

Chronic cork oak decline and water status: new insights

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Abstract Chronic decline and Sudden death are two syndromes of cork oak (*Quercus suber*) dieback. Mortality is associated with water stress, but underlying physiological mechanisms are poorly understood. Here, we investigated the physiological performance of declining trees during the summer drought. Leaf water potential, gas-exchange, fluorescence of photosystem II and leaf and root starch concentration were compared in healthy (asymptomatic) and declining trees. Low annual cork increment in declining trees indicated tree decline for several years. All trees showed similar water status in spring. In summer, declining trees showed lower predawn leaf water potential (−2.0 vs. −0.8 MPa), but unexpectedly higher midday leaf water potential than healthy trees (−2.8 vs. −3.3 MPa). The higher midday water potential was linked to by means of strongly reduced stomatal conductance and, consequently, transpiration. This study is pioneer showing that declining trees had high midday water potential. A tendency for lower sap flow driving force (the difference between predawn and midday water potential) in declining trees was also associated with reduced photosynthesis, suggesting that chronic dieback may be associated with low carbon uptake. However, starch in roots and leaves was very low and not correlated to the health status of trees. Declining trees showed lower water-use efficiency and non-photochemical quenching in summer, indicating less resistance to drought. Contrarily to chronic decline, one tree that underwent sudden death presented predawn leaf water potential below the cavitation threshold.

Keywords *Quercus suber* · Mortality · Dieback · Sap flow driving force · Sudden death

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Introduction

Forest decline and tree mortality associated with water stress are widespread and some of the world's forested ecosystems may already be responding to climate changes. Several studies suggest that forests may become increasingly vulnerable to higher background tree mortality rates and die-off in response to future warming and drought (see Allen et al. 2010). Mediterranean regions are already characterized by recurrent droughts and long dry summers with most of annual precipitation falling in the six cool season months (Rundel et al. 1995). Mediterranean trees have evolved with summer drought as one of the major environmental constraints (Sala and Tenhunen 1994; David et al. 2004; Otiemo et al. 2007). However, 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 and Lionello 2008). Cork oak (*Quercus suber* L.) is a Mediterranean tree of high conservation and socioeconomic value. Cork is the outer layer that covers the tree trunk and branches and is removed every 9 or 10 years for commercial purposes. It is the main product obtained from this system with its global production reaching 201.428 ton in 2010 and cork stoppers correspond to 70% of cork's market value (APCOR 2011). The silvopastoral systems derived from mediterranean oaks (the Portuguese montados, the Spanish dehesas or Italian pascolo arbolato) conjoins several other production activities - agriculture, pasture, grazing, animal stock, etc. that share the same growing space (Ribeiro and Surovy 2011). Cork oak Montados alternate cork extraction with multi-purpose farmland systems high in natural value.

High mortality events in cork trees have occurred in the Mediterranean basin since the beginning of the twentieth century, but the severity of cork oak decline increased during the 80s (Baeta Neves 1949, 1954; Natividade 1950; Macara 1975; Cabral and Sardinha 1992; Brasier et al. 1993; Carvalho 1993; Moreira 2001; Sousa et al. 2007). Two main types tree death syndromes have been observed (Cobos et al. 1992; Tuset et al. 1996; Gallego et al. 1999; CAMA 2001; Moreira 2001; Ruiiu 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; (2) a progressive decline with gradual loss of foliage, drying of the tree top and sprouting of epicormic shoots. Cork oak decline is considered a complex multifactorial phenomenon involving the combination of several factors acting together (Camilo-Alves et al. 2013), which may be aggravated by climate change. Factors associated with decline are mainly drought (Macara 1975; Cabral et al. 1992; Lloret and Siscart 1995; Peuelas et al. 2001), soil characteristics that may limit root expansion (Bernardo et al. 1992; Ribeiro and Surovy 2007; Costa et al. 2010; Dinis et al. 2015), root rot caused by the soilborne root pathogen *Phytophthora cinnamomi* (Cobos et al. 1992; Brasier et al. 1993; Robin and Desprez-Loustau 1998; Sanchez et al. 2002; Moreira and Martins 2005) and root destruction caused by soil disking practices (Ribeiro et al. 2010, Dinis 2014). Studies regarding physiological responses of cork oaks to environmental stress have focused on healthy trees under short-term summer droughts, (e.g., Faria et al. 1996, 1998; Garcia-Plazaola et al. 1997; Otiemo et al. 2006, 2007; Passarinho et al. 2006; David et al. 2007; Grant et al. 2010; Vaz et al. 2011; Pinto et al. 2012; Kurz-Besson et al. 2014). These studies show the isohydric behavior of healthy trees facing summer droughts: a decline in predawn leaf water potential correlated to soil moisture and a steady midday leaf water potential by means of strong stomatal control. While much progress has been achieved in understanding the effects of episodic events of water stress on tree performance, there is little knowledge on tree's physiology under permanent stress and/or in trees that already

lost their recovery capacity. Oak decline around the world is a known phenomenon and studies under field conditions on adult oak trees with chronic stress are necessary to shed light on the physiological processes preceding death. Analyzing trees that are far from optimal conditions for long periods is a first step in understanding the physiological parameters underlying tree death, the limitations of trees to overcome stress events, and also to predict mortality events. Thus, the objective of our study was to analyze what and how physiological processes change when trees are already suffering chronic decline. Our approach was based on the conceptual framework proposed by McDowell et al. (2008) of the functional mechanisms underlying plant mortality. Two physiological processes were suggested by these authors: (1) hydraulic failure—massive xylem embolism occurring when soil water content does not meet transpiration demand; (2) carbon starvation—negative carbon balance after prolonged stomatal closure. The carbon-starvation hypothesis predicts that mortality occurs in isohydric trees that strongly regulate stomatal conductance to prevent cavitation resulting in insufficient carbon assimilation under prolonged non-lethal drought. A complementary mechanism for this hypothesis is that trees may die before carbon depletion due to the slowdown in long-distance phloem transport limiting carbon translocation within the tree (Sala et al. 2010). This study compared the seasonal water relations of *Q. suber* trees showing decline symptoms with the water status of healthy (asymptomatic) trees, under natural conditions. Tree water dynamic was analyzed through measurement of leaf water potential at predawn and midday. The daily difference between both water potential values—the sap flow driving force—is related to the water flow rate through the plant. Maximum xylem water flow is well correlated to maximum stomatal opening and, therefore, to minimal diffusional limitations to photosynthesis (Thomas et al. 2003). When sap flow is reduced, not only root nutrients uptake is limited, but also photosynthesis. Therefore, leaf water potential and sap flow driving force are good indicators of tree hydraulic status and carbon assimilation. Leaf gas exchange, chlorophyll fluorescence and starch concentration were also measured to better understand the decline of cork oak and to assess the implication of water potential results. The hypothesis was that trees under chronic decline—thus under suboptimal conditions for a long period—already have physiological dysfunctions to respond to seasonal water stress. In view of that, the specific questions of this work are: Do healthy and declining cork oaks respond physiologically differently to periods of low water availability? Is cork oak, an isohydric species, dying from carbon starvation?

Materials and methods

Study site

Since 1995 a set of 64 permanent plots were installed in montados in the center of Portugal (39°6'N8°22'W, 130–150 m a.s.l., 16 °C mean annual air temperature, 640 mm mean annual precipitation) with the assistance of forest producers associations. Plots are circular with 80 m in diameter and located on undulating terrain. Tree density varies between 60 and 140 trees/ha and canopy coverage varies between 35 and 60%. Cork oaks are systematically monitored in regards to tree growth and cork production (Ribeiro et al. 2003a, b) which is linked with information regarding site characteristics, stand structure, human management and meteorology. Spatial tree growth models and mortality models (CORKFITS, Ribeiro et al. 2004, 2006) and a decision support system (ECCORK,

Pinheiro et al. 2008) were produced with the information gathered in the permanent plots, both being useful tools to help forest producers in their management practices decisions.

Eight managed cork oak stands, located on Cambisols and gleyc lxisols soils were selected from the permanent plots for the study. Five stands have no known limitations to cork oak growth and the remaining three stands, located on fluvisols soils, show deficient water drainage and present excess water holding capacity for cork oak growth. Five to six trees per stand, in a total of 43 trees were selected for water status and cork growth measurements. Dendrometric measurements have been performed in those trees since 1995 and cork is extracted every 9 years. In 2010, diameter at breast height (DBH) was about 33.76 ± 8.4 cm and trees were 9.18 ± 1.54 m high with no statistical differences between healthy and declining trees. By that time about half of the trees presented decline symptoms such as upper dead branches and epicormic shoots. The remaining trees were asymptomatic. In each plot, there were both declining and asymptomatic trees. Physiological measurements were carried between June and September 2010. During the winter and early spring preceding the measurements, precipitation was higher than the long-term mean in Portugal (1971–2000, Fig. 1) and cumulative precipitation between October 2009 and March 2010 was 40% higher than average for the region (IPMA 2010, 2011).

Cork growth

Last cork extraction was in 2009 for 27 of the sampled trees and in 2008 for the remaining 16 trees. Perimeter at breast height was measured before (PBHb) and after (PBHa) cork extraction. Cork thickness was determined using the formula: $(PBHb - PBHa) / 2\pi$. Cork samples of 20×20 cm were obtained at breast height and in the same southern orientation during the stripping of the trees. To analyze rings width the fresh cork was dried under ambient conditions. Radial cork strips used for growth evaluation by means of image analysis were ca. 3–4 cm thick in axial direction, about 2 cm wide and smoothed on their cross-sectional surfaces with a microtome knife profile “c” (Reichert-Jung, Leica Instruments, Nußloch, Germany). Annual cork increment (between winters) was measured with Windendro[®] Software (Regent Instruments Inc.) in 32 of the studied trees. The first growth ring (since cork extraction until the following spring) was ignored from the analyses.

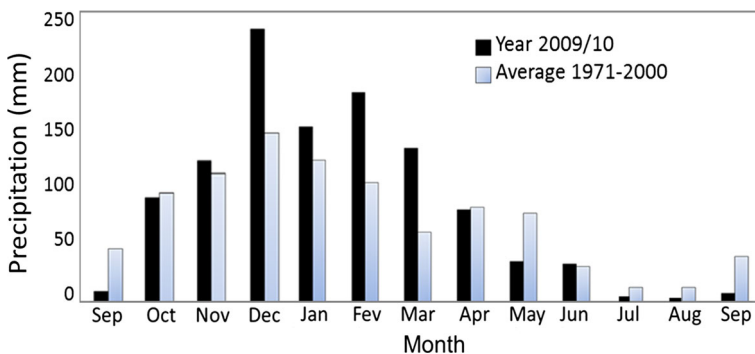


Fig. 1 Monthly values of total rainfall observed for the region in 2010 and long-term averages (1971–2000)

Water potential

To analyze tree water status according to decline symptoms, 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 and 4 m high were sampled just prior to sunrise and at midday (12–13 h, local time). Samples were taken at a similar height above ground to avoid variability due to hydrostatic pressure and leaf water potential was measured immediately after cutting. Measurements were done during 2 weeks in spring (early June) and during 2 weeks in summer (end August/September, before fall rains), all on sunny days. Each tree was measured five times in each season. In figurative representations, average values per tree by season were computed.

Physiological measurements

Instantaneous gas exchange and chlorophyll fluorescence measurements, chlorophyll and starch quantification were performed in a subsample of 12 trees located in four adjacent plots. Two plots were located on gleyc lixisols and the other two on cambisol soils. Each plot had declining and asymptomatic trees. All measurements were taken from young, fully sun expanded leaves at 4 m high. This information was obtained only in summer, a critical period when drought and high temperatures lead to high evaporative demand and low soil water availability (Tenhunen et al. 1990).

Instantaneous gas exchange and chlorophyll fluorescence measurements

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.), at PPFD 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with a CO_2 concentration in the leaf cuvette of 360 ppm CO_2 . Block temperature was kept at 30 °C. Measurements were done in summer during the morning period (10.00–11.00 h, local time) on four sun-exposed leaves per tree.

Chlorophyll quantification

Relative chlorophyll content was measured with a portable chlorophyll analyzer (Hansatech chlorophyll meter CL-01) and, for calibration propose, 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, quick-freeze in liquid nitrogen and then kept in the freezer (−20 °C) until laboratory analyses. The disks were placed into 8 ml of 100% methanol, and pigments were allowed to extract in the dark at 30 °C 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 a 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} :

$$\begin{aligned} \text{Chlorophyll a} &= 18.22 * A^{665.2} - 9.55 * A^{652.0}; \text{Chlorophyll b} = 33.78 \\ &* A^{652.0} - 14.96 * A^{665.2}; \text{Chlorophyll a} + \text{b} = 24.23 \\ &* A^{652.0} - 3.26 * A^{665.2} \end{aligned}$$

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

Starch quantification

Since starch is considered the most important carbohydrate reserve (Kozłowski 1992), it was measured in the source (leaves) and in one sink (roots) in August: Leaves were collected during the morning period (10.00–11.00 h, local time) and roots were collected in four locations distant 1 m from the trunk base of each tree, during the afternoon. All samples were stored in $-20 \text{ }^\circ\text{C}$ until laboratory analyses. Roots were sieved from the soil and washed with tap water. Turnover roots were discarded and structural roots (that can reserve starch) with $\approx 5 \text{ mm}$ were selected. Bark and heartwood were discarded and sapwood tissues used for starch measurements. Leaves and root sapwood samples were oven dried at $85 \text{ }^\circ\text{C}$ for 48 h, ground and analyzed for starch reserves in duplicates using the amyloglucosidase α -amylase method (total starch kit, megazyme[®]). Initially, all glucose and maltodextrins were first removed from the samples with an 80% ethanol solution at $85 \text{ }^\circ\text{C}$ for 5 min and resistant starch was pre-dissolved by stirring the samples with dimethyl sulphoxide at $100 \text{ }^\circ\text{C}$. 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 of each sample was read at 510 nm in a spectrophotometer. Starch was estimated according to the following equation: $\text{Starch (g/100 g)} = \Delta A * F * 1.8$; where ΔA is the absorbance (reaction) read against the reagent blank and F is the conversion from absorbance to μg ($F = 100 \mu\text{g of D-glucose/absorbance for } 100 \mu\text{g of glucose}$).

Statistical analyses

The permanent plots are monitored periodically. In the year following the physiological measurements, it was observed that seven of the declining trees analyzed in this study were dead. Moreover, one of those trees presented symptoms of sudden death. It was then decided to split the group of the declining trees into three groups. Therefore, for statistical analyses and graphics there were four groups, according to health status: health status 0—no declining symptoms (21 trees); health status 1—declining trees alive in 2011 (15 trees); health status 2—declining trees dead in 2011 (six trees); health status 3—trees with sudden death symptoms by late summer 2010 (one tree). Health status 3 was removed from all the statistical analyses due to the absence of replicates.

Statistical analyses were made using the SPSS v.11 software package. Linear Mixed models were used to analyze (1) if tree water status varied along seasons and if it was related to health status (2) if total cork thickness was related to tree health status and (3) the relation between annual cork increment and tree health status. In all the referred analyses “plot” was considered a random factor. The period of cork growth was also a categorical factor in the last model. Bivariate associations between variables were assessed by Chi

square tests. For the analyses in the subsample of 12 trees, there was only two health status categories: 0—no declining symptoms; and 1—declining trees. Thus, two-sample *T* Test with unequal variance was used to compare significant differences between photosynthetic parameters according to tree health status. All measurements shown are the mean \pm standard deviation.

Results

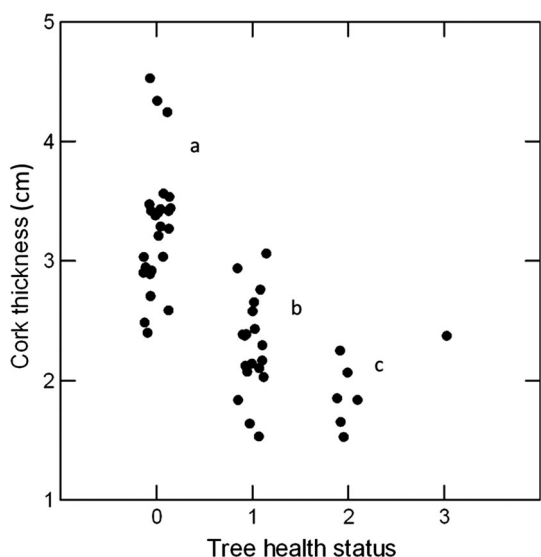
Cork growth

The 9 year-old thickest cork was observed in healthy trees (health status 0, $p < 0.001$, Fig. 2). This trend was observed through time (Fig. 3; “Appendix”). The thinnest cork occurred in trees with health status 2, both when considering total cork thickness ($p = 0.04$) or annual cork increment ($p = 0.015$). Total cork thickness was 32.45 ± 5.26 mm in trees with health status 0; 22.96 ± 4.29 mm in trees with health status 1 and 18.67 ± 2.29 mm in trees with health status 2. Cork thickness of the tree with sudden death symptom (health status 3) was 23.57 mm.

Tree water status

Variations in water potential related to tree health status were observed only in summer (Table 1). In late spring, all trees showed high Ψ_{pd} and leaf water status decreased equally at midday, resulting in similar sap flow driving force ($\Psi_{pd} - \Psi_{md}$; David et al. 2007) regardless of tree health status. In summer, trees were not able to maintain the same Ψ_{pd} observed in spring and reduction was stronger in declining trees ($p < 0.001$; Fig. 4; Table 1). On the other hand, significant variation in Ψ_{md} between spring and summer was only observed in healthy trees, showing lower Ψ_{md} in summer and maintaining the same sap flow driving force along seasons (Table 1). Therefore, contrary to what was expected,

Fig. 2 Relationship between thickness of the 9 years old cork extracted in 2008 and 2009 ($n = 43$) and tree health status 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 by late summer 2010. Different letters denote statistically significant differences at the 5% level



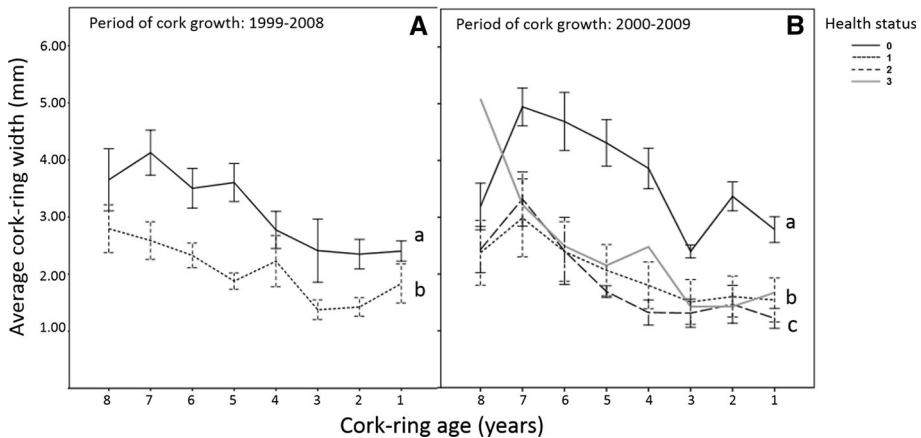


Fig. 3 Profile plots of the means standard errors of annual cork-ring increment during the 9 years of cork growth. **a** trees debarked in 2008, $N = 12$. Trees debarked in 2009, $N = 20$. Trees health status classification in 2010: 0-asymptomatic, 1-trees with upper branch dieback and epicormic shoots, 2-symptomatic trees considered dead in 2011, 3-tree that suffers from sudden death by late summer 2010. Different letters denote statistically significant differences at the 5% level

trees showing symptoms of water stress showed less sap tension at midday summer than asymptomatic trees (Fig. 4). As a consequence of a combination of lower Ψ_{pd} and higher Ψ_{md} , sap flow driving force was strongly reduced in declining trees (Table 1; Fig. 5).

In 2011 six of the declining trees were considered dead and cut down (health status 2). We assessed if in 2010 it was already possible to observe differences in water status between those dying trees and the other trees under chronic decline yet alive in 2011. Statistically, no significant differences were observed between both tree types in summer water status (Figs. 4, 5). However, sap flow driving force tended to decrease as decline aggravated (Fig. 5).

Another declining tree suffered from sudden death by late summer 2010 (health status 3). 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 of all studies individuals ($\Psi_{pd} = -3.62$; $\Psi_{md} = -3.75$ MPa, Fig. 4).

Photosynthetic parameters and starch concentration

In the subsample with 12 trees, there was asymptomatic trees and declining trees that remained alive in the year after measurements (health status 1). Physiological analysis realized in summer on the subsample showed that declining trees had lower stomatal conductance rate and lower net CO_2 assimilation rate, accompanied by declines in maximum efficiency of PSII (F_v/F_m'), photochemical efficiency of photosystem II (ϕPSII), non-photochemical quenching ($q\text{NP}$) and leaf chlorophyll content (Table 2).

Starch concentration was low in both roots and leaves (8.4 ± 1.3 and 12.2 ± 3.8 mg g^{-1} respectively) and it was not statistically related to tree health status or tree water status ($p > 0.05$).

Table 1 Average water status of *Quercus suber* trees according to decline symptoms (N = 43)

Parameter	Spring				Summer			
	Health status				Health status			
	0	1	2	3	0	1	2	3
Ψ_{pd} (MPa)	-0.44 ± 0.23 ^a	-0.41 ± 0.17 ^a	-0.46 ± 0.71 ^a	-0.51	-0.80 ± 0.42 ^b	-1.81 ± 0.51 ^c	-2.17 ± .040 ^c	-3.62
Ψ_{md} (MPa)	-2.92 ± 0.48 ^a	-2.78 ± 0.82 ^a	-2.83 ± 0.91 ^a	-3.33	-3.28 ± 0.51 ^b	-2.83 ± 0.41 ^a	-2.62 ± 0.20 ^a	-3.75
$\Psi_{pd} - \Psi_{md}$ (MPa)	2.48 ± 0.5 ^a	2.37 ± 0.36 ^a	2.37 ± 0.64 ^a	2.82	2.48 ± 0.71 ^a	1.01 ± 0.78 ^b	0.45 ± 0.33 ^b	0.13

Health status: 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

Ψ_{pd} Predawn leaf water potential, Ψ_{md} midday leaf water potential, $\Psi_{pd} - \Psi_{md}$ sapflow driving force

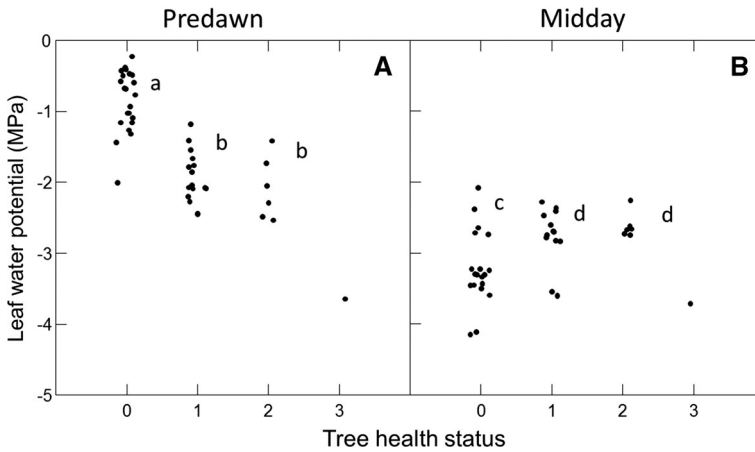


Fig. 4 Water status of *Quercus suber* trees (n = 43) at **a** predawn and **b** midday measured between Aug 30th and Sep 10th 2010, according to tree health status. Decline symptoms: 0-asymptomatic, 1-trees with upper branch dieback and epicormic shoots, 2-symptomatic trees considered dead in 2011, 3-tree that suffers from sudden death by late summer 2010. Each point denotes mean values for each individual tree. Different letters denote statistically significant differences at the 5% level

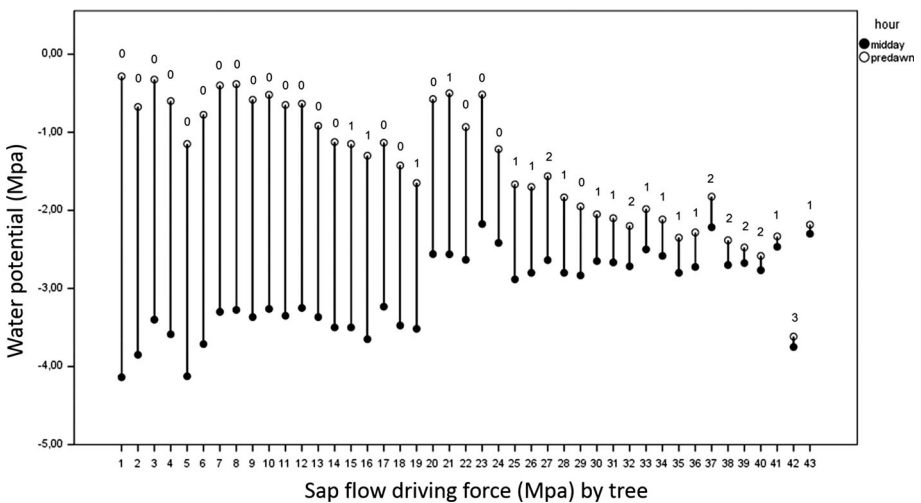


Fig. 5 Predawn and midday water potential of the *Quercus suber* trees (n = 43) measured between Aug 30th and Sep 10th 2010, ranked from higher to lower sap flow driving force: 0-asymptomatic, 1-trees with upper branch dieback and epicormic shoots, 2-symptomatic trees considered dead in 2011, 3-tree that suffers from sudden death by late summer 2010

Discussion

Symptoms of chronic decline

Dieback of branches and epicormic shoots are unspecific symptoms that can be associated with changes in soil moisture or virulent pathogens (Ciesla and Donaubaer 1994).

Table 2 Average physiologic parameters of *Quercus suber* trees according to decline symptoms (N = 12)

Parameter	Health status	
	0	1
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)	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
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	0.37 ± 0.07 ^a	0.32 ± 0.05 ^a
qNP	2.75 ± 2.30 ^a	1.79 ± 0.16 ^b
Chlorophyll (μmol m ⁻²)	401.6 ± 41.0 ^a	300.9 ± 30.5 ^b

Health status: 0-asymptomatic, 1-trees with upper branch dieback and epicormic shoots. Different letters denote statistically significant differences at the 5% level

gs stomatal conductance, A photosynthetic rate, WUE intrinsic water-use efficiency, E transpiration rate, F_v/F_M maximum efficiency of photosystem II, φ_{PSII} quantum yield of photosystem II, qP photochemical quenching, qNP non-photochemical quenching

Epicormic shoots, common in oaks, are often stimulated by sudden stems exposure to light (Kerr and Harmer 2001; Kozłowski and Pallardy 2010) which, in turn, may be a consequence of water stress-induced defoliation (Larcher 1995). Dieback of the most upper twigs usually indicates tree water stress. 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 the greater hydraulic resistance over long distances (Tyree and Sperry 1989). As the decline in water supply intensifies, embolism preferentially occurs in branches where xylem tensions are greatest (Tyree and Sperry 1989; Rust and Rolof 2002). Trees reduce in height after branch shedding (Ribeiro 2006) but reduce water use (Tyree and Sperry 1989). Branch sacrifice provides a means for reducing water stress during drought events (Rood et al. 2000).

Cork thickness is also an indicator of tree water stress, particularly chronic stress (Ben Jamâa et al. 2005; Caritat et al. 2000; Costa et al. 2003; Ribeiro et al. 2003b; Surový et al. 2009) and ecological processes that interfere with tree water availability, such as intraspecific competition (Ribeiro 2006; Sánchez-González et al. 2007). The lower annual cork increments in declining trees reinforce the idea that those trees are under chronic decline for several years. In this study, asymptomatic trees present higher annual cork increment than declining trees during the 9 years period of cork growth (Fig. 3).

Tree water status and chronic decline

In general, leaf Ψ_{pd} in cork oak can reach values lower than -2 MPa during summer drought (e.g., Chaves et al. 2002; Otieno et al. 2007; David et al. 2007; Vaz et al. 2010; Pinto et al. 2012; Kurz-Besson et al. 2014), reflecting soil water potential at dawn (David et al. 2007). However, in this study asymptomatic trees were well hydrated as indicated by high Ψ_{pd} by the end of the dry season (Table 1; Fig. 4): summer Ψ_{pd} declined little along the summer and was higher than -1 MPa. This result suggests that groundwater resources

were still available for trees root uptake. In fact, although summer precipitation was low during the study, the accumulated precipitation in previous seasons was higher than long-term mean (Fig. 1).

In spring, all the studied trees had similar water status but at the end of the drought period differences were visible: first, declining trees did not recover their water status at predawn—maybe they have limited access to groundwater or they are facing impairment in water absorption due to root destruction by *P. cinnamomi* or soil disking—then declining trees showed higher midday water status. High Ψ_{md} indicates strong stomatal control and reduced transpiration, which was confirmed by leaf level gas-exchange measurements (Table 2). In other studies (David et al. 2004; Otieno et al. 2006; Pinto et al. 2012; Kurz-Besson et al. 2014), several healthy cork oaks under summer drought (with Ψ_{pd} lower than -2 MPa) presented high sap flow driving force due to low Ψ_{md} . Those trees, as well as the healthy trees from this study, dropped their Ψ_{md} to values < -3.0 MPa, operating below water potential values that may cause 50% loss in hydraulic conductivity (-2.9 MPa; Pinto et al. 2012). Interestingly, the small but statistically significant variation in Ψ_{md} of healthy cork oaks indicates a less strict isohydric behavior across the continuum of stomatal regulation of water status, compared with that of declining trees. Moreover, midday water status was related to chronic water stress and, contrary to our expectations, was higher in symptomatic trees (Fig. 4). On the contrary, predawn water status is associated with soil water potential and does not indicate the health status of trees. This result is novel and ecophysiologicaly relevant since it shows what functional parameters should be analyzed to detect tree decline. It also indicates that chronic decline is associated with a reduced sap flow driving force resulting from strong control of transpiration. Reduction in sap flow driving force seems to increase as tree decline aggravates (although not statistically significant: health status 1–2, Fig. 5). Therefore, our results showed that chronic decline is associated with a stronger avoidance of water loss by means of stomata closure, with implications in water and nutrients root uptake, photosynthesis and transport of photosynthates within the plant.

Photosynthetic parameters and chronic decline

In our study, cork oaks showing chronic stress symptoms were able to maintain high sap flow driving force during the growing season (spring, Table 1), a pattern also observed in holm oak (*Q. rotundifolia*, *Q. ilex*) affected trees (Sala and Tenhunen 1994). However, plants with favorable water status in the rainy season may already have some physiological functions, such as photosynthesis, affected by chronic stress. This was observed in holm oak seedlings, where well hydrated plants that had been subjected to repeated drought cycles presented 80% of photosynthesis of control plants, low stomatal and mesophyll conductances to CO_2 (Galle et al. 2011). Similar observations were referred in Blackman et al. (2009): after intense water stress, plants quickly recovered leaf water potential, but stomatal conductance remained limited. Niinemets (2015) refers that in the case of stomatal conductance and photosynthesis, the way recovery occurs often depends on past stress level, where a more severe stress is associated with slow recovery and undercompensation. Other responses, like chronic photoinhibition or down-regulation of photosynthesis, may also occur following drought events associated with high temperatures and excess of light (Osmond 1994; Ripullone et al. 2009). These responses were also observed in our study. Summer reduction in the photosynthetic apparatus was stronger in declining trees, presenting less chlorophyll content and a decrease in photochemical efficiency. In asymptomatic and declining trees, F_v/F_m values were 0.63 and 0.43,

respectively, which were lower than values for healthy plants—between 0.75 and 0.85. Accordingly, in studies concerning healthy trees suffering seasonal drought events a decrease in the pool and efficiency of the photosystem II open centers, driven by low chlorophyll content, was also observed (Faria et al. 1998; Chaves et al. 2002; Grant et al. 2010; Vaz et al. 2011). However, plant water use efficiency is expected to increase with water stress, as well as plant ability to dissipate excitation energy by mechanisms other than photosynthetic C-metabolism (non-photochemical quenching). In contrast to previous reports, we observed that trees under chronic decline showed less water use efficiency and less non-photochemical quenching. These observations indicate that declining trees have less physiological protection against drought stress, particularly against potential photo-oxidative damage (Müller et al. 2001). Moreover, the observed reduction in water use efficiency in trees under chronic decline also suggests a role of non-stomatal limitation of photosynthesis under increasing drought conditions (Shardendu et al. 2011).

Starch and chronic decline

The low leaf starch concentration found in all the trees may be a consequence of an eventual starch conversion into soluble sugars for leaf osmoregulation (Morgan 1984; Chaves 1991; Quick et al. 1992; Pinheiro et al. 2001) and/or starch depletion after an increase in respiration due to summer high temperatures (Adams et al. 2009). Starch stored in roots was also low and had no relation to tree health status. It is considered that starch reserves in oaks are mainly stored in roots, however, stems or lignotubers may also account for preferential starch pools (Molina and Verdaguer 1993; Dickson and Tomlinson 1996). Measurements of all nonstructural carbon reserves in stems and lignotuber, as well as in roots and leaves, will provide more insights on tree carbon availability and its relation to dieback. Our results were inconclusive in relation to the role of starch in decline. More analyses are needed to assess if chronic decline is related to carbon depletion or to limited carbon translocation associated with low sap driving force.

Chronic decline versus sudden death

In chronic decline we observed that dying trees close stomata, likely avoiding hydraulic failure. Sap flow driving force tends to be gradually reduced and, consequently, CO₂ uptake is limited. The physiological events linked to chronic decline were similar to the physiological events involved in the carbon starvation hypothesis (McDowell et al. 2008). On the contrary, “sudden death” appeared more likely to occur after runaway embolism, which is the main physiological process underlying the hydraulic failure hypothesis. This is expected to occur rapidly leading to fast mortality in plants that keep their stomata open during drought (usually observed in relatively anisohydric plants; McDowell et al. 2008, Sevanto et al. 2014). This symptomatology frequently occurs in apparent healthy cork and holm oaks trees (Brasier et al. 1993), though in our study it occurred in a tree already under chronic decline. Unfortunately, we have no replicates since it is a matter of chance to measure their water relations before death. However, such low water potential at predawn (−3.62 MPa, Fig. 4) was not observed in the other trees that died during the study. Cork oak leaf Ψ values that induces 50% loss hydraulic conductivity is −3 MPa, i.e., cavitation threshold (Tyree and Cochard 1996; Cruziat et al. 2002). Healthy trees may also exhibit a drop of water potential below −3 MPa at midday, but their xylem is probably less vulnerable to cavitation and/or have higher capacity for embolism repairing.

Cork oaks are isohydric trees, controlling tissue dehydration via stomatal closure and deep rooting to avoid drought-induced hydraulic failure (David et al. 2007, Vaz et al. 2010). Therefore, they are most likely suffering from chronic decline than sudden death. Further studies on the drivers of sudden death in cork oaks would help to understand the patterns of cork oak decline and to predict future dieback events.

Conclusions

This study shows that declining and healthy trees respond physiologically different to water stress. Trees under chronic decline have lower Ψ_{pd} and higher Ψ_{md} , resulting in lower sap flow driving force. They also have stronger down-regulation of photosynthesis when compared with that of asymptomatic trees subjected to seasonal water stress. Consequently, declining trees have lower assimilation capacity than healthy trees making them more susceptible to carbon starvation (McDowell et al. 2008). However, no relation was observed between leaf or root starch concentration and the health status of trees. Declining trees also showed lower water use efficiency and less protection against photo-oxidative damage, indicating a loss of resistance to drought. Finally, chronic decline was associated with a reduction in water flow rather than to dehydration, since water potential did not drop below critical values. On the contrary, sudden death appears to be a consequence of tree dehydration, evidenced by a drop of water potential below cavitation threshold, though more replicates are needed to confirm the assumption.

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Appendix

Mixed model analysis: for tree water status and health status

Model dimension

		Number of levels	Covariance structure	Number of parameters
Fixed effects	Intercept	1		1
	Health_status	3		2
	Season	2		1
Random effects	Tree (plot)	42	Variance components	1
Residual				1
Total		48		6

Results for the dependent variable “Predawn leaf water potential”

Type III tests of fixed effects

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	84	357.240	>0.001
Health_status	2	84	20.179	>0.001
Season	1	84	97.919	>0.001

Results for the dependent variable “Midday leaf water potential”

Type III tests of fixed effects

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	42	2947.598	>0.001
Health_status	2	42	6.067	0.005
Season	1	42	10.320	0.003

Results for the dependent variable “Sap flow driving force”

Type III tests of fixed effects

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	80	469.615	>0.001
Health_status	2	80	24.145	>0.001
Season	1	80	19.225	>0.001

Mixed model analysis: for cork thickness and health status**Dependent variable: cork thickness**

Model dimension

		Number of levels	Covariance structure	Number of parameters
Fixed effects	Intercept	1		1
	Health_status	3		2
Random effects	Tree (plot)	42	Variance Components	1
Residual			1	
Total		46		5

Type III tests of fixed effects

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	39	789.962	>0.001
Health_status	2	39	24.580	>0.001

Estimates of fixed effects

Parameter	Estimate	SE	df	t	Sig.	95% Confidence interval	
						Lower bound	Upper bound
Intercept	1.867	0.206	39	9.062	>0.001	1.450	2.284
[Health_status = 0]	1.407	0.234	39	6.022	>0.001	0.934	1.879
[Health_status = 1]	0.510	0.244	39	2.094	0.043	0.0174	1.003
[Health_status = 2]	0	0

^b This parameter is set to zero because it is redundant

Mixed model analysis for annual cork increment and health status

Dependent variable: ring_width

Model dimension

		Number of levels	Covariance structure	Number of parameters	Subject variables	Number of subjects
Fixed effects	Intercept	1		1		
	Health_status	3		2		
	Period_cork_growth	2		1		
Random effects	Intercept	1	Identity	1	Plot	
Repeated effects	Ring_N	8	First-order ante-dependence	15	Tree * plot	30
Total		15		20		

Type III tests of fixed effects

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	6.696	237.606	>0.001
Health_status	2	34.810	22.306	>0.001
Year_decork	1	9.035	3.232	0.106

Estimates of fixed effects

Parameter	Estimate	se	df	t	Sig.	95% Confidence interval	
						Lower bound	Upper bound
Intercept	1.345	0.253	23.052	5.316	>0.001	0.821	1.868
[Health_status = 0]	1.677	0.281	35.631	5.964	>0.001	1.107	2.248
[Health_status = 1]	0.765	0.299	35.503	2.555	0.015	.158	1.373
[Health_status = 2]	0	0
[Year_decork = 2008]	-0.389	0.216	9.035	-1.798	0.106	-0.878	0.100
[Year_decork = 2009]	0 ^a	0

^a This parameter is set to zero because it is redundant

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