Physiological effects of *Phytophthora cinnamomi* infestations on *Quercus suber* seedlings

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Abstract

Oak decline is among the more serious environmental problems in the Mediterranean Basin. *Phytophthora cinnamomi* Rands has been identified as the main pathogen involved in the decay and death of holm and cork oaks in the southwest of the Iberian Peninsula. In this work, we examined the variation of physiological parameters in two-year-old plants of cork oak infested by *P. cinnamomi* fungi. Seedlings from 10 different families were grown in 300 cm^3 forest pots containing a commercially substrate (peat + perlite) or a natural one (forest soil + perlite). During their second year of life, half of the plants were inoculated with *P. cinnamomi* mycelia. Survival, gas-exchange parameters (photosynthesis, transpiration), water relations (water potential of leaves, relative water content, stomatal aperture) and status of the photosynthetic system were monitored on a periodic basis. Four months after inoculation, stem hydraulic conductance was measured in a plant sample. The results revealed considerable susceptibility of cork oak to the fungus. Thirty-eight days after inoculation, the plants exhibited significant differences in water status between treatments, but not between substrates or families. The water potential of inoculated plants exceeded that of non-inoculated plants by 43%. These differences in plant water status were not echoed by any significant differences in photosynthesis or transpiration rate, however. Hydraulic conductance was higher in plants grown in natural soilthan in those grown in the commercial substrate (peat + perlite). There were significant differences in maximum specific conductance referred to leaf area between inoculation treatments; thus, non-inoculated plants exceeded inoculated plants by 21% in this respect.

Keywords: oak decline, gas exchange, water potential, hydraulic conductance, disease effects.

Introduction

Since the early XX century, *Quercus* species all over Europe have gone through several stress episodes some of which have resulted in tree decay and death (Brasier, 1996). In fact, *Quercus* populations in the Mediterranean Basin have decayed severely since the 1980s. As in central Europe, a number of factors including severe, recurrent droughts, flooding, atmospheric pollution, changes in traditional dehesa uses, attacks by trunk boring insects and canker fungi, have caused the decay of Mediterranean *Quercus* trees (Montoya, 1992; Ragazzi *et al*, 1989; Tuset *et al*, 1996; Sánchez *et al*, 2000, 2002).

Tree decay has a complex origin. Initially, it was ascribed to the action of a variable number of biotic and non-biotic factors causing gradual, extensive deterioration of trees leading to their death. Typically, tree decay involves several factors none of which by itself can account for every symptom observed in the field. Also, the factors are exchangeable, act in a non-specific manner, and can vary in time and space but still produce the same symptoms (Sinclair, 1965; Manion, 1991). In western Andalusia (Huelva, Andévalo), the tree damage pattern involves small foci with a medium proportion of affected trees, a random distribution in land and a prevalence of progressive death. Damage in this area has been unequivocally associated to the presence of *Phytophthora cinnamomi* (Rupérez and Muñoz, 1980; Brasier *et al*, 1993a, 1993b; Tuset *et al.*, 1996; Gallego *et al*, 1999; Sánchez *et al*, 2000, 2002; Ferraz *et al*, 2003; Navarro *et al*, 2004), which causes root rot in a variety of hosts including *Quercus* spp. This fungus results in massive death of absorbing roots, thereby reducing the ability of trees to absorb water and nutrients. The symptoms observed in the aerial part of trees are similar to those of drought and result in gradual decay, but have occasionally led to sudden death in some trees (Luque *et al*, 1999, 2002; Sánchez *et al*, 2000,2002; Luisi *et al.*, 1993; Agrios, 1997). Biological baits have allowed the fungus to be isolated in most of the plots sampled in the province of Huelva (Sánchez *et al.*, 2000; 2002).

The resistance/tolerance mechanism for the plant remain obscure. Unlike other species of this fungal genus (viz. *Phytophthora quercina*, Jung *et al*, 1999), there is no evidence of toxins released by *P. cinnamomi* reaching other parts of the plant. The most plausible hypotheses in this respect are as follows: (a) general resistance mechanisms for water stress allowing plants to survive with a partly damaged root system as if they were under a severe drought; (b) a high root regeneration ability; and (c) differences in biochemical composition of the roots hindering penetration and colonization by the fungus. There is evidence that water stress in plants infested by fungal pathogens is associated to a loss of xylem conductance (Vanni and Valentín, 1994; Solla-Hach, 2000), which depends on the ease of cavitation of the vessels.

Also, adequate availability of nutrients (particularly potassium) helps overcome water stress and influences the amount of phenols and tannins present in roots, and hence their disease resistance (Shaw *et al*, 1998; Marschner, 1995).

In order to monitor the effects of *P. cinnamomi* infestation on decline in holm oak (*Quercus ilex* L.), in this work we examined various physiological aspects including photo-synthetic behaviour, water status, gas exchange and hydraulic conductance in fungus-inoculated two-year-old plants under typical nursery conditions and compared the results with those for non-inoculated plants.

MATERIAL AND METHODS

Plants were produced from *Quercus suber* L. seeds that were collected from various areas in the province of Huelva (viz. Hinojos, Almonte, Doñana National Park and San Bartolomé). Once germinated, seedlings were planted in 300 cm polyethylene pots containing a commercial substrate (PP) consisting of 5:1 peat/perlite or a natural substrate (NS) consisting of 4:1:1 natural soil/sand/perlite. The natural soil was obtained from a holm oak dehesa in El Villar (Huelva).

The pots were placed in 35-cup trays in accordance with a randomized block design. Plants were grown in two steps. The first, in a shade nursery, spanned the period from June 2003 to May 2004. Plants were watered as required to ensure and optimum status for vegetative growth. No differences in health status or development between substrates were observed during this step. The second step, in a hothouse, spanned from May to September 2004. At the beginning, half of the plants were artificially infested with a mixture of 5 provenly pathogenic *P. cinnamomi* strains and placed on two separate tables under constant humidity conditions. A complete factor design involving 12 trays (2 substrates x 2 inoculation treatments x 3 trays per treatment) was used. The seedlings employed in the experiment were selected from families exhibiting 4-6 plants in each type of substrate; therefore, a total of 135 oaks from 10 families were studied. The families were distributed among groups in such a way that each tray would only contain one plant per family.

The inoculum vehicle consisted of millet seeds (Moreira, 2001). The plants were one-year-old at the time of inoculation (June 1, 2004). A perforating punch was used to make three holes 0.5 cm in diameter at a depth of 5-8 cm in the ball near the stem. The holes were used to insert 1.5 g of colonized millet seeds that were then covered with the previously extracted substrate. The plants were supplied with dechlorinated water on a daily basis in order to preserve water saturation. After inoculation, the inoculum was monitored at random in the two substrates in order to check its viability as previously proposed by Edwin and Ribeiro (1996).

Plants were assessed by making various measurements of the status of the photosynthetic system (chlorophyll fluorescence), water potential, gas exchange variables and stem hydraulic conductance during the experiment. The chlorophyll fluorescence parameters determined included initial fluorescence (F_o), variable fluorescence (F_v), maximum fluorescence (F_m), mean time for variable fluorescence to be reached (T_m) and variable-to-maximum fluorescence ratio (F_v/F_m). The water potential (Ψ) was determined by using the pressure chamber

developed by Schölander *et al.* (1965) and commercially available as model 1000 (Corvallis, Oregon). Carbon dioxide uptake was measured with a portable CO_2 IR analyser (LCi, ADC®, UK). Hydraulic conductance was determined by cutting stems into pieces 2-3 cm long and placing them in a hydraulic circuit in order to measure the amount of water passing through the segment during a preset time. The applied pressure was 0.065 bar and that used to reverse embolism 1.5 bar. After each stem piece was measured (initial conductance: Ci), potential cavitation was reversed by applying an overpressure (maximal conductance: Cmax). The loss of hydraulic conductance (LHC) was obtained as the percent difference between the previous two values: % LHC = 100 (Cmax - Ci)/Cmax. These values were referred to the conducting xylem cross-section, leaf area and weight of transpiring leaves supplied with water from this flow in order to calculate the specific xylem conductance (C_{ex}) (kg/m^2 s MPa), specific conductance per leaf area (C_{ela}) (kg/m^2 s MPa) and specific conductance per leaf weight (C_{elw}, kg/g s MPa). Measurements were made in 6 inoculated plants and 6 non-inoculated plants. Comparisons between the two substrates were based on the same procedure and involved 6 plants grown in PP and another 6 grown in NS. Table 1 lists the measurement dates for the fluorescence, water potential and gas exchange in addition to the inoculation time. Because conductance measurements were destructive, they were made at the end of the experiment (September 2004). The time elapsed between fungal inoculation and measurement was 115-120 days.

Days after artificial infestation	Water potential	IRGA	Fluorimeter
Before infestation	25/05/2004	25/05/2004	25/05/2004
8-19	08/07/2004	19/06/2004	18/06/2004
33-39	30/07/2004	02/07/2004	02/07/2004
45-60		14/07/2004	14/07/2004
80-85	20/08/2004	20/08/2004	25/08/2004

Table 1. Dates of the physiological status measurements. The first column lists the time elapsed after inoculation with P. cinnamomi.

The test results were statistically analysed with the software SPSS v. 12. The significance of each factor was determined by using the linear general model and means were compared via Tukey's test.

Results

Chlorophyll fluorescence

The statistical analysis of the initial fluorescence (F_o) revealed significant differences between dates and the date*substrate*family interaction only. The results obtained in both substrates were similar and decreased with time (see Table 2). This was also the case with the variable fluorescence (F_v) and maximum fluorescence (F_m). The F_v and F_m values for the Control treatment decreased with time; on the other hand, those for the inoculated plants initially decreased and then increased in the last measurement.

	Date	25/05/2004	18/06/2004	02/07/2004	14/07/2004	25/08/2004
	Days after inoc.	-5	18	33	45	85
F_o		448,12±24,82	393,91±6,97	376,61±6,81	366,71±7,27	375,74±3,88
F_v	Control	1749,72±35,79	1706,33±44,57	1706,23±37,10	1693,86±66,44	1494,02±23,52
	Inoculated	1846,13±48,35	1556,00±38,45	1730,50±41,50	1710,40±63,85	1802,74±23,16
F_m	Control	2175,64±44,70	2103,17±48,93	2073,62±42,01	2055,71±67,86	1875,61±26,54
	Inoculated	2277,93±61,41	1946,40±45,42	2119,10±47,84	2083,90±67,99	2172,63±26,25

Table 2. Mean values and standard error of initial fluorescence (F_o), variable fluorescence (F_v) and maximum fluorescence (F_m) on each studied date.

The mean time required to reach variable fluorescence (T_m) differed significantly between dates and substrates, and also for the date*treatment interaction. The Control treatment performed better: its plants took less time to reach variable fluorescence, the difference peaking at the end of the experiment (Table 3). The ratio of variable fluorescence to maximum fluorescence exhibited significant differences between treatments ($p = 0.054$), and so did the date*treatment and date*treatment*substrate*family interactions. Until July 7, the F_v/F_m ratio was higher for the Control treatment than for the inoculated plants; the two nearly converged, but the ratio for the Control treatment decreased whereas that of the inoculated plants increased during the last period.

	Date		25/05/2004	18/06/2004	02/07/2004	14/07/2004	25/08/2004
	Days after inoc.		-5	18	33	45	85
Tm	Control	NS	328,42±17,64	270,50±8,85	282,50±15,12	250,17±14,14	206,86±10,65
		PP	295,46±16,80	262,33±12,88	292,14±16,66	251,25±9,20	205,07±7,57
	Inoculated	NS	353,50±26,28	339,14±33,83	360,17±29,73	254,33±11,69	314,40±9,14
		PP	294,43±36,56	293,33±38,32	348,75±25,98	253,25±3,96	319,89±12,14
Fm/Fv	Control		0,804±0,003	0,810±0,005	0,822±0,003	0,820±0,008	0,795±0,003
	Inoculated		0,815±0,002	0,798±0,003	0,816±0,003	0,819±0,007	0,829±0,001

Table 3. Mean values and standard error of the time needed to reach variable fluorescence (Tm), and the variable-to-maximum fluorescence ratio (F_v/F_m) for the two types of treatment and substrate on each studied date. (NS: natural soil and PP: peat +perlite commercial substrate)

Water potential

The water potential, **Ψ**, exhibited significant differences between treatments and dates. Prior to inoculation, both treatments started with similar values; from the second measurement to the last, however, inoculated plants exhibited much lower **Ψ** values than did control plants (see Fig. 1). Differences were significant from the second measurement after inoculation. After 35 days, the water potential of inoculated plants was 1.4 times lower than that of control plants, the difference amounting to 1.5 times after 80 days.

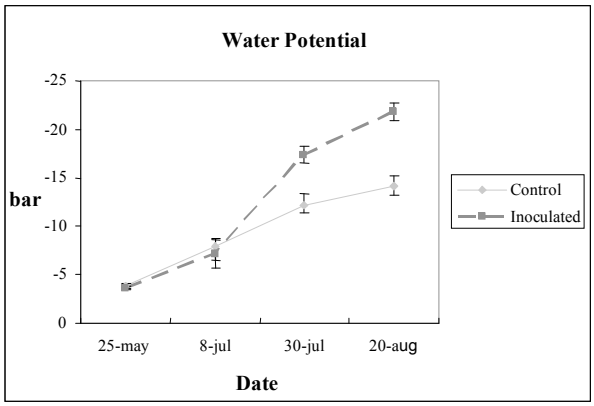

Figure 1. Variation of the water potential and standard deviation for cork oaks with treatment and date.

Gas exchange

Transpiration, stomatal conductance and photosynthesis values as referred to leaf área (m^2) and leaf weight (g) (Table 4) differed between dates, but not between treatments. Both plant groups exhibited similar values prior to inoculation that decreased in the middle of the studied period but regained their initial levels by the end.

		31/05/2004	19/06/2004	02/07/2004	14/07/2004	20/08/2004
Transpiration (mmol H_2O m^{-2} s^{-1})	C+T	5,41±0,21	2,71±0,17	3,99±0,29	5,77±0,29	6,33±0,17
Stomatal conductance (mmol H_2O m^{-2} s^{-1})	C+T	237,67±10,3	129,01±10,5	176,30±16,3	318,16±23,5	290,48±11,4
Photosynthesis (µmol CO_2 m^{-2} s^{-1})	C+T	11,03±0,45	6,58±0,43	9,62±0,60	14,85±0,69	14,37±0,44
A/E (µmol CO_2/mmol H_2O)	C+T	2,06±0,05	2,44±0,07	2,47±0,09	2,60±0,07	2,28±0,04
A/gs (µmol CO_2/mol H_2O)	Control	46,74±1,86	51,62±3,27	57,26±3,73	47,69±1,74	50,56±1,75
	Inoculated	48,56±1,96	55,28±3,61	60,14±4,32	49,33±3,25	54,29±1,47

Table 4. Variation of the gas exchange parameters [transpiration relative to leaf area (mmol H_2O m^{-2} s^{-1}), stomatal conductance relative to leaf area (mmol H_2O m^{-2} s^{-1}) and photosynthesis-to-transpiration ratio (µmol CO_2/mmol H_2O)] in cork oaks between dates and treatments.

Hydraulic conductance

The statistical analysis of the initial conductance (Ci), initial xylem conductance (Ci_{ex}), maximum conductance (Cmax) and maximum specific xylem conductance ($Cmax_x$) revealed significant differences between substrates and the natural substrate (NS) to exhibit better conductance than the commercial substrate (PP) (see Table 5). The maximum specific conductance as referred to dry leaf weight and leaf area exhibited significant differences between treatments; thus, the plants in the Control group exhibited an increased ability to supply water to their leaves relative to the inoculated plants (0.0095 vs. 0.007 kg/m Kg/m^2 s MPa).

The statistical analysis of the loss of hydraulic conductance (LHC) revealed differences between treatments. Control plants experienced greater losses than did inoculated plants (33.75 ± 4.2 vs. 20.3 ± 2.7%); however, the values for each treatment were consistent with typical LHCs -even with those for healthy, well-watered plants (10-30%).

		Substrate	
		Natural soil (NS)	Peat+perlite (PP)
C.i. (Kg/s MPa)		$4{,}490\ 10^{-5}\pm6{,}436\ 10^{-6}$	$2{,}030\ 10^{-5}\pm4{,}740\ 10^{-6}$
C.i. $_{ex}$ (Kg/m^2 s MPa)		10,69±0,71	6,00±0,91
C.i. $_{elaf}$ (Kg/m^2 s MPa)		$6{,}87\ 10^{-3}\pm4{,}31\ 10^{-4}$	$5{,}58\ 10^{-3}\pm1{,}03\ 10^{-3}$
C.max. (Kg/s MPa)		$5{,}660\ 10^{-5}\pm8{,}178\ 10^{-6}$	$2{,}680\ 10^{-5}\pm5{,}004\ 10^{-6}$
C.max. $_{ex}$ (Kg/m^2 s MPa)		13,51±1,12	8,09±0,95
C.max. $_{ela}$ (Kg/m^2 s MPa)	Control	$0{,}009\pm0{,}61\ 10^{-3}$	0,010±0,002
	Inoculado	0,008±0,001	0,006±0,02

Table 5. Mean hydraulic conductance of plants grown in a mixture of natural soil, sand and vermiculite (NS) and a commercial substrate consisting of peat and perlite (PP). Ci = initial conductance, Ci_{ex} = initial specific xylem conductance referred to the conducting xylem cross-sectional area (m^2), Cmax = maximum conductance following reversal of potential cavitations; $Cmax_{ex}$ = maximum specific xylem conductance referred to the conducting xylem cross-sectional area (m^2). $Cmax_{ela}$ = maximum specific conductance referred to leaf area in each type of substrate.

There were no significant differences in any studied physiological parameter between families.

Discussion

The results obtained in this work help us to describe the infestation/attack process in terms of plant water status, gas exchange parameters and secretion of defense substances such as phenols and tannins. This can help improve available knowledge about the process and the relationships with plant status (Luque *et al*, 1999; Sánchez *et al*, 2002).

Cork oaks maintained a good physiological status -better than that exhibited by holm oaks (Tapias *et al*, 2005b)- throughout the studied period. This suggests an increased resistance or tolerance of the former species, even though it was inoculated at a lower rate (1 g/plant vs 1.5 g/plant). This is consistent with previous results (Sánchez *et al*, 2002; Tapias *et al*., 2005a; 2008). Thus, Tapias *et al*. (2005a; 2008) found mean annual survival rates of 50% in holm oaks and as high as 70% in cork oaks grown in various types of substrates; rates fell to 2-33% in the substrates bearing the highest pathogen concentrations, however.

The physiological parameters most markedly influenced by the pathogen were the water potential and, to a lesser extent, those related to gas exchange. As regards chlorophyll fluorescence, the strongest influence was exerted by the mean time required to reach variable fluorescence (T_m).

After 35 days, the water potential of inoculated plants was 1.4 times higher than that of control plants, the difference rising to 1.5 times after 80 days. The differences were smaller

and occurred later than they did in holm oaks, which exhibited significant differences and potentials twice lower in their inoculated plants after only 18 days (Tapias *et al*, 2005b).

The gas exchange values obtained for cork oaks were essentially similar to those found in a simultaneous test on control holm oaks (Tapias *et al*., 2005b). The values for the control plants of this species clearly surpassed those of infested plants after 33 days and doubled the photosynthesis-related values by the end of the experiment.

The effect of inoculation on hydraulic conductance was weaker than were those of substrate and species. The lack of an effect of the treatment factor was a result of the active conducting cross-section of stems forming over long periods (years) preceding evaluation and infestation with the pathogen. Cork oaks can supply or transport water to their leaves over 3 times more easily than holm oaks. Also, plants grown in the natural substrate (NS) were more efficient in supplying or transporting water to their leaves than are those grown in peat substrate (PP). This may be the result of the natural substrate leading to more fertile plants growing to a thicker diameter and exhibiting a disparate ratio of the bark thickness and stem medulla surface to stem total surface. The stems of the plants grown in the natural substrate (NS) exhibited lower proportions of bark and marrow with respect to the total surface than did those of the plants grown in the peat substrate (PP); as a result, the stems grown in NS possessed an increased water conducting ability relative to those grown in PP.

Infestation with *P. cinnamomi* worsens the water status of the aerial portion of the plant (stem and leaves), especially in the more susceptible species (after 18-30 days). Likewise, the photosynthetic system exhibits an increased delay in electron transfer between photosystems. Only when water stress is strong -as found in holm oaks- does it affect gas exchange rates. Stem conductance can exhibit differences in the long run if infestation is present during a substantial portion of the time needed for the active conducting section to form and leads to thinner vessels. Surprisingly, however, the loss of hydraulic conductance (LHC) was not higher in inoculated plants, which were under stronger water stress. This may have been the result of the fungus having some effect on the vessel sealing ability of the plants.

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