *Quercus sp*.) may eventually be amplified, under conditions where both plant and fungal DNA are present fungal DNA is amplified to the apparent exclusion of plant DNA (Gardes & Bruns 1993).

Primers sequence:

ITS1-F: CTT GGT CAT TTA GAG GAA GTA A

ITS4-B: CAG GAG ACT TGT ACA CGG TCC AG

Reaction:

Amplification was carried out in 50 µl reactions with Supreme NZYTaq Green Master Mix (50 mM Tris-HCl, pH 9.0, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP, dTTP, 5 U Taq DNA polymerase).

Reaction mixture combined the following components, for 50 µl:

- 25 µl Supreme NZYTaq 2× Green Master Mix
- 0.2 µM each primer
- 1 µl template DNA
- Nuclease-free water up to 50 µl

A negative control was added in each set of reactions. Template DNA was replaced by 1 µl of nuclease-free water in order to test contamination of reagents and reaction mixtures. The thermal cycling program was run in a programmable heat block (marca).

Amplification:

1) initial denaturation step of 94ºC for 120 s
2) 35 amplification cycles:

2A) first 13 cycles:

2A1) melting temperature at 95ºC for 35 s

2A2) annealing at 55ºC for 55 s

2A3) extension at 72ºC FOR 45 s

2B) next 14-26 cycles:

2B1) melting temperature at 95ºC for 35 s

2B2) annealing at 55ºC for 55 s

2B3) extension at 72ºC FOR 120 s

2C) next 14-26 cycles:

2C1) melting temperature at 95ºC for 35 s

2C2) annealing at 55ºC for 55 s

2C3) extension at 72ºC FOR 180 s

3) Additional incubation for 10 min at 72ºC.

Agarose gel electrophoresis of diagnostic PCR products

5 µl of each PCR reaction was loaded into a 1% (wt/vol) agarose gel in 1x Tris-acetate-EDTA (TAE) buffer. Gels were stained by immersion in an ethidium bromide solution for 10 min and gel images digitally recorded under UV light (EDAS 120, Kodak Digital Science). Fragment sizes were determined by comparison with a 1 kp molecular weight standard (Fischer Biotech)

DNA Sequencing of the amplified products

PCR products were purified using QIAquick Gel PCR purification kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's instructions and a sample of 8 fragments were sequenced using the ITS1-F primer in Macrogen Standard Sequencing Service (Amsterdam, The Netherlands). DNA sequences of the ECM samples analyzed were compared with the sequences in the National Center of Biotechnology Information

nucleotide databases (Genbank; Zhang et al. 2000, Morgulis et al. 2008; using the BLASTN search: http://blast.ncbi.nlm.nih.gov/Blast.cgi)

6.3.1.5- Statistical analyses

Statistical analyses were made using the Systat v.13.1 software package. Two-way ANOVA was used to test significance of each ECM species and treatment in root tip colonization variation. Significant differences between treatments means were evaluated with Tukey's HSD tests with $P<0.05$. Before applying ANOVA tests, data were tested for normality by using Kolmogorov-Smirnov D test at the significance level of 0.05.

6.3.2- EXPERIMENT 2:

6.3.2.1- Study area

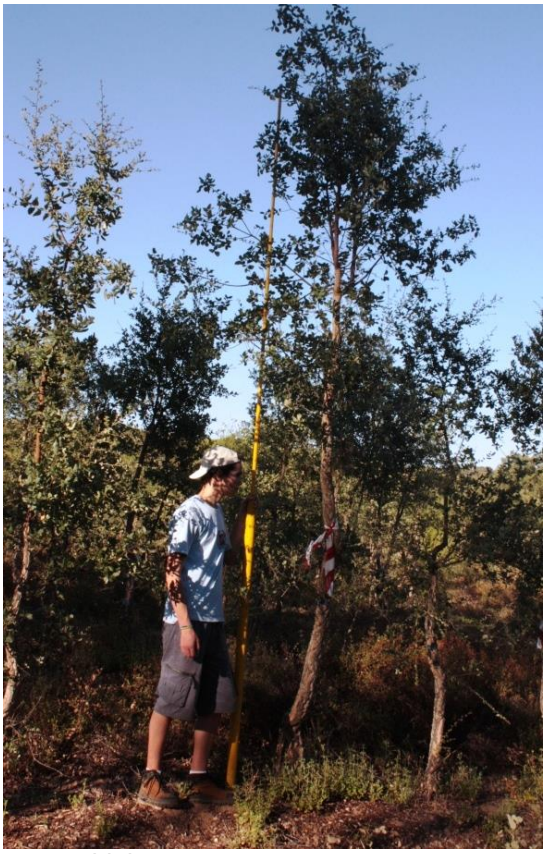
The study took place, at the "Herdade da Mitra" experimental stand (38º32'N, 8º01'W, 243m a.s.l.) located near Évora, Portugal between September 2012 and August 2013. The experimental stand consists on a cork oak forest plantation with 5 ha comprising 16 years old trees located distantly 1 m from each other. Access to the stand is restricted to livestock and shrub management is periodically realized by cutting practices with no soil tillage. The soil is a very shallow (30 cm deep) sandy Cambisol (FAO, 1988) overlying a fractured gneiss rock (David *et al.* 2004). The trees selected to the study

were distributed homogeneously along 8 lines parallel to each other, distantly 3 m from each other. The selected study area only comprised young cork oaks with the same age.

6.3.2.2- Experimental design

40 trees were selected for this experiment, distant from each other by about 4 m. Between each selected tree there was 2 to 3 trees not considered in this study, therefore all the selected trees were separated from each other by other trees, reducing the possibility of root contact between selected trees. On September 2012, after first autumn rains, dendrometric measurements were realized: tree high, canopy projection in each quadrant (4 parameters), diameter of the trunk at the base (2 perpendicular measurements) and perimeter at 1 m from the base. Application of commercial mixture ECTOVIT (Symbiom Ltd.) was realized in 20 of the selected trees, alternating ECM application between treated trees and control trees. In each treated tree, ECM inoculation was made through 8 soil injections distant 50 cm of the base trunk and 20 cm deep (after removing soil litter) each with 60 ml of the product, with a microinjection machine (fig. 6.1). 11 months after, in August, dendrometric measurements were realized again and tree increment was calculated for each parameter.

Figure 6.1: Study site at Herdade da Mitra, Évora



A: Tree high measurements.

B: Filling the microinjection machine with the ECM product

C, D: Injecting ECM product in the tree rhizosphere

6.3.2.3- Statistical analyses

Statistical analyses were made using the Systat v.13.1 software package. Absolute and relative increments were calculated for each dendrometric parameter. Absolute increment is the difference between the two measurements realized for each parameter (in September 2012 and August 2013) and refers to the total increase during the study period. Relative increment is the absolute increment divided by the initial value of the parameter and refers to the percentage of increase in relation to initial tree dimensions. Relative increment was calculated for the study period and also for the formerly period (between September 2011 and September 2012).

To compare differential absolute and relative increment during the study period between control and treated trees, it was used the two-sample T-test with separate variance for each dendrometric parameter. To test if the effect of the treatment was related to the relative increment in the previous year, it was applied a general linear model, to estimate a multivariate general linear model with two independent factors, a linear factor (relative increment in the previous period) and a factorial one (treatment).

6.4- RESULTS

6.4.1- EXPERIMENT 1:

6.4.1.1- Description and identification of the ectomycorrhizal morphotypes

Two main morphotype of ECM were observed in the root samples and vestigial old

ECM (shrank, dark mantle), but about 99% of the total ECM belonged to only 2 morphotypes. PCR was realized with DNA extracted from 40 ECM samples, resulting in three DNA fragments. 8 randomly selected DNA fragments were sequenced.

Morphotype I:

Macroscopic features:

ECM tips not branched or monopodial pinnate with unramified ends straight or sinuous. Mantle surface dark-yellow, smooth. No rhizomorphs and emanating hyphae observed (fig. 6.2).

Molecular analyses:

PCR amplification using ITS1-F and ITS4-B primers resulted in a DNA fragment with 793 bp. DNA sequencing was strongly aligned (identical sequence superior to 95%) with *Thelephora terrestris* (fig. 6.2, fig. 6.6).

Figure 6.2: Morphological features of morphotype I. DNA fragment matched *Thelephora terrestris.*

Morphotype II:

Macroscopic features:

ECM tips not branched, monopodial pinnate irregular or pyramidal with tortuous or sinuous unramified ends; mantle surface yellow-white, shiny; frequent rhizomorphs connected to the mantle at distinct points (fig. 6.3). Anatomical features of a few samples showed the outer mantle surface with ring-like arrangement and inflated hyphae and highly differentiated rhizomorph (fig. 6.4, 6.5).

Molecular analyses:

PCR amplification using ITS1-F and ITS4-B primers resulted in two DNA fragment with 869 bp and 805 bp. DNA sequencing of 4 random samples of the larger fragment strongly aligned (identical sequences superior to 95%) on Genbank with *Scleroderma cepa, S. verrucosum* or *S. aerolatum*. Some were strongly aligned with more than one species (table 6.1). The smaller DNA fragment strongly aligned with *S. citrinum* (fig. 6.6).

Figure 6.3: Morphological features of morphotype II: Ectomycorrhizal root tips. DNA fragment aligned with *Scleroderma sp.*

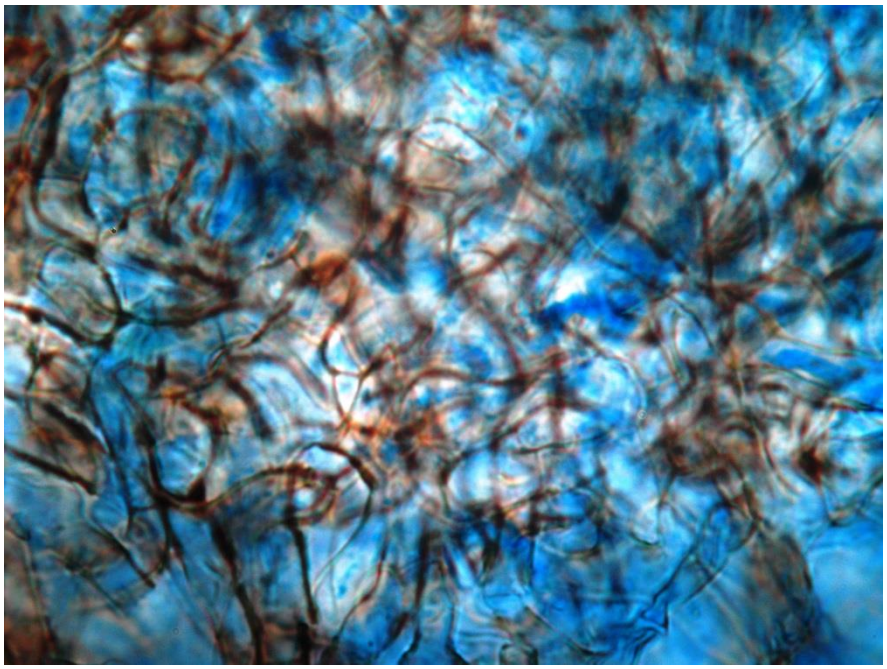


Figure 6.4: Anatomical features of morphotype II, *Scleroderma sp.* : plectenchymatous mantle with ring like arrangement and inflated hyphae

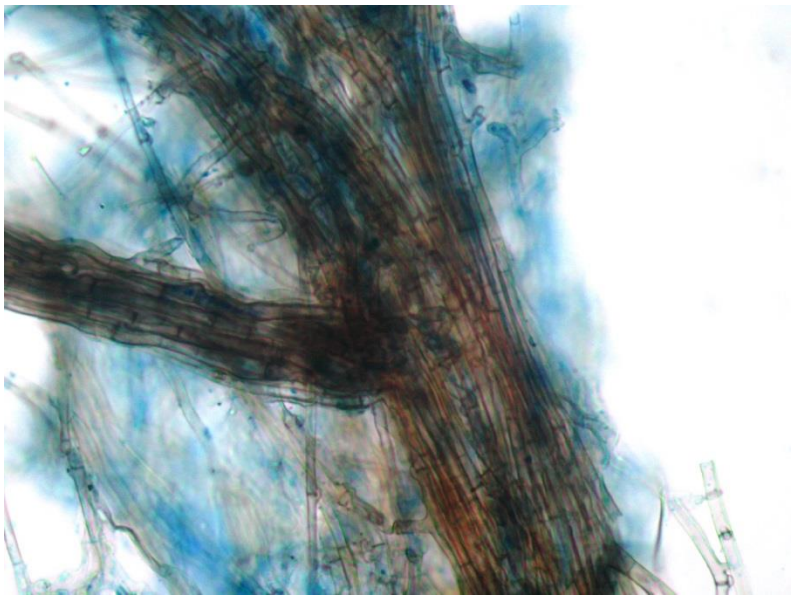


Figure 6.5: Anatomical features of morphotype II, *Scleroderma sp.*: branching of a highly differentiated rhizomorphs with thick hyphae.

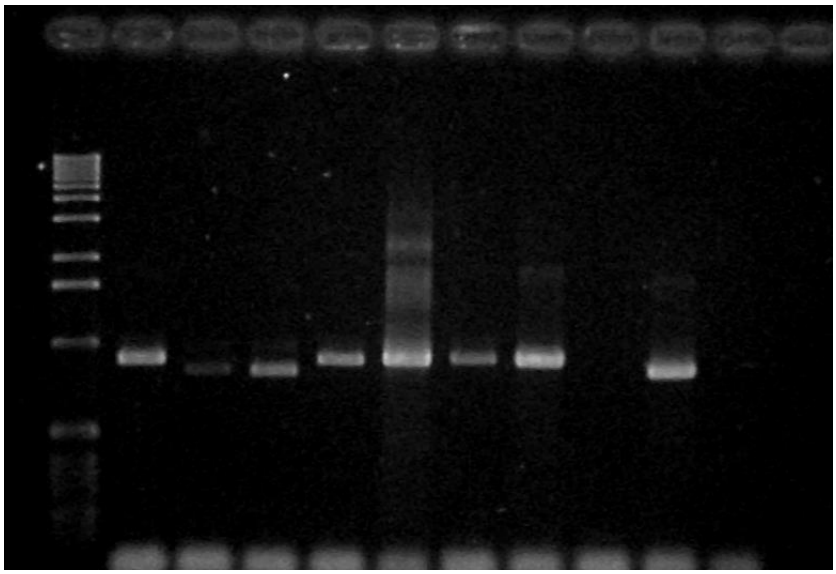


Figure 6.6: Difference between DNA fragments of morphotype I and II obtained by PCR using ITS1-F and ITS4-B primers. The fragments were separated on a 1 % agarose TAE gel

Lane 1: 1 kb ladder as marker

Lane 2, 5, 6, 7, 8: 869 bp DNA fragment obtained from morphotype II

Lane 3, 4, 10: 793 bp DNA fragment obtained from morphotype I.

Lane 11: Negative control

6.4.1.2- Effect of the treatments on ectomycorrhizal root tip colonization

The percentage of seedlings ECM colonization varied from 14% to 86% and was on average 50 ± 23 %. Root tips were more colonized by morphotype II (Anova: $F_{(40,1)}$: 11.43, p = 0.01; morphotype I colonization rate: 14 ± 17 %; morphotype II colonization rate: 37 ± 29 %, fig. 6.7); treatment was not significantly related with colonization rate (Anova: $F_{(40,4)}$: 0.803, p = 0.53; fig. 6.8) and there was no interaction between treatment and morphotype (Anova: $F_{(40,4)}$: 2.22, p = 0.11).

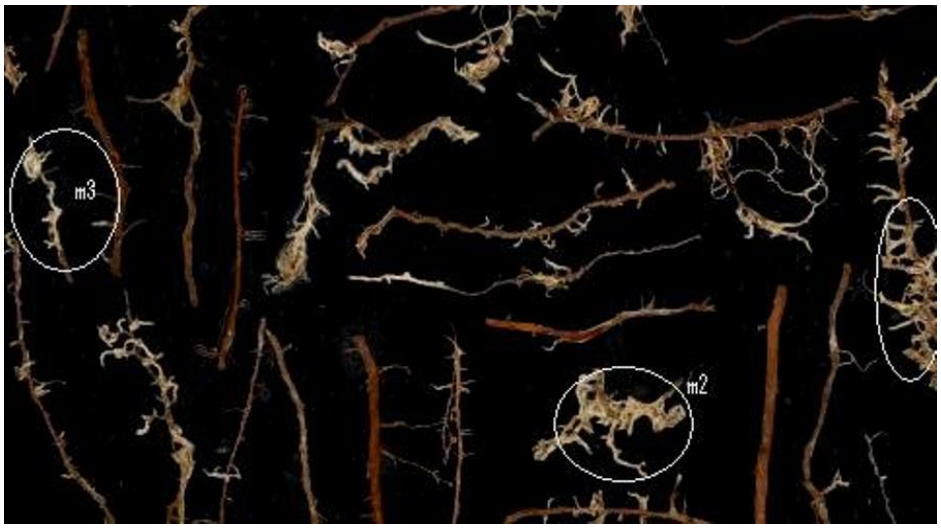


Figure 6.7: Example of digitalized sample roots from FA treatment with ectomycorrhizae selected for molecular analyses**.**

Table 6.1: Total root tips counted in each treatment, and quantity of root tips colonized by each morphotypes.

Treatment	Total root tips (n.)	Morphotype I tips (n.)	Morphotype II tips (n.)
TC	1229 ± 817	486 ± 857	67 ± 105
TF	1597 ± 927	76 ± 111	242 ± 186
TFM	1783 ± 788	69 ± 117	413 ± 471
TFA	2798 ± 1706	40 ± 89	983 ± 552
TFAM	1811 ± 1284	327 ± 458	274 ± 423

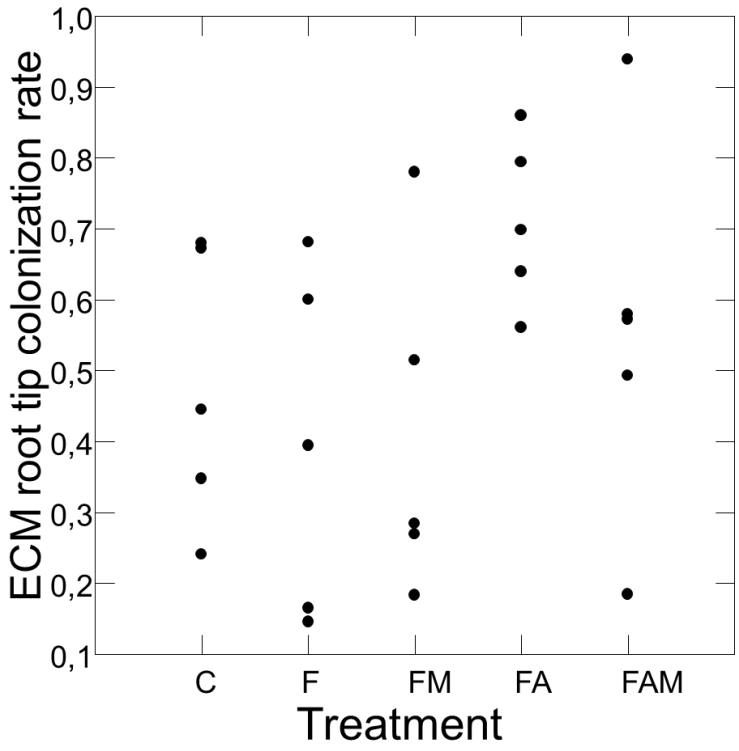


Figure 6.8: Relative ectomycorrhizal fungi (ECM) root tip colonization according to treatment. C: control; F: fertilizers; FM: fertilizers and ECM; FA: fertilizers and amino-acids; FAM: fertilizers, amino-acids and ECM.

6.4.2- Experiment 2

In general trees grew less during the study period than in the period before, and total and relative tree increment occurred between September 2012 and August 2013 was not related to ECM application (p >0.05 in all the tree growth parameters, table 6.2). However, relative increment in tree high during the study period was slightly but significantly higher in trees that showed relatively less increment in the previous year (GLM*:* $F_{(37,2)} = 5.12$, $p = 0.03$). All the other parameters were not affected by treatment (fig. 6.9).

Table 6.2: Total and relative increment of cork oak trees in the study field at Herdade da Mitra, Évora, between September 2012 and August 2013

Dendrometric parameter	**Total increment (average ± s.d.)**	**Relative increment (average ± s.d.)**
Tree high	19.49 ± 22.42 cm	3.65 ± 3.42 %
Crown horizontal projection	76.75 ± 39.53 cm^2	12.91 ± 35.06 %
Perimeter at the trunk base	18.84 ± 16.40 cm	4.75 ± 4.84 %
Perimeter 1 m from the base	17.98 ± 7.72 cm	6.42 ± 3.51 %

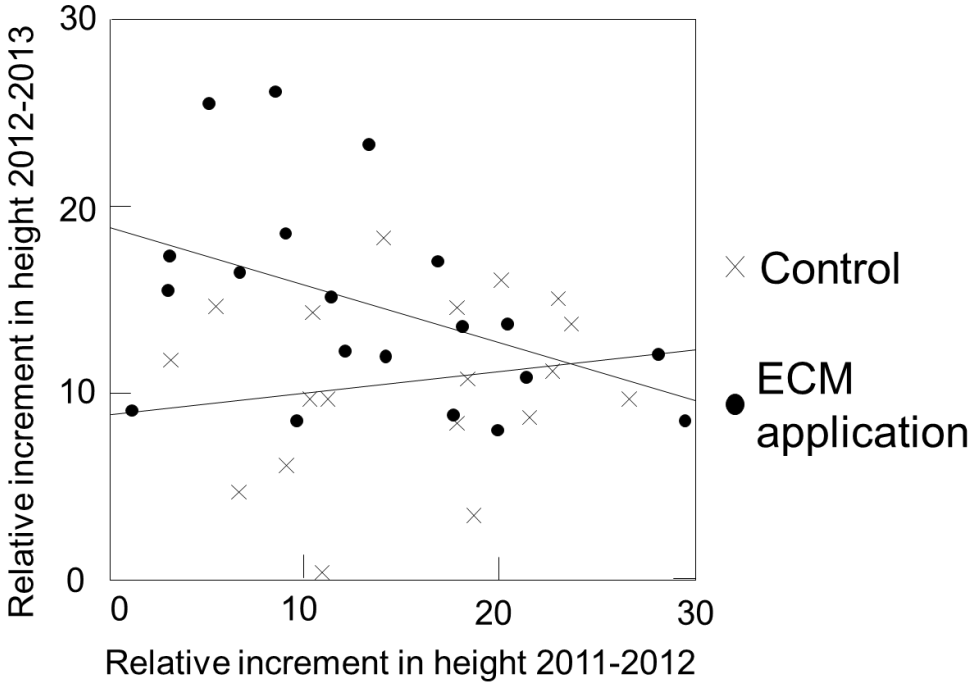


Figure 6.9: Difference in the relative increase in height of young cork oaks (*Quercus suber*) between the year prior to the study (2011-2012) and the year after artificial ectomycorrhizal application (2012-2013), in the study field at Herdade da Mitra, Évora.

6.5- DISCUSSION

6.5.1- Experiment 1:

It was clear in these results that ECM application (in treatments FM and FAM) did not influence ECM colonization. The two dominant morphotypes belong to other species than the strains from the commercial mixture and, moreover, qualitative and quantitative colonization was not related to ECM treatments. It was observed more morphotype II infection, but it may be a result of its tendency to enhance root tips ramification and enlargement in relation to morphotype I. In general, root colonization rate was as high as in some artificial inoculations in controlled conditions (Ortega et al. 2004, Oliveira et al 2010, Sousa et al. 2012), suggesting redundancy of ECM applications in natural conditions.

Nursery inoculations of forest tree seedlings with ECM fungi are usually carried out with few fungal species in sterilized conditions. However, this study shows that artificial inoculation in non-sterilized substrate may not be effective in root colonization against native species. Large variation in response to inoculation arises from factors such as the degree of host–fungus compatibility, mycorrhizal dependency of the host, fungal effectiveness in relation to biotic and abiotic site conditions, and the abundance and effectiveness of indigenous fungi (Ortega et al. 2004). In this study it was selected a commercial mixture specific for oak trees, thus compatible with the host and with species tolerant to diverse soil conditions. For example, the applied ECM *C. geophilum* is known for is wide hosts and habitat range and has been suggested to provide isolate-dependent drought protection to fine roots (Jany et al. 2002). This nearly ubiquitous and often abundant species was systematically observed in fine roots of adult cork oaks

(Azul 2002). Being the dominant ECM species in oak woodlands (Dickie et al. 2005), has strong interspecific competitive capacity during colonization processes (Villeneuve et al. 1991) and is not affected by application of fertilizers (Oliveira et al. 2007). In a sterilized substrate, *C. geophilum* inoculation resulted in about 20 to 40% root tips of holm oaks seedlings colonized by this species (Oliveira et al. 2010); Other applied ECM species, *P. tinctorius*, has a worldwide geographic distribution and is found in forests, pecan orchards, urban settings and on adverse sites, such as severely eroded soils and mined lands; it occurs in both cold and warm climates on a broad range of tree hosts and is one of the best examples of ecological adaptation (Marx 1977, Marx et al. 2002), maintaining its viability after application of fertilizers (Hatchell et al. 1985). Nevertheless, none of *C. geophilum* and *P. tinctorius* (neither the other species) were able to colonize the analyzed root tips in this experiment. Moreover, seedlings inoculated with ECM product (FM and FAM treatments), which includes not only the ECM inoculum but also ingredients supporting the development of mycorrhizas, did not show the highest colonization rate and half of the seedlings had less than 50% colonized root tips.

It is usually considered that high amounts of P and N may change ECM diversity, composition and abundance (Björkman 1942, Slankis 1974, Buscot et al. 2000, Peter et al. 2001, Lilleskov et al. 2002). Nutrient demand and response to nutrient supplements vary among fungi, thus fertilization can have different effects on ECM establishment, enhancing or inhibiting root colonization (Molina & Chamard 1983, Castellano & Molina 1989, Rincón et al., 2007, Liu et al. 2008, Vaario et al. 2009). Moreover, in natural soils the effect of inorganic nutrients on ECM formation is complex, presumably due to the effect of soil microorganisms or other plant competition for mineral nutrients (Slankis 1974). Addiction of fertilizers in this study was towards *Q. suber* requirements

and the applied quantities did not affect ECM composition and abundance. Other studies also reported no effect of fertilization in ECM colonization (Conjeaud et al. 1996, Hawley 2006), including *C. geophilum* colonization of holm oaks roots (Oliveira et al. 2007). Application of about 10-fold more N in a study with oak tended to reduce ECM abundance or colonization, but this effect was more significant in controlled experiments instead of seedlings growing in the natural conditions (Newton & Pigott 1991).

Soil substrate was obtained in a traditional but degraded *montado* which is expected to have low ECM inoculum due to lack of hosts. However, the species observed in the treatment, *Scleroderma spp.* and *T. terrestris*, are known for they capacity in contaminating plant nurseries since cultural procedures used to produce seedlings in bare-root or container nurseries create environmental conditions that select naturally occurring ECM fungi adapted to these conditions; producing mushrooms or puffballs that release many spores that are wind disseminated to nursery soils (Marx et al. 2002).

T. terrestris is considered an early stage fungus with medium-distance exploration (Last et al. 1987, Agerer, 2001), appears to naturally dominate the roots of most pines, oaks and spruce grown in nursery soil and containers (Marx et al. 1982, 1984a) and is considered the first symbiotic species colonizing fumigated nursery soils (Marx & Bryan 1969). Researchers involved in ECM inoculation programs undoubtedly have cursed this vigorously growing pioneer species because it easily outcompetes with their inoculant ECM species (Colpaert 1999). They benefit to the host is not so evident comparing with other species like *P. tinctorius*, as it is not efficient in nutrient uptake when external nutrient concentrations are low but this mycobiont is well adapted to the environmental conditions in modern nurseries and there is evidence that it confers some advantages to its young host plants in these nurseries (Colpaert et al. 1999), improving

survival and growth of some transplanted seedlings (Tomazello & Krügner 1982), particularly under fertilization treatments (Sousa et al. 2012).

Scleroderma is a common and widespread gasteromycete genus; most of the species are pioneer in colonizing bare roots and are capable to colonize both highly stressed sites (Jeffries 1999; Marx et al. 2002) and nutrient rich soils (Newton & Pigott 1991). ECM producing abundant rhizomorphs, such as *Scleroderma spp.*, may increase plant tolerance to drought (Duddridge et al. 1980, Ortega et al. 2010). The genus produces ECM with highly differentiated rhizomorphs, which have been shown to facilitate water transport over ecologically significant distances (Duddridge et al. 1980, Foster 1981, Brownlee et al. 1983, Read and Boyd 1986, Cairney 1992). There are reports of overall improvement of physiologic status and /or biomass of several host species after *Scleroderma spp.* application (Ortega et al 2004, Duñabeitia et al. 2004a, Caravaca et al. 2005, Chen et al. 2006). Moreover, some *Scleroderma spp.* inhibit some root pathogens like *Phytophthora spp.* (Marx 1973). *S. citrinum*, S. *verrucosum* and S. *aerolatum* are very common species; on the other hand, *S. cepa* are occasionally found under oaks on sandy soil (Storey 2009). The observed dominant species are known to contaminate forest nurseries and their presences are not associated to the use of natural soils in nurseries, since they occur even when using sterile substrate, particularly in large-scale production of seedlings.

One explanation for the inefficacy of ECM application could be the unviability of ECM spores and mycelium caused by eventual maintenance of the product in inappropriate conditions, like adverse temperature conditions. However, several applications from several ECM packages were made, reducing the probability that all the applications were made with unviable ECM.

Historically, the most widely used natural inoculum is obtained from not sterilized forest soils, despite of its disadvantages (Marx et al. 2002). Established nurseries often do not inoculate at all, relying on natural soil populations, and inoculum material is usually collected from these sites (ex: http://www.nzffa.org.nz). Although in other experiments it was observed favorable results with artificial inoculation in non-sterile soils (ex: Baum et al. 2002, Chen et al. 2006, Sousa et al. 2012), this study suggests that ECM application in large-scale may be an inefficient procedure because of contaminations by ECM producing large amount of spores. On the other hand, substrate sterilization is resource consuming and may not prevent further contaminations in the nurseries. Therefore, it should be evaluate the possible economic advantage of ECM inoculation in forest nurseries. ECM inoculation cannot become a routine practice unless viable and inexpensive inoculum is available (Ortega et al. 2004) and root colonization is efficient. Artificial inoculation in sterilized conditions requires several procedures that are associated to a large investment of resources (Menkis et al. 2007) and may not be feasible for large reforestation campaigns. An alternative would be to explore existing natural mycorrhizae in forest nurseries by selecting a cultivation system that may yield seedling material with a high extent of ECMs. The cultural procedures used to produce seedlings in nurseries could be oriented to create environmental conditions that select naturally occurring ECM adapted to these conditions, like substrate selection (Chen et al. 2006) and appropriate cultivation systems (Menkis et al. 2011). These authors demonstrated that selection of proper cultivation system might result in similar or higher mycorrhization and survival rates of outplanted seedlings than achieved by expensive and laborious artificial mycorrhization.

6.5.2- **Experiment 2:**

The same ECM product used in this study was already tested on adult cork oaks in another study (symbio docs): the product was injected into the soil, together with natural slow release fertilizer and, in general, cork oaks health status improved significantly. However, it was not reported if ECM effectively colonized fine-roots and if oaks were favored by fertilizers, ECM or both. In contrast, all the trees from this present study were in good sanitary conditions regardless of intra-competition and the purpose was to test differences in tree increment.

All trees in the study area have the same age but show different size, due to their own intrinsic conditions but also due to environmental conditions, for example, access to nutrient and water resources that may be limited by neighboring dominant trees. This study showed that ECM application was not effective in improving tree size on trees that previously showed the highest relative increments, but favored trees with less relative increment. It is referred that beneficial effects of ECM fungi are likely to be most pronounced on poor fertility planting sites under harsh environmental conditions (Smith & Read 1996). Cork oaks with less access to nutrient and water supply, as a result of tree competition or other environmental cause, could be favored by an increase, or a shift, in ECM colonization, increasing by this way nutrient and water availability. Mycorrhiza-mediated nutrient uptake patterns may be important mechanisms in competition between plants (Aerts 2002). Interestingly, ECM application had significant effect only in tree vertical growth and not in horizontal growth. Upper part of the canopy is usually subjected to the lowest tree water potential, as a result of the greater effect of the gravity (Larcher 1995) and hydraulic architecture of the tree (Tyree & Sperry 1989). As trees grow taller, a larger soil-to-leaf water potential gradient is

required to overcome the effect of gravity and the increased hydraulic resistance of a longer flow pathway; maintenance of favorable water status by stomatal closure might progressively slow height growth by reducing photosynthetic carbon gain (hydraulic limitation hypothesis, Ryan & Yoder 1997). Moreover, in nutrient poor sites, trees tend to have low rates of photosynthesis and consequently narrower tracheids, thus less permeable xylem which increases hydraulic resistance (Pothier et al. 1989b, Schulze et al. 1994, Ryan & Yoder 1997). Vertical growth is thus limited by water and nutrient supply: trees grow tall when resources are abundant, stresses are minor, and competition for light places a premium on height growth (Koch et al. 2004). Consequently, if tree growth in height is limited by nutrient and water availability, one will likely be favored by symbioses with ECM. It is known that ECM favors host nutrient acquisition and mycorrhizal plants often have higher nutrient contents than non-mycorrhizal plants especially when grown in soils with low nutrient availability (Simard et al. 2002). In relation to water uptake by adult trees, ECM may not be their major pathway, but may play a critical role at times of stress (Lehto & Zwiazek 2011). Actually, an experiment with mature live oak trees (*Quercus virginiana*) estimated to be between 150 and 250 years old, successfully introduced ECM in stressed environment increased significantly root absorbing potential of the roots (Marx et al. 1997). In other study, ECM growth stimulation was more marked on years with a dry summer (Garbaye & Churin 1997). These reasons may explain why vertical growth was favored by ECM instead of horizontal growth (trunk width and canopy projection).

Most of the research related to management of ECM in forestry are focused on cultural practices in nurseries to support ECM colonization of the seedlings and on their growth and survival after transplantation to the field; in several of those studies, artificial mycorrhizal seedlings were able to maintain the symbiotic association with

inoculated species in the field for about two years (Beckjord & McIntosh 1984, Castellano & Molina 1989, Marx et al. 1985, 1991, Thomson et al. 1996, Parladé et al. 1999, Selosse et al. 2000, Battista et al. 2002, Núñez et al. 2006; Duponnois et al. 2007, Oliveira et al. 2010) suggesting that artificial ECM inoculations may be effective when realized previously to field transplantation, particularly in areas with low native ECM inoculum. However, in other studies transplanted ectomycorrhizal seedlings showed limited survival indicating that fungal community formation in root systems was governed mainly by environmental factors (Loopstra et al. 1988, Maestre et al. 2002, Gilman 2001, Menkis et al. 2007). Few artificial mycorrhization experiments were performed after plants transplantation to the field or with established trees. In some studies, ECM were applied during seedlings outplanting and it was reported successful root colonization, at least in the first year (Garbaye & Churin 1996, Baum et al. 2002, Duñabeitia et al. 2004b). Problems that may arise with ECM application in the field are competition with native ECM or environmental influences, such as drought periods in the summer that can reduce the spreading of the inoculated strain and cause a lack of inoculation effects (Feil et al. 1988, Nilsen et al. 1998, Baum et al. 2002). In this study, ECM application was realized in an established cork oak plantation that was likely to have appropriate ECM species. Enhancement in tree height, observed in trees with previous low relative increment, indirectly indicates the success in artificial ECM inoculation. It may enhance root ECM colonization by the input in ECM inoculum or by favoring natural ECM with ingredients supporting the development of mycorrhiza. However, it is needed to analyze ECM colonization of root samples in the study trees for a better comprehension of the mechanisms underlying the positive effect of this artificial inoculation.

6.6 - CONCLUSIONS

Artificial ECM application was not successful in the nursery experiment with non-sterile conditions but it was beneficial in field experiment. In the latter, further studies are needed to understand if artificial ECM application was effective in root colonization or, on the other hand, only enhanced natural ECM colonization. Tree seedlings are usually considered to perform better in reforestation when mycorrhized, but this is associated with a large investment of resources that may not be effective in large-scale production if nurseries are prone to be contaminated by ECM producing large amount of spores. Moreover, growth and survival of outplanted seedlings is not consistently improved by ECM application. Cultural practices oriented in enhancing natural ECM colonization in nurseries appear to be an alternative to artificial inoculation. Application of artificial ECM in the field after reforestation may also be an interesting alternative, though it requires more studies. The field experiment is still undergoing and surveys are already schedule for posterior examination of ECM abundance and diversity in cork oak root tips according to treatments.

7 - FINAL CONCLUSIONS AND GENERAL PERSPECTIVES

In this work it was performed an extensive analysis of former studies, highlighting that cork and holm oak decline is a phenomenon without a single cause, resulting in complexes interactions between abiotic and biotic factors, acting in synergism and in long-term, and reducing trees resilience until trees are no longer able to recover from imposed stress. For accurate studies concerning the association between *P. cinnamomi* and cork and holm oak decline, a reliable method for pathogen diagnose is imperative. In this study it was demonstrated that, contrary to what is widely accepted, limitations in baiting-selective method are more related with contaminations by other fast-growing organisms instead of sampling size and, on the contrary, limitations in PCR-based method are more related to sampling size and less to contaminations by soil inhibitors. All procedures from the first method can be optimized to prevent undesirable contaminations until one can consider that negative results confidently indicate lack of inoculum in the samples. Moreover, baiting-selective methods are not constraining by the amount of soil sample to be analyzed, though it is in general a time consuming method, particularly if using larger soil samples. Nevertheless, analyzes with reduced sampling size are biased to *P. cinnamomi* detection only from soils with high inoculum amount. On the other hand, the development of DNA purification techniques allowed, in theory, that PCR-based approaches could be applied in soil samples. However, failure in detecting the pathogen from naturally infested soil samples associated to positive detection from artificially infested soils or, in the case of this study, positive results with *P. cinnamomi* DNA added to PCR reactions, strongly suggests that the main obstacle found in these methods is the reduced

amount of target DNA in the soil combined with extremely low amounts of substrate used for DNA extraction.

This research showed that the two unspecific decline symptoms, also reported in trees affected by *P. cinnamomi*, are linked to different tree water status. In field measurements it was underlined that sapflow is reduced in chronic decline and, consequently, reduces nutrient absorption and photosynthesis. On the other hand, if sapflow is not reduced and trees are not able to absorb water, xylem water potential may fall until vessels embolism. The last symptom has been extensively reported to occur in areas were *P. cinnamomi* is active (but not exclusively), particularly in young holm oaks, suggesting a straight association with root rot, differences in the root system related to tree age – for example, reduced root depth - and specific physiologic responses to water stress – it is referred that holm oaks are more drought tolerant than cork oaks, allowing a lower whole-tree hydraulic conductance and midday leaf water potential, however, their xylem vulnerability to embolism is similar to cork oaks (David et al. 2007, Pinto et al. 2012). Conditions associated to sudden death, for example, a rapid destruction of roots associated to a synergism between *P. cinnamomi* and soil waterlogging, limitations in root depth and water potentials working near the limits of safety margins, should be explore for a better understanding of the phenomenon.

This study also showed that trees under chronic decline are able to maintain appropriated water status with favorable environmental conditions, like during the rainy season. Former studies related to physiologic and biochemical alterations during seasons were only applied in asymptomatic trees, but further studies focused on declining trees will allow a better comprehension of the physiologic meaning of chronic decline and, moreover, if trees are able to recover when not restricted by soil water. This

knowledge could open opportunities to identify possible mechanisms for health status improvement and decline reversion.

Other important result from this study was the association between high midday leaf water potential and chronic decline. Studies with seedlings did not report this association, since midday water potential decrease with water stress and eventually the plant will die from runaway embolism. Chronic decline is only observed in adult trees and relative measurements of midday water status indicate whether tree is suffering on not from sapflow reduction.

P. cinnamomi inoculation in cork and holm seedlings under appropriated abiotic conditions have already indicated their moderate susceptibility to infection; fine-roots are destroyed by the pathogen but plants may eventually replace them or support some root pruning before showing above symptoms. In this study it was found an association between fine-roots and adult tree water status, but more studies in the field should be underwent to understand the differential contribution of sink and fine roots in tree water status and resilience to fine-root destruction. The questions raised were: adult trees located in soils with no limitations in access to deep groundwater are able to support some fine-root destruction without sharp decline in tree water status? In addition, what is the role of ectomycorrhizae in protection of fine-roots and in overall tree benefit? Analyzed trees from areas affected by *P. cinnamomi* also showed high ECM root tips, irrespective of health status (Bloom et al. 2009). This could be related to the physical barrier around fine roots, protecting them against root pathogens: roots infected by ECM were able to survive and, on the contrary, roots infected by the pathogen showed reduced longevity – thus, percentage of ECM root tips is as high as in non-affected trees. However, both fine-roots and ECM root tips have high turnover and their longevity may last only one or two seasons. In areas of *P. cinnamomi* occurrence, where

natural ECM are not able to produce chemical compounds inhibiting pathogen growth, both may compete for root tip colonization. Therefore, in the presence of high ECM inoculum, the fungi may have competitive advantage for root colonization in detriment of the oomycete. In this study, application of ECM in established and healthy adult cork oaks, likely to have natural ECM colonizing their root tips, resulted in a positive outcome motivating further studies on artificial ECM applications for *P. cinnamomi* prevention through direct competition on root infection, or for overall tree health improvement.

Finally, the role of starch – and non-structural carbon compounds in general - in tree decline would also be also an interesting approach to be taken. If *P. cinnamomi* cause impaired phloem transport, it may have important implications in tree response to infection, not only in relation to plant defense against root pathogens that are mediated by secondary metabolites, but also in relation to ECM root infection – favoring species with less carbon requirements but maybe not so efficient in protecting against root pathogens.

8- FINAL CONSIDERATIONS

This research was realized with the support of ICAAM (standing for Institute of Mediterranean Agricultural and Environmental Sciences) funding but it was not inserted in a broad project about the functional association between *P. cinnamomi* and cork oak decline, as it was expected in the beginning of the study. Consequently, there were some restrictions in the practical work, limiting the scope of the study. On the other hand, a deep analysis of former studies, complemented with observations realized in the study field, highlighted some aspects related to the association between *P. cinnamomi* and cork oak decline that should be explored more accurately. Therefore, this research moved to the direction of better comprehension of the physiologic status of adult trees under decline. It was then recognized that additional studies about decline processes should be undertaken in field conditions. Although experiences under controlled conditions allow a better understanding of the relation between each variable in plant responses, results obtained with this procedure cannot be extrapolated for adult trees, which are more complex, structurally and functionally, than young seedlings.

Finally, for further studies, it would be interesting to cover the aspects between tree health status, *P. cinnamomi* occurrence, relevant edaphic factors and human management in the *montado* system. Such approach would be a further step to the ultimate objective of all these researches, which is decline reversion and improvement in health status of those trees that play an essential role in the conservation of biodiversity, ecological processes, such as water retention, soil conservation or carbon storage, and in the socio-economy of the rural, disadvantaged areas in the Mediterranean basin, being a successful example of a balancing conservation and development for the benefit of people and nature.

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