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Effect of Soil Water Content and Soil Texture on *Phytophthora cinnamomic* Infection on Cork and Holm Oak

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Abstract. *Phytophthora cinnamomi* is an important soil borne plant pathogen, associated to decline of cork and holm oak stands in Iberian Peninsula. This decline results from a complex of biotic and abiotic interactions culminating in root infection. Field observations in cork and holm oak sites suggested an enhancement of soil moisture and texture in decline processes. Greenhouse assays were developed to evaluate the impact of soil moisture and texture on the severity of root infection on both species. Seedlings were potted in *P. cinnamomi* infested soils with different textures (loamy-sand, clay and silty-loam) combined with different watering regimes (flooding, normal irrigation, and irrigation till wilting and field capacity). The impact of *P. cinnamomi* infection was assessed through plant biomass, water consumption and root severity. The infection caused either losses of plant biomass (root and shoot) as plants water consumption, in both species, depending on soil moisture and texture. Holm oak plants were more susceptible to *P. cinnamomi* infection than cork oak, with higher mortality and root degradation. To minimize the risk of infection, watering managing appeared to be an essential condition to optimize plant growth and survival taking also into account the texture and moisture of the soil.

Key words: *Phytophthora cinnamomic,* decline, soil texture, soil moisture, biomass, water consumption

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Efeito do Teor de Água e da Textura do Solo na Infeção Causada por *Phytophthora cinnamomi* em Sobreiro e Azinheira

Sumário. *Phytophthora cinnamomi* é um patogénio do solo, associado ao declínio dos sobreiros e azinheiras na Península Ibérica. Este declínio resulta de um complexo de interações bióticas e abióticas causando a infecção do sistema radicular das plantas. Observações de campo sugerem que a textura e o nível de humidade do solo podem ter grande influência no processo de declínio. Os ensaios em estufa permitiram avaliar o impacto da humidade e textura do solo na severidade da infecção em plantas de sobreiro e azinheira. O impacto da infecção foi avaliado em plantas envasadas em solos de diferentes texturas (areno-argilosa, argiloso e argilo-limoso) combinados com diferentes regimes de rega (encharcamento, rega normal, capacidade de campo e perto de emurchecimento) através da biomassas (raiz e parte aérea), do consumo de água e da severidade da infecção na raiz. As plantas infectadas em ambas as espécies mostraram perda de biomassa e redução do consumo de água. A azinheira mostrou-se mais susceptível à infeção do que o sobreiro, com maior mortalidade e degradação das raízes. Para minimizar o risco da infecção, a gestão da água, parece ser uma condição essencial para otimizar o crescimento e a sobrevivência das plantas, tendo em atenção a textura do solo.

Palavras-chave: *Phytophthora cinnamomi*, declínio, textura do solo, humidade do solo, biomassa, consumo de água

Effet de la Contenu de l'Eau et de la Texture du Sol sur l'Infection par *Phytophthora cinnamomi* sur Chêne-Liège et Chêne Vert

Résumé. *Phytophthora cinnamomi* est un phytopathogène du sol, associé au dépèrissement des peuplements du chêne-liège et du chêne vert dans la Péninsule Ibérique. Ce dépèrissement résulte d'interactions biotiques et abiotiques qui cause l'infection des racines. Des essais en serre ont été développés pour évaluer l'impact de l'humidité et de la texture du sol sur l'infection des racines sur les plantes des deux espèces. Les plants étaient dans des sols infestés de *P. cinnamomi* avec différentes textures (sable loameux, argile et loam limoneux) combinés avec différents régimes d'arrosage (inondation, irrigation normale et irrigation jusqu'au flétrissement et capacité au champ). L'impact de l'infection a été évalué à travers de: la biomasse, la consommation d'eau et de la sévérité des racines. L'infection a causé la réduction de biomasse et de consommation d'eau par les plantes en fonction de l'humidité et de la texture du sol. Les plants de chêne vert étaient plus sensibles à l'infection que le chêne-liège, avec un renforcement de mortalité et de dégradation des racines. Pour minimiser le risque d'infection, la gestion de l'arrosage resemble à une condition essentielle pour optimiser la croissance et la survie des plantes en tenant compte également de la texture et de l'humidité du sol.

Mots-clés: *Phytophthora cinnamomi*, dépèrissement, texture du sol, humidité du sol, biomasse, consommation d'eau

Introduction

In Iberian Península, cork oak (*Quercus suber* L.) and holm oak (*Quercus rotundifolia* Lam.) are two important oak species, forming the evergreen oak stands, the so-called *Montados*. In Portugal, cork and holm oak decline has been observed since the 1890's (ALMEIDA, 1898) but it was only during the last decades (BRASIER *et al.,* 1993a) that the mortality of these oaks reached high proportions, particularly in the central and southern regions of the country. Oak decline is considered a multifactorial disease with a complex aetiology. MANION and LACHANCE (1993) proposed a model, that may explain the decline, wherein for each side a succession of several biotic and abiotic factors interact, according with type, intensity and frequency.

Phytophthora cinnamomi Rands is an oomycete that, under environmental favourable conditions, causes polycyclic diseases in successive cycles of inoculum production and infection, during the same season (FRY, 1982). In this way, the population of *P. cinnamomi* constantly increases the number of infectious propagules and consequently the number of foci of infection. The development of these diseases depends on the cumulative effect of multiple infections on the host root system and on the factors that influence the behaviour of the inoculum outside the host (DUNIWAY, 1983). In areas where the soil is soaked or moist through most of the year, the antagonistic microbial population can be inactive (NESBITT *et al*., 1979), whilst *P. cinnamomi* can survive on these soils for more than three years (ZENTMYER and MIRCECITH, 1966). Several studies support a relation between *P. cinnamomi* and oak decline, evidenced for example in central and southern of Portugal and Spain (MOREIRA and MARTINS, 2005;TUSET *et al*, 1996; BRASIER *et al* 1993b; SÁNCHEZ *et al,* 2002; SÁNCHEZ *et al* 2005; CAMILO-ALVES *et al*. 2013) pointing this pathogen as a very important contributor factor to the rapid decline of cork and holm oak trees.

Iberian Peninsula is mostly characterized by a Mediterranean-type climate, with short winters and long dry seasons culminating in hot summers. In recent years, *Montado* areas have been subject to cyclic droughts and rain episodes and floods, with a particular drastic impact in shallow soils and hydromorphic edaphic conditions where cork and holm oak decline and death are more evident. These abiotic factors may occur nowadays more frequently as result of climate change.

In Mediterranean areas, flooding rain episodes alternating with droughts, alongside with poor soils and inadequate cultural practices in association to the soil pathogens, have been indicate as the main factors responsible for the decline

and death of cork and holm oak trees (DINIZ, 1994; BRASIER, 1993; BRASIER, 1996; GALLEGO *et al.,* 1999; MOREIRA and MARTINS, 2005). Studies indicated that soil moisture regime and permeability, among other edaphic properties, such as texture, soil nutrient contents, pH and depth were important factors that could influence the activity and development of *P. cinnamomi* and consequently the root infection (STERN *et al*, 1977; WESTE and MARKS, 1987; MOREIRA and MARTINS, 2005; CORCOBADO *et al*., 2013).

Soil has an important role in the dynamics of the populations of the microorganisms that inhabit it. Field observations on cork and holm oak stands, showed that negative incidence of *P. cinnamomi* might be influenced by some edaphic and physiographic factors. Databases indicated that soils having higher clay and silt content usually yielded higher frequencies of *P. cinnamomi* in roots (CORCOBADO *et al.,* 2013, JÖNSSON *et al.,* 2005, JUNG *et al.,* 2000), and that topography might also contribute to increase the probability of *P. cinnamomi* occurrence (MOREIRA and MARTINS, 2005; CARDILLO *et al*., 2018). In fact, GÓMEZ-APARÍCIO *et al.* (2012), RODRIGUEZ-MOLINA *et al*., (2005) and HERNÁNDEZ-LAMBRAÑO *et al*., (2018) found that *P. cinnamomi* distribution and abundance in the Iberian forest soils is not random, but exhibits a clustered distribution influenced by soil texture what is related to the structure and availability of water. Soil moisture regime may specifically condition the activity of *P. cinnamomi*, by promoting successive cycles of infection spores (zoospores) and the activity of the remaining soil microflora (bacteria and other microorganisms) to interact or not with this pathogen (ERWIN and RIBEIRO, 1996).

In this whole context, the present study aimed analysing, under controlled conditions, the influence of different soil textures in combination with distinct watering regimes and duration periods, on the severity of *P. cinnamomi* infection on cork and holm oak seedlings. Our goal was to contribute to a better understanding of how the abiotic factors described above interact with *P. cinnamomi*, predisposing oak young plants to the decline disease in Portugal. Intended results could also be useful for collateral environmental analyses of the impact of climate change dynamic in plant growth, insofar that at least in Mediterranean areas, soil watering management can be also considered as representative of that dynamic.

Material and methods

Biological material

Cork and holm oak materials used in the three greenhouse assays, were acquired in a commercial forest nursery and transported to the experimental location, two months before the beginning of assays for acclimation. All the plants were selected individually in order to present the least possible variability.

Inoculum preparation

Inoculum with *P. cinnamomi* isolates from roots of cork oak trees from field plots in Corgas Bravas, Barranco do Velho and Ameixeira, S. Brás de Alportel, Portugal, was prepared with seeds of millet (*Panicum miliaceum* L.). The isolates were mating type A2. The preparation consisted in colonization of the seeds by the isolates during three weeks at 25ºC in dark, as described elsewhere (MOREIRA-MARCELINO, 2001). The inoculated millet seeds were mixed with soil in assays 1 and 3.

Soil preparation

Soil samples used in the assays 1 and 3, described below, were based on clayey soil samples, free of *P. cinnamomi* infestation, collected in the superficial layer up to 30 cm deep in an agricultural field located in Quinta do Marquês, Oeiras, Portugal. Two distinct textures were tested (i)"clay"- clay soil; (ii) A mixture of dry clayey soil with washed sand, in a ratio of 1:4, and with 10% of a green compost was used representing a loamy-sand soil. The obtained soil samples were dried, sieved and infested with the pathogen inoculum.

For assay 2, described below, a silty-loam soil sampled in field was collected from a cork oak woodland, located in Corgas Bravas, Caldeirão mountain (37.209N; -7.940W) Portugal. The sampled soil was naturally infested with *P. cinnamomi.* The soil was collected from the top 20 cm layer, under an adult cork oak tree canopy located downhill a very steep valley, close to a water line and showing evident symptoms of decline with a high degree of defoliation (GD-3) (CADAHIA *et al*, 1991). The presence of *P. cinnamomi* on roots and on soil samples was detected as described elsewhere (MOREIRA-MARCELINO, 2001).

Physical and chemical analyses of the soils used in the three assays are shown in Table 1.

Table 1 - Physical and chemical properties of soils in the three assays

Texture (1)	Csand (%)	Fsand (%)	Lime (%)	Clay (%)	pH	N (g Kg^{-1})	P_2O_5 (mg Kg^{-1})	K_2O (mg Kg^{-1})	OM (%)
Clay	16.4	14.4	21.5	47.7	7.7	2.42	288.7	518.2	6.7
Loamy-sand	79.8	8.2	3.4	8.6	6.4	1.12	113.6	294.0	5.3
Silty-loam	13.1	35.2	29.4	22.1	5.5	1.7	3.0	132	5.2

(1) According International Classification for soil texture; Csand- Coarse sand; Fsand-fine sand; OM- organic matter

Plant response evaluation

After the assays, plants were removed from pots, and roots washed with water, blotted and dried with paper towels. Root systems were then thoroughly examined for evidence of necrosis and fine root death.

P. cinnamomi was isolated from the roots in the infested and non-infested pots using the method described by MOREIRA and MARTINS (2005). Root damage symptoms were visually estimated by an empirical root severity score. The severity scale, adapted from TRAPERO and JIMENÉZ, 1985, ranged between 0 and 4, accordingly with the percentages of necrotic root tissue and the dead fine roots, in comparison with control, as follows: 0- no damage or necrosis, 1- 1 to 33%, 2- 34 to 66%, 3- 67 to 99%, and 4- 100% necrotic/dead tissue. Thereafter, root and shoot biomass of each plant were oven dried at 60°C, for a week and weighted. Plants dead before harvest date were assessed using the same parameters described above.

Assay 1- Effects of two watering regimes on severity of cork and holm oak plants over time using an artificial infested soil

Cork and holm oak seedlings (ninety for each species) with eight month-old were potted in plastic containers with 5 x 5 x 20 cm of capacity and filled with the loamy-sand soil (Table 1) infested with colonised seeds of millet, 60 g for each pot, prepared as described above. Control plants, 90 of cork oak and 90 of holm oak, were prepared in the same way receiving only sterilised seeds of millet and placed on separate trays. Infested and control pots were placed on separate trays to avoid contamination.

Thereafter, plants were subjected to the watering treatments: (i) flooding episodes simulated by saturation of soil with water for five days followed by 13 days without any water input; (ii) "normal irrigation" pots irrigated every three days according to their needs. Soil saturation was carried out by flooding the trays until the water level was 2-3 cm below the soil surface in the pots. After this period, pots were removed from the trays and left to drain until they are flooded again following 13 days. Fifteen seedlings of cork and holm oak and from each treatment (flooding/no flooding; infested /non-infested) were randomly chosen every three months, till the end of the assay. The root severity score was assessed for each plant through the parameters described above. The assay lasted 9 months and plants were kept in the greenhouse, with the temperature between 15ºC – 26ºC. Between March and June (6^{th} - 9^{th} month pos-soil infestation) water consumed by each plant in the flooding treatment, was weighted. Root severity score and root and shoot biomass for each plant were measured, in the term of the assay, accordingly with the above described.

This assay was set up in a completely randomized block design in pots, with two levels of infection status (infested vs non-infested; control); and two watering patterns (flooding and normal irrigation) with fifteen replicate seedlings per treatment, per species. The control concept is applied here only to not-infected plants for comparison with infected plants under two watering regimes. Seedling responses and *Phytophthora* infection were considered as dependent variables for the independent treatments.

Assay 2- Effects of two watering regimes on root damage of cork and holm oak plants using naturally infested soil

Twenty-four seedlings for each species with eight month-old were removed carefully from their original pots and replanted in plastic containers with 10 x 10 x 20 cm filled with naturally infested soil, collected in an affected site and described above, proceeding simultaneously with the potting of the plants to be tested. The naturally infested soil used in the control plants, was transported to the laboratory and autoclaved at 121ºC for a period of 1h in three consecutive days to kill *P. cinnamomi* population. Twenty-four plants for each species, cork and holm oak, were potted, with autoclaved soil, in separate trays. Thereafter, plants were subjected to the same watering treatments described in the assay 1 (flooding and normal irrigation).

Nine months after plantation, plants were removed and the root severity score for each plant was assessed, through the methodology described above.

Root and shoot biomass for each plant were measured, in the term of assay, accordingly with the above described.

This assay was set up in a completely randomized block design, considering the same treatments as described for assay 1, but, as aforementioned, in a naturally infested silty-loam soil with characteristics shown in Table 2 with twelve replicate seedlings per treatment, for each species.

Assay 3- Effects of two watering regimes and two soils textures on severity of cork and holm oak plants

In this assay were tested two soil textures (clay vs. loamy-sand) combined with two watering regimes. The watering regimes applied were: (i): irrigation till the field capacity (FC) for each soil defined as the stationary soil water content remaining after drainage during 24 to 48 hours, from saturation. For the clay soil sample, the water content in field capacity was about 38% (volume basis), corresponding to a 30 kPa suction. For loamy-sand soil sample it was about 12% corresponding to a 6 kPa suction; and (ii): irrigation until near wilting point (WP), corresponding to a 1.5 MPa suction and to about 28% and 7% moisture for the clay and loamy-sand soils, respectively.

Cork and holm oak eleven month-old seedlings were placed in 25 x 12.7 x 12.5 cm plastic pots. Infested and control pots were placed in separate trays (23 x 48 x 36 cm). During the first three months, infested and non-infested pots were subjected to flooding, as described above, for encouraging disease development, following with watering till FC and WP. Watering treatments were mimicking the water content by weight. Plants were kept in the greenhouse during 9 months (March-November), with the temperature between 15-26^{o}C. At the end of assay 3, the root severity score was assessed for each plant through the parameters described above. Between May and November (3rd-9th month after soil infestation) the water consumed by each plant in the field capacity water treatment, was measured by weight. Root and shoot biomass for each plant were measured, in the term of the assay, accordingly with the above described.

The bioassay was designed a split-plot experimental design in pots with treatments (infested vs. non-infested-control) and combination of two soil textures (Table 1) / watering schemes applied in sub-plots. Eight replicate seedlings were tested per treatment and per each species.At the end, infected and uninfected roots were observed under a microscope to detect changes in tissues. For this, small pieces about 1 cm long of roots segments were selected, washed and then fixed in Carnoy's 1: 1 solution (glacial acetic acid: 96% ethanol)

and kept at –20ºC. Later, some material was sectioned in a freezing microtome "Leica Biosystems Germany". The tissue sections were immediately placed in potassium phosphate buffer (0.07M K_2HPO_4), stained with Lactophenol cotton blue solution and mounted on Lactophenol. Fresh material (not included in resin or wax) roots segments of 20 month-old seedlings from cork and holm oak exhibited hard consistency, not allowing cutting sections with less than 55-60 µm width, more difficult to characterize and observe. Furthermore, during cutting, epidermis of necrotic roots was detached from the remaining tissues.

Statistical analyses

The variation on the effects of treatments on response variables (root and shoot dry biomass, root severity score and only in the assay 3 the plant water consumption) were analysed with a two-way ANOVA. LSD pairwise tests ($p \leq 0.05$ significance level) were carried out to evaluate significant treatment differences. All the statistical analyses were performed with package SPSS 8.0.4 for Windows.

Results

Assay 1- Effects of two watering regimes on root damage and on root and shoot biomasses of cork and holm oak plants, using an artificial infested soil

The analysis of variance confirmed that the watering factor ($F= 8.02$; $p< 0.01$) and the interaction *P. cinnamomi*-infection x watering x time, influenced significantly ($F= 3.72$; $p< 0.05$) the development of the cork oak plants evaluated in this study. Observations of infected plants six months after soil infestation, under flooding, showed lower height growth, slight crown symptoms and dark-brown root coloration, by comparison with not-infected plants. Other specific symptoms were taproot necrosis and loss of fine roots. Six months after soil infestation, the infected plants were not very affected in the general development by comparison to the non-infected plants. In infected plant new roots compensated the dead ones, indicating a moderate infection and a slow progression. At the end of the assay the prolonged flooding caused a significant ($p<0.05$) decrease in root (25%) and shoot (63%) biomass of infected cork oak plants by comparison of not-infected (Table 2). Extensive root necrosis with necrotic areas resulted in a significant root severity score (3.7), and a high loss of

rootlets relative to smaller plants (39%) was observed on tested plants after 9 months.

During the whole 9-month assay with normal irrigation, cork oak infected plants showed no visible symptoms of decline. The production of new roots in these plants throughout the assay occurred as a reaction to the infection, because infected plants were not enough stressed to inhibit a response to the infection.

Table 2 - Influence of two watering regimes (assay 1) on root severity score and on root and shoot biomass of cork oak plants in an artificially infested loamy-sand soil by *P. cinnamomic*

		Root				Shoot		Dead Plants (%)	
	Months	Not-infected (Control)		Infected		Not-infected (Control)	Infected		
Watering regimes		Biomass (g)	Rsev	Biomass (g)	Rsev	Biomass (g)	Biomass (g)	Not-infected (Control)	Infected
Flooding	3	3.5 bc	0.0 g	3.7 bc	0.5 g	2.6 bc	2.0 cde	0.0	0.0
	6	3.1 cd	1.0 f	3.8 abc	1.1 f	3.6 a	2.8 b	0.0	0.0
	9	3.6 bc	1.2 f	2.7 d	3.7 e	3.9 a	1.5 e	0.0	0.0
Normal irrigation	3	3.9 ab	0.0 g	3.9 ab	0.0 g	1.9 de	2.4 bcd	0.0	0.0
	6	3.3 bcd	0.0 g	3.9 ab	0.0 g	2.3 bcd	2.4 bcd	0.0	0.0
	9	3.6 bc	0.0 g	4.4 a	0.0 g	2.6 bcd	2.6 bc	0.0	0.0

The dry weights concern means of 15 plants per treatment; Rsev: Root severity score; numbers followed by the same letters (inside each area variable) are not significantly different ($p > 0.05$).

For holm oak plants, *P. cinnamomi*-infection ($F=51.11$; $p<0.001$) and time ($F=3.99$; $p<0.05$) factors were statistically significant. Infected plants under both watering regimes (Table 3) showed a high decrease in shoot and root biomass and a high root severity score over time, compared to the controls. Following induced flooding, 80% of the infected plants exhibited deep and extensive root necrosis (score of 3.5) three months after infestation and plants showed slower development with reductions of 27 % in plant heights. At the end, all the plants showed deep necrosis and high loss of rootlets with a dead incidence in 25% of plants (Table 3). However, surviving plants would be dead in a few days after the end of the assay, since their root systems exhibited a high degree of

destruction, insofar that more than 2/3 of taproots were necrotic, and 95% of the roots (fine and thick) had disappeared or were dead.

Table 3 - Influence of two watering regimes (assay 1) on root severity score and on root and shoot biomass of holm oak plants in an artificially infested loamy-sand soil by *P. cinnamomi*

		Root				Shoot		Dead Plants (%)	
	Months	Not-infected (Control)		Infected		Not-infected (Control)	Infected		
Watering regimes		Biomass (g)	Rsev	Biomass (g)	Rsev	Biomass (g)	Biomass (g)	Not-infected (Control)	Infected
Flooding	3	2.9 ^{bcd}	0.0 ^{h}	2.6 ^{d}	3.5 ^{e}	3.0 ^{nop}	3.1 ^{mnop}	0.0	0.0
	6	4.3 ^{a}	1.0 ^{h}	2.6 ^{d}	3.9 ^{e}	6.7 ^{i}	3.4 ^{lm}	0.0	0.0
	9	4.1 ^{a}	1.0 ^{g}	2.6 ^{d}	4.0 ^{e}	6.7 ^{i}	2.5 ^{pq}	0.0	25.0
Normal irrigation	3	3.8 ^{a}	0.0 ^{h}	2.6 ^{d}	2.8 ^{f}	3.4 ^{lm}	2.9 ^{op}	0.0	6.7
	6	3.8 ^{a}	0.0 ^{h}	2.8 ^{cd}	3.0 ^{ef}	4.9 ^{k}	3.0 ^{nop}	0.0	0.0
	9	3.8 ^{a}	0.0 ^{h}	2.9 ^{bcd}	3.8 ^{e}	5.7 ^{j}	3.8 ^{lm}	0.0	16.7

The dry weights concern means of 15 plants per treatment; Rsev: Root severity score; numbers followed by the same letters (inside each area variable) are not significantly different ($p > 0.05$).

Under normal irrigation, dead incidence in holm oak plants was 16.7%, (Table 3) and all the surviving plants showed a high injury on fine roots with significant necrosis with a score of 3.8 and a decrease in root weight, alongside with crown symptoms. These results showed that the high susceptibility of holm oak plants to *P. cinnamomi* infection is somewhat independent of soil water conditions. In assay 1, only holm oak plants showed mortality, under flooding at ninth month, and under normal irrigation at third and ninth months (Tables 2 and 3). The above ground parts of infected plants showed discolouration and partial defoliation whereas control plants lacked such symptoms. For both species in both treatments *P. cinnamomi* was reisolated from necrotic root tissues on infected plants, but not from controls.

During the last four months of flooding in assay 1, the water consumed by infected and not-infected plants of both species was assessed by weight. An evident difference of water consumption was observed in plants of both species (Figure 1), with greater relevance for the holm oaks. High water consumption was recorded for not-infected plants of both species, by comparison with

infected, with greater propensity of this tendency for not-infected holm oak plants. These results are indicative that either physiology as growth of young plants of both species could possibly be decreased by *P. cinnamomi* infection.

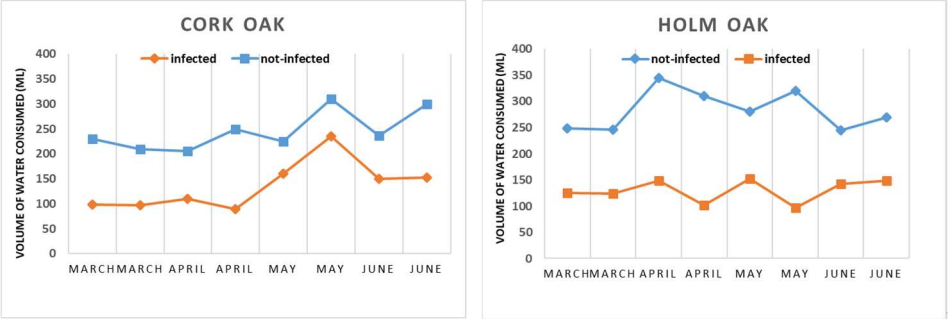

Figure 1 - Water consumed by infected and not-infected cork and holm oak plants during flooding watering in an artificially infested loamy-sand soil (assay 1) between March and June (6^{th} - 9^{th} month post- soil infestation)

Assay 2- Effects of two watering regimes on root damage and root and shoot biomasses of cork and holm oak plants in a naturally infested soil

Quantification of cork oak root biomass by analysis of variance ($F = 1.17$; $p > 0.01$) showed small differences, not statistically significant, between infected and not-infected plants, watering levels and in the interaction *P. cinnamomi*-infection x watering. However, for shoot biomass, infected plants of cork oak showed a significant decrease ($F = 57.48$; $p < 0.001$) of 43 % and 48% reflecting a smaller development by comparison with not-infected plants (Figure 2-A). The LSD between watering treatments, for shoots and roots of infected cork oak plants, were not significant (Table 4 and Figure 2-C).

Under flooding > 60% of infected plants exhibited lesions on root system, mature and lignified roots evidenced small necrotic lesions underneath external surface. Root severity score was 1.6, however not reflected by the decrease of root biomass, maybe because we assessed only the total of root biomass.

Under normal watering, infected plants showed a root severity score of 1.3 (Table 4), somewhat lower than under flooding, with only 20% (data not shown) of the plants exhibiting root lesions. The infected plants in both watering regime exhibited many new fine roots to compensate the dead ones, which explained their lower severity compared to the assay 1, as well as the slow progression of the infection. Root necrosis, were not observed on cork oak controls of the normal watering regime.

Table 4 - Influence of two watering regimes (assay 2) on root damage and on root and shoot biomasses (dry weight) of cork oak plants in a naturally infested silty-loam soil by *P. cinnamomi*

	Root				Shoot		Dead Plants (%)	
	Not-infected (Control)		Infected		Not-infected (Control)	Infected		
Watering regimes	Biomass (g)	Rsev	Biomass (g)	Rsev	Biomass (g)	Biomass (g)	Not-Infected (Control)	Infected
Flooding	9.1 a	1.0 b	9.0 a	1.6 b	9.4 d	5.4 d	0.0	0.0
Normal irrigation	9.9 a	0.0 c	8.4 a	1.3 b	12.2 d	6.4 d	0.0	0.0

The dry weights concern means of 12 plants per treatment; Rsev: Root severity score; numbers followed by the same letters (inside each area variable) are not significantly different (p> 0.05).

In this assay, holm oak infected plants by comparison with not-infected plants showed by analysis of variance a statistically significant reduction both to shoot ($F=36.60$; $p<0.001$) as to root biomass ($F=10.41$; $p<0.001$) concerning either *P. cinnamomi*-infection as well as watering factors (Figure 2-B). The LSD between watering treatments, for shoots and roots of infected holm oak plants, were not significant (Table 5 and Figure 2-D).

Table 5 - Influence of two watering regimes (assay 2) on root damage and on root and shoot biomasses of holm oak plants in a naturally infested silty-loam soil by *P. cinnamomi*

	Root				Shoot		Dead Plants (%)	
	Not-infected (Control)		Infected		Not-infected (Control)	Infected		
Watering regimes	Biomass (g)	Rsev	Biomass (g)	Rsev	Biomass (g)	Biomass (g)	Not-infected (Control)	Infected
Flooding	4.9 a	1.0 d	2.1 a	3.0 b	8.4 e	2.8 e	0.0	58.0
Normal irrigation	5.2 a	1.0 d	4.5 a	1.6 c	9.9 e	4.9 e	0.0	0.0

The dry weights concern means of 12 plants per treatment; Rsev: Root severity score; number followed by the same letters (inside each area variable) are not significantly different (p> 0.05).

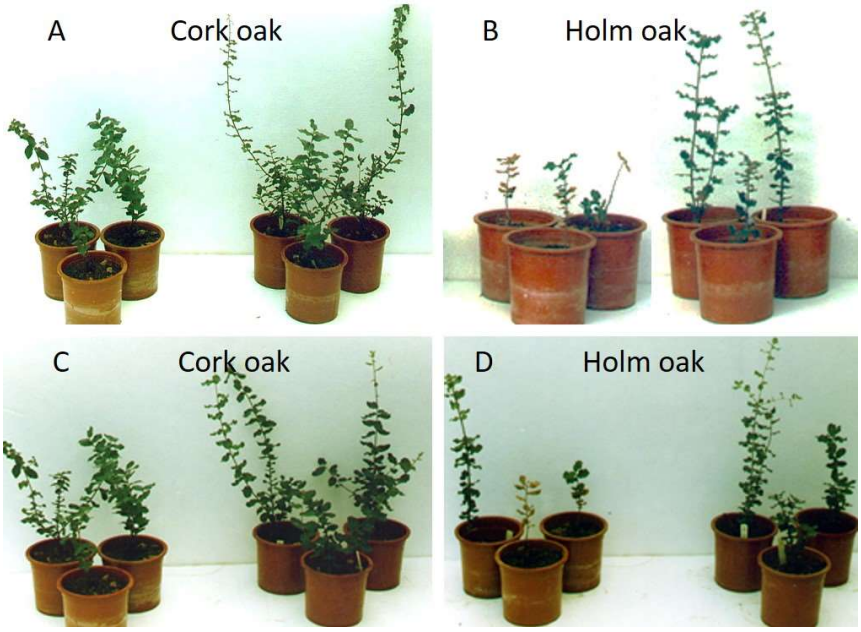

Figure 2 - Aspect of cork and holm oak plants in a naturally infested and autoclaved silty-loam soil after 9 months after soil infestation (assay 2). Cork oak: A and C; holm oak: B and D. (i) Plants submitted to the flooding regime on infested and autoclaved soil: A - infected plants on left and plants on autoclaved soil on right; B- infected plants on left and plants on autoclaved soil on right. (ii) Infected plants submitted to both water regimes: C- plants under flooding on left and under normal irrigation on right; D- plants under flooding on left and under normal irrigation on right

Under flooding, all infected plants exhibited a higher root severity score and a higher percentage of dead roots, especially the fine roots, by comparison with plants under normal watering. Biomass reduction of holm oak plants under flooding was of 67% and 55% on shoots and roots, corresponding to a mortality of 58% (Table 5).

Under normal watering few infected holm oak plants (20%) showed root lesions. The infected plants developed new roots showing a lower and slower infection progress than on flooding. No mortality was observed under this watering regime.

The naturally infested soil, showed a slowdown in the progress of the *P. cinnamomi* infection on plants of both species, more evident in holm oaks under normal irrigation. This is evident through the development of the aerial part of the plants (Figure 2). The statistical analyses of shoot biomass, in both species indicated that *P. cinnamomi*-infection and watering factors showed significant differences ($p<0.001$), between infected and not-infected plants. The slower progression of the infection in both species suggests being consequence of a smaller amount of potential inoculum of *P. cinnamomi* combined with low soil moisture. At the end, *P. cinnamomi* was recovered from necrotic root tissues of plants of both species in infested soil, in both watering regimes, but not from the autoclaved soil.

Assay 3- Effects of two watering regimes and two soil textures on root damage and on root and shoot biomasses of cork and holm oak plants

The analysis of variance showed that factors *P. cinnamomi*-infection and watering/soil texture and their interaction, influenced significantly ($F= 4.05$; $p< 0.05$) the behaviour of cork oak infected plants. There were no dead cork oaks (Table 6), although infected root systems, as in previous assays, exhibited taproot necrosis in both watering and soil texture factors, with the higher root severity scores (> 2.0) and root biomass reductions of 49% and 41%, in plants under watering till field capacity. On contrary, plants watered till wilting point in both soil textures showed lower reductions in root biomass (18% and 19%) and smaller root severity scores (Table 6) with less root injuries in infected plants (Figure 4).

For cork oaks watering pots until wilting point, in both soils, induced smaller plants, either in infected as in not-infected plants, with thicker and darker green leaves, by comparison with plants watered until field capacity. In clay soil, all inoculated plants, showed a decrease in height, particularly in FC/clay (46.5%). In the soil with lower moisture content, the root infection seemed less intense (Figure 4-C) and the number of infected roots was lower, however, the decrease of shoot biomass was higher than on field capacity irrigation. Water shortage resulted in fewer secondary infections and infected roots, but allowed the tissue colonization, perhaps due to the tissue weakening for lack of water leading to the non-functional roots.

On holm oak plants, the interactions watering x *P. cinnamomi*-infection and *P. cinnamomi*-infection x watering x soil texture, affected significantly the root and shoot biomass ($F=23.91$; $p< 0.001$). All the infected plants showed higher

severity values and a higher root and shoot biomass decrease under field capacity water conditions, under equal conditions, by comparison with not-infected plants. Root damage include, in general, extensive lesions and cell necrosis in xylem (Figure 4). At the end, 100% of the holm oaks on FC/clay soil conditions were dead by comparison with a nil mortality of cork oak plants (Table 7). Water shortage conditioned the development of the plants, which resulted in similar root and shoot biomasses for infected and control plants (Table 7), especially in the clayey soil. The water shortage resulted in dieback of fine roots and taproots necrosis observed in particular in clay soil (Table 7).

Table 6 - Influence of two watering regimes (assay 3) and two different soils artificially infested with *P. cinnamomi* on root severity score, root and shoot biomasses of cork oak plants

	Root				**Shoot**		**Dead plants (%)**	
	Not-infected (Control)		**Infected**		**Not-infected (Control)**	**Infected**		
Watering regimes/soil textures	**Biomass (g)**	**Rsev**	**Biomass (g)**	**Rsev**	**Biomass (g)**	**Biomass (g)**	**Not-Infected (Control)**	**Infected**
FC/C	21.1 ^{a}	1.0 ^{e}	10.9 ^{b}	2.4 ^{c}	19.8 ^{f}	15.9 ^{fg}	0.0	0.0
WP/C	13.1 ^{b}	1.4 ^{d}	10.7 ^{b}	1.9 ^{d}	15.4 ^{fg}	9.5 ^{h}	0.0	0.0
FC/LS	20.7 ^{a}	1.0 ^{e}	12.4 ^{b}	2.6 ^{c}	12.9 ^{g}	10.4 ^{gh}	0.0	0.0
WP/LS	14.7 ^{b}	1.0 ^{e}	12.2 ^{b}	1.3 ^{d}	12.5 ^{g}	9.5 ^{h}	0.0	0.0

The dry weights concern means of 8 plants per treatment; FC: field capacity; WP: wilting point; C: clay soil; LS: Loamy-sand soil; Rsev: Root severity score; numbers followed by the same letters (root: infected vs not-infected; shoot: infected vs not-infected) are not significantly different ($p > 0.05$).

Some control plants in all the watering regimes revealed also some necrotic lesions on roots and death of fine roots, although in smaller proportions than in infected plants in which dieback and root necrosis were more severe. This could be due to infections other than *Phytophthora* as result of water stress (combination of irrigation/soil texture) once plants were potted in a non-sterilized soil which microbiome could interact with the plants. The pathogen was isolated only from roots of infected plants of both species.

Analysis of variance for water consumption by plants showed statistically significant influence of interactions *P. cinnamomi*-infection x watering x soil texture for cork oak plants in assay 3, ($F = 22.85$; $p < 0.05$) in the same way as with biomass and root severity scores. This result allowed concluding about the

joint relevance of the three factors in the dynamics of plant growth of both species. This tendency for holm oak plants was the same with this interaction showing still higher significant differences (F=23.91; $p<0.001$) in water consumption between infected and not-infected plants under all watering regime and soil textures.

Table 7 - Influence of two watering regimes (assay 3) and two different soils artificially infested with *P. cinnamomi*, on root severity score and on root and shoot biomasses of holm oak plants

	Root				Shoot		Dead plants (%)	
	Not-infected (Control)		Infected		Not-infected (Control)	Infected		
Watering regimes/soil textures	Biomass (g)	Rsev	Biomass (g)	Rsev	Biomass (g)	Biomass (g)	Not-infected (Control)	Infected
FC/C	10.3 ^{a}	1.0 ^{f}	4.4 ^{c}	3.9 ^{d}	11.8 ^{h}	6.8 ^{j}	0.0	100.0
WP/C	7.8 ^{abc}	1.0 ^{f}	7.6 ^{abc}	2.8 ^{e}	8.9 ^{ij}	8.9 ^{ij}	0.0	0.0
FC/LS	10.4 ^{a}	1.0 ^{f}	4.3 ^{c}	2.9 ^{e}	15.7 g	6.4 ^{i}	0.0	0.0
WP/LS	8.8 ^{ab}	1.0 ^{f}	5.6 ^{bc}	2.3 ^{e}	10.7 hi	6.0 ^{i}	0.0	0.0

The dry weights concern means of 8 plants per treatment; FC: field capacity; WP: wilting point; C: clay soil; LS: Loamy-sand soil; Rsev: Root severity score; number followed by the same letters (inside each area variable) are not significantly different ($p> 0.05$).

Figure 3 shows that water consumption was higher in not-infected plants of both species, for soil at field capacity by comparison with soil at wilting point, especially for clay soils. This is in line with the decrease in root biomass. For loamy-sand soil the same tendency is more notorious for holm oak from May to September, and especially in the peak summer months. In addition, the differences in water consumption between not-infected and infected holm oak plants are greater than for the correspondent for cork oak plants, either in clayey as in loamy-sand soils. These results showed that in clay texture soil, a higher level of soil water is an influential factor to *P. cinnamomi* infection, by comparison with wilting point (Figure 3). The results also confirm that under high soil water amounts, the infection has a different effect on the two species, insofar that holm oak plants showed as more vulnerable to the infection than cork oaks. The pathogen activity was stronger in soils at field capacity moisture, inhibiting the water consumption of holm oak plants in a higher extent than the one of cork oak plants. These effects are more evident in fine soils, and

inclusively for cork oak plants in loamy-sand soil the difference of water consumption between infected and not-infected plants was very small.

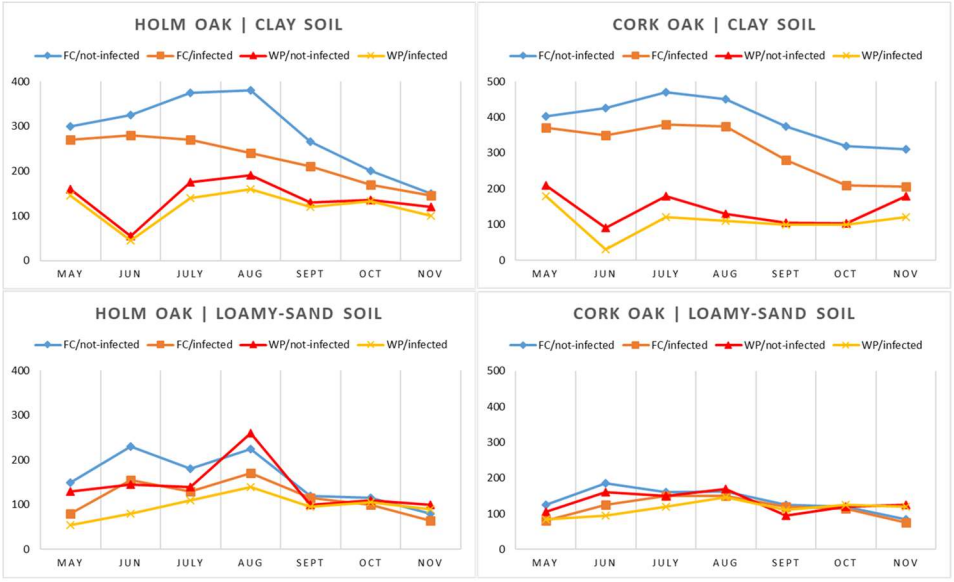

Figure 3 - Volume of water consumption by cork oak and holm oak plants, infected and not-infected, in clay and loamy-sand artificially infested soils in field capacity and wilt point moisture conditions between May and November (3rd - 9th month post-soil infestation) (legend-FC: field capacity; WP: wilting point)

At the end of the assay 3, microscopic observations of transversal sections of infected cork and holm oak roots segments took place, and showed *P. cinnamomi* hyphae in all tissues and confirmed the intense root colonization by the pathogen (Figure 4). Hyphae invaded the root cortical parenchyma and the central cylinder. The observations confirmed that in all the treatments the negative effect of the infection was more notorious under high soil water availability at field capacity, associated to fine textures (clay). In most affected roots, areas of the central cylinder showed necrotic vascular vessels very destroyed and sometimes surrounded by hyphae. The observations indicated that the infection progressed more in the thinner roots than in the larger ones. However, roots with high diameter (>7 mm in diameter) were also affected. These observations were similar in infected roots in all treatments. Differences observed on root damage between treatments were associated to the number of infected/dead roots in high moisture conditions. The penetration and

intercellular progression of the pathogen through the cortical parenchyma and central cylinder were similar in both species and quicker in infected plants under high moisture conditions.

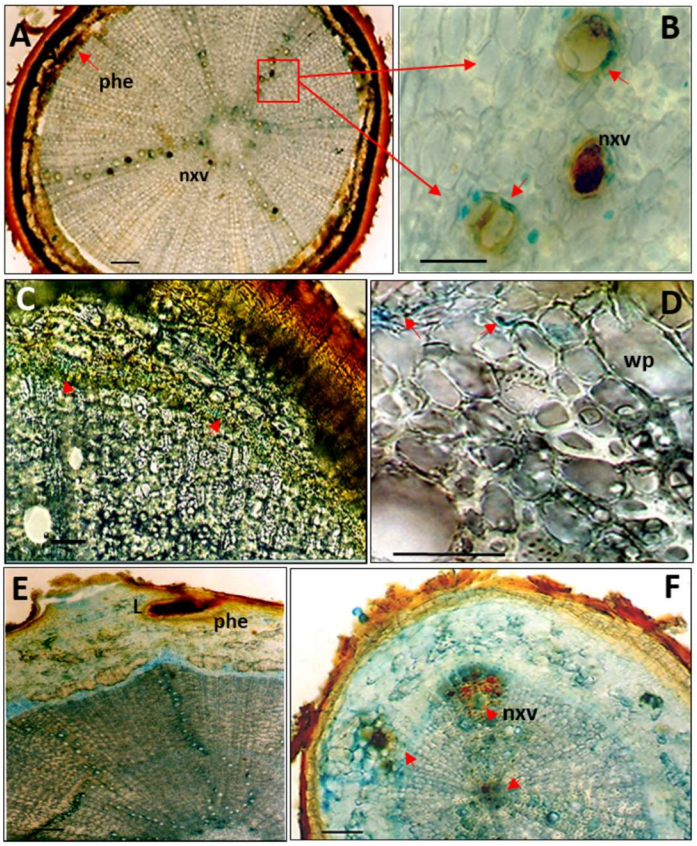

Figure 4 - Transversal sections of secondary cork and holm oak roots segments infected by *P. cinnamomi* and submitted to different water conditions. A-D- cork oak roots. A- root section of plant grown on clay soil with a high moisture content-field capacity (FC/clay) evidencing necrotic phelloderm (phe) and necrotic xylem vessels (nxv). Bar =100 µm. B- detail of necrotic xylem vessels (nxv) (arrows) and hyphae. Bar = 50 µm; C- section of a root in loamy-soil with water near the wilting point (WP/loamy- sand soil),few hyphae were observed near the phloem (arrows); D-(detail) cross section of a thin root under clay soil with shortage water (WP), showing intercellular fungus hyphae (arrows) Bar = 50 µm; E-F- infected holm oak roots under clay soil with a high moisture content-field capacity (FC/clay): E- > 4mm in diameter, small lesion in the cortical parenchyma (L) delimited by phelloderm, hyphae were found in the

phloem and in the woody parenchyma. Bar =100 µm; F- young and small root < 2mm in diameter with necrotic cells and necrotic xylemic vessels (nxv) in the cylinder central. Bar = 100 µm

Overall, this study showed an interaction between soil moisture and the *P. cinnamomi* infection on the root systems of cork and holm oak potted seedlings, under controlled conditions. The severity of root damage caused by the infection on young plants of these oak species differed among soil moisture levels. Indeed, the high negative impact of the *P. cinnammomi* infection was observed with high levels of soil water content (FC and flooding). Soil texture was shown also to interact with root infection insofar that fine textured soils, with water retention, led to an increase in the pathogen's inoculum favouring the root infections.

Discussion

This study showed that the infection impact was reflected in decreases in shoot and root biomasses with fine root loss, production of new roots, and in damage of root systems with diverse degrees of severity as described in previous studies (BRASIER *et al*., 1993b; SÁNCHEZ *et al*., 2002; TAPIAS *et al*., 2004). This global picture, common to considered soil textures and watering as indicative of the negative impact of the pathogen on the plant development and physiology of both species under controlled conditions, is in line with what occurs in field stands (e.g., MOREIRA and MARTINS, 2005; CAETANO, 2007; MORALEJO *et al*., 2009; MOREIRA *et al*., 2018).

Our observations showed that the pathogen intercellular progression in seedlings of both species begins in the root epidermis, following through the cortical parenchyma and reaches the root central cylinder. From the central cylinder the infection supposedly spreads to the whole root systems. These observations are similar to those found in other studies (RUIZ *et al*., 2015). In this context, PIRES *et al.* (2005) referred that necrosis on young roots of both species result of invasion of phloem and xylem vessels by *P. cinnamomi* hyphae shortly after infection.

Holm oak plants were more susceptible to *P. cinnamomi* than cork oaks, with higher mortality rates, greater root damaging and higher severity scores, particularly in summer time. Higher decreases of root and shoot biomass and higher differences of water consumption between not-infected and infected plants during summer periods were also noticed by other authors (e.g., TUSET *et*

al., 1996; ROBIN *et al*., 1998; MOREIRA-MARCELINO, 2001; SANCHEZ *et al*., 2002; TUSET and SÁNCHEZ, 2004; PIRES *et al*., 2005; LÉON *et al*., 2017).

The cumulative negative impact of multiscale processes, such as invasion of vascular tissues by *P. cinnamomi* hyphae (Figure 4) consisted in fine root death, with loss of biomass both in roots and shoots, surely delivering restrictions in plant growth and physiology (ROBIN *et al*., 2001; MAUREL *et al*., 2001; SGHAIER-HAMMAMI *et al*., 2013; RUIZ-GÓMEZ *et al*., 2015). The root system degradation induce a severe reduction of water supply and increase in plant hydric stress, causing plant mortality (RUIZ-GÓMEZ *et al*., 2015). These circumstances were also reflected on high root severity scores in both species and were shown also in other studies (e.g., WILCOX and MIRCETICH, 1985; DUNCAN and KENNEDY, 1989; RISTAINO and DUNIWAY, 1991; RUIZ-GÓMEZ *et al*., 2015; HOMET *et al*., 2019).

This negative impact of flooding, regarding mortality and root damage, was higher in holm oak seedlings probably due to a greater sensitivity to a decrease of soil aeration and lower oxygen level, interfering negatively with the ability of plants to respond to infection inhibiting the emission of new roots to replace the dead ones. High soil moisture provides contribute also for inoculum build-up of *P. cinnamomi*, increasing thereby the capacity of the pathogen to cause root damage (ZENTMYER, 1980; DUNIWAY, 1983).

As expected, under normal irrigation, the negative impact of *P. cinnamomi* infection in plants of both species were mitigated with fewer aerial symptoms in both naturally and artificially infested soils. Cork oak plants showed inclusively some stimulus in root and shoot biomass in the artificially infested soil. In this context, it was also observed that dead fine roots following *P. cinnamomi* infection were compensated by new roots emerging, akin with a pruning effect, and leading to an increase of root and shoot biomass. This reaction has been observed in others species. PHILLIPS and WESTE (1984) also observed this situation considering this stimulus as a reaction of the plant to infection by *P. cinnamomi*. HOMET *et al*. (2019) observed a similar response in inoculated cork oak seedlings, with increasing net photosynthetic rates in response to *P. cinnamomi* infection, in order to counteract loss of root functionality. The balancing effect reported by these authors in cork oak seedlings was significant even with average pathogen density, with the condition that plant tolerable environmental and *P. cinnamomi* inoculum density thresholds be respected.

Under both watering regimes, root damage evaluated through severity scores, was not as drastic in natural infested silty-loam soil as in artificially infested loamy-sand soil. This effect was more pronounced in holm oak

seedlings. This was perhaps because the natural soil samples presented a lower density of *P. cinnamomi* from the beginning. In addition, this soil with a finer texture could deliver good hydric conditions for a robust expansion of new fine roots, which invigorated plants to better resist to the pathogen infection. In fact, HOMET *et al.* (2019) verified that the soil inoculum density required to cause significant root damage, with holm oak potted seedlings, decreased as soil moisture increased.

The Analyses of the impact on pathogen infection alongside with the watering of the two soils until FC and WP levels combined with the soil texture, revealed the significant interactions of these three factors. The infected cork and holm oak plants with highest root infection were the ones associated with soil moisture at field capacity in both soil textures. On contrary, seedlings of both species under wilting point in loamy-sand soil exhibited slower root and shoot growth and less infection. A similar effect was observed by other authors (TIPPETT *et al*., 1987; MARÇAIS *et al.*, 1993; HOMET *et al*., 2019). On the other hand, CORCOBADO *et al.* (2014) and RUIZ-GÓMEZ *et al.* (2018) found that holm oak seedlings were very sensitive to conditions wherein root infection was combined with water shortage conditions. Growth reduction is a common mechanism to compensate for the low amount of water available, as result of less photosynthetic activity. A decrease in the normal water supplement is reflected in low plant growth due to the corresponding decrease in the production of auxins (OGAYA and PEÑUELAS, 2003).

The three-way interactions were reflected by distinct shoot biomass variations with soil moisture in comparison with control. While for clayey soils shoot biomasses of infected holm oak plants increased from FC to WP moisture thresholds, for loamy-sand soils the tendency was opposite. On the hand, for cork oak infected plants shoot biomasses increased with soil moisture for both textures.

Dissimilarities occurred also with biomasses of root systems in infected plants. Indeed, on one hand for holm oak in both soils, these biomasses increased with soil moisture from FC to WP moisture amounts. Under reduced moisture conditions for both soils a slight decrease of root biomass occurred for infected cork oaks.

The results of ANOVA of water consumption of infected and not infected plants as a function of soil watering, soil texture and infection with *P. cinnamomi* in the period between May and November showed also a three-way significant interaction, wherein infected plants of both species exhibited lower water consumption by comparison with not-infected plants.

This was verified mainly for clayey soils at field capacity (Figure 3). Indeed, for infected and not-infected plants of both species the patterns of water consumption were more or less parallel at WP and FC soil moisture contents in the whole six-month period, with a higher water consumption by not-infected plants. On the other hand, for holm oak plants the main differences in water consumption were verified at field capacity, and especially between June and September when water stress is higher. As abovementioned, water consumption under flood watering between March and June of infected plants of both species in loamy-sand soil. The minor water consumption of infected seedlings of both species seem to confirm the aforementioned restrictive role of infection in plant physiology, with relation with shoot and root biomasses and damage of root systems. This restrictive role was more evident for clayey soils and was presumably, caused by a loss of fine roots coupled with a blockage of xylem through hyphal obstruction and deposition of plant materials inhibiting vertical water movement from root systems to shoots (RUIZ GÓMEZ *et al*., 2015).

Conclusions

The results of this work showed the interconnectedness between of soil texture and watering in the dynamics of *P. cinnamomi* infection in cork and holm oak seedlings potted in controlled conditions. These dynamics concerning biomass balances of root systems and shoots, alongside with the water plant consumption, reflected significant three-way interactions among the main factors involved. The infection caused biomass losses of plant roots and shoots of both species and root damages at different levels. Holm oak plants, with higher mortality and root degradation, were shown as more sensitive to pathogen infection than cork oaks. Infected plants of both species consumed more water per month by comparison with their not-infected counterparts, particularly on fine texture soils. Monthly variation of infected vs. not-infect plant water consumption appeared thereby to be a credible proxy of the level of infection and degradation of plant physiologic and growth profiles.

The climate change dynamics is a current challenge that turns necessary to assess the effects of severe rain episodes and droughts on the dynamics of the *P. cinnamomi* population on the cork and holm oak agroforest systems. Extreme rain events could saturate the soil causing waterlogging, especially in clayey and shallow soils, which will favour *P. cinnamomi* infections. The primary infection of pathogen in these species could be however, slowed down more by frequent drought events in Mediterranen regions, notwithstanding the tendency

for enhanced conditions of tree weakness, which would reduce its resistance. The predicted alternating extreme events of drought and flooding could contribute to the negative impact of the infection on these species. The results were indicative about the convenience of considering management strategies for optimizing soil physical conditions for ensuring good plant development and vigour. Canopy management should by tailored to control the density of the pathogen inoculum in the soil, as low as possible, for mitigation of the *P. cinnamomi* impact in already infested forest canopies as in un-infested areas.

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