Summer and winter sensitivity of leaves and xylem to minimum freezing temperatures: a comparison of co-occurring Mediterranean oaks that differ in leaf lifespan

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Summary

- Freezing sensitivity of leaves and xylem was examined in four co-occurring Mediterranean oaks (*Quercus* spp.) grown in a common garden to determine whether freezing responses of leaves and xylem were coordinated and could be predicted by leaf lifespan.
- Freezing-induced embolism and loss of photosynthetic function were measured after overnight exposure to a range of subzero temperatures in both summer and winter.
- Both measures were found to be dependent on minimum freezing temperature and were correlated with leaf lifespan and vessel diameter. The dependence of xylem embolism on minimum freezing temperature may result from the decline in water potential with ice temperature that influences the redistribution of water during freezing and leads to an increase in xylem tension. Winter acclimatization had a relatively small effect on the vulnerability to freezing-induced embolism, although leaf photosynthetic function showed a strong acclimatization response, particularly in the two evergreen species.
- *Quercus ilex*, the species with the longest leaf lifespan and narrowest vessel diameters, showed the highest freezing tolerance. This helps explain its ability to inhabit a broad range throughout the Mediterranean region. By contrast, the inability of the deciduous oaks to maintain photosynthetic and vascular function throughout the winter indicates a competitive disadvantage that may prevent them from expanding their ranges.

Key words: cold acclimatization, decline in F_V/F_M, freezing-induced embolism, leaf lifespan, minimum temperatures, xylem anatomy.

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Introduction

Freezing temperatures can cause lethal injuries in living plant tissues (Sakai & Larcher, 1987; Wisniewski *et al.*, 1991; Guy, 2003; Kuroda *et al.*, 2003) and are a major factor limiting the long distance transport of water in the xylem (Améglio *et al.*, 2002; Cochard & Tyree, 1990; Améglio & Cruiziat, 1992; Sperry *et al.*, 1994; Sperry, 1995; Améglio *et al.*, 2001a; Feild & Brodribb, 2001; Cavender-Bares, 2005). The ability of different species to avoid or tolerate freezing stress through various mechanisms can go a long way in explaining their geographic distributions (Burke *et al.*, 1976; Parker, 1963; Sakai, 1970; George *et al.*, 1974; Sakai *et al.*, 1981). Both drought and freezing are hypothesized to be important stress factors affecting the ecology and evolution of plants in the Mediterranean region (Damesin & Rambal, 1995; Mitrakos, 1980; Lo Gullo & Salleo, 1993; Damesin *et al.*, 1998; Garcia-Plazaola *et al.*, 1999), although the adaptation of plants to drought has traditionally been a larger research focus (Mooney & Dunn, 1970). Freezing stress, however, is more

Table 1 Leaf lifespans (days) and Northern range limits (degrees N, latitude) of four Mediterranean oak (*Quercus*) species

	Leaf lifespan	N range limit
Quercus afares	235	37.0
Quercus faginea	250	43.5
Quercus ilex	735	47.0
Quercus suber	464	45.5

N range limits are from Quézel & Médail (2003). Leaf lifespans for *Q. afares* and *Q. faginea* are estimated from leaf emergence and leaf fall dates in the common garden. Leaf lifespans for *Q. ilex* and *Q. suber* are from Mediavilla and Escudero (2003).

likely to limit the ranges of Mediterranean plants, including the numerous oak species of this region (Larcher, 1981; Tognetti *et al.*, 1998; Nardini *et al.*, 2000; Quézel & Médail, 2003). In this study, we compare the freezing sensitivity of four Mediterranean oak species growing in a garden in southern France, in terms of xylem embolism and photosynthetic function of leaves. These species vary in leaf lifespan (Table 1) and latitudinal distribution (Fig. 1).

Winter nights in the Mediterranean region of southern Europe and North Africa frequently reach freezing temperatures during winter months (December, January and February) but only rarely extend below −10°C. Both daily water transport through the xylem and leaf photochemistry are known to be impaired by night-time freezing temperatures as a result of freeze-thaw embolism and impairment of enzymatic processes involved in photosynthesis.

Xylem embolism formation in response to freezing stress has been shown to be dependent on vessel diameter (Davis *et al.*, 1999; LoGullo & Salleo, 1993; Sperry, 1995; Pittermann & Sperry, 2003). The degree of xylem embolism is also sensitive to the minimum temperature experienced during freezing (Pockman & Sperry, 1997), although the effect of decreasing temperature may only be pronounced in some species (Pitterman & Sperry, 2003). Currently, it is unknown whether these contrasting patterns correspond to leaf phenology, although it has long been observed that hydraulic architecture and leaf phenology are coordinated, and it has been hypothesized that they evolve in concert (Lechowicz, 1984). In a study of hydraulic properties and freezing-induced xylem embolism in 17 oak species in north central Florida, USA, Cavender-Bares & Holbrook (2001) found that evergreen species tended to have smaller maximum vessel diameters and diffuse porous anatomy relative to co-occurring deciduous species with large diameter early wood vessels and ring porous anatomy. Loss of xylem conductivity after freezing was correlated with leaf phenology indicating that hydraulic architecture and phenology may be coevolved traits in oaks. Leaves of deciduous species have been shown to possess lower tolerance to chilling and freezing stress compared with evergreen species (Sakai *et al.*, 1981; Adams *et al.*, 1995; Cavender-Bares *et al.*, 1999). Acclimatization to cold temperatures in overwintering evergreen species, including in oaks, has been linked to increases in antioxidants and xanthophyll pigments (Adams *et al.*, 1995; Garcia-Plazaola *et al.*, 1997; Garcia-Plazaola *et al.*, 1999), as well as changes in the composition of photosystem II antenna and increases in cyclic electron transport that allow increased quenching of absorbed light (Öquist & Huner, 2003). Deciduous leaves are programmed to senesce and abscise in response to cold temperatures, potentially obviating the need for mechanisms to protect the photosynthetic apparatus during freezing. Evergreen leaves, on the other hand, even of species occurring in relatively warm climates such as northern Florida or Mediterranean Europe, would be expected to benefit in terms of carbon gain by maintaining function under mild freezing stress. Maintenance of leaf function should be greatest in the species with the longest leaf lifespan. However, maintaining functional leaves without functional xylem should be of little benefit. It is likely therefore that losses of function in the leaves and xylem are coordinated.

We hypothesized that the degree of sensitivity of the xylem to freezing temperatures should be related to leaf habit (evergreen and deciduous) or leaf lifespan and coupled to the sensitivity of the photosynthetic apparatus to freezing. We took advantage of a previously established common garden in Montpellier, France (Acherar & Rambal, 1992) where young trees of four Mediterranean oak species, two evergreen (*Quercus ilex* and *Quercus suber*) and two deciduous (*Quercus afares* and *Quercus faginea*), had been growing under the same conditions for 17 yr. Current ranges of each species are shown in Fig. 1 (based on Quézel & Médail, 2003). We examined the responses of leaves and stems of whole shoots to experimental overnight freezing and asked the following questions: (1) Is leaf longevity correlated with hydraulic traits and freezing sensitivity of leaves and xylem among closely related species? (2) Does loss of function in both xylem and photosynthesis of leaves increase with declining minimum temperatures during freezing? (3) Is there evidence for a cold acclimatization response in the xylem as well as in the photosynthetic apparatus, and does it differ between evergreen and deciduous species?

Materials and Methods

Common garden

We made use of a common garden established in 1986 on the grounds of the Centre d'Ecologie Fonctionelle et Evolutive, CNRS, in Montpellier, France (Acherar *et al.*, 1991; Acherar & Rambal, 1992). Seedlings were grown from seeds collected from native populations of *Q. ilex* L., *Q. suber* L., *Q. afares* Pomel, and *Q. faginea* Lam. in eastern Algeria in the massif of Akfadou (36.48° N, 4.30° E) and transported to Montpellier in 1986. The soil in the garden is a loamy clay soil. The surface layer (0–50 cm) is 35% clay increasing with depth to 55% at 1.5–2 m. Freezing temperatures occur in the garden every year,

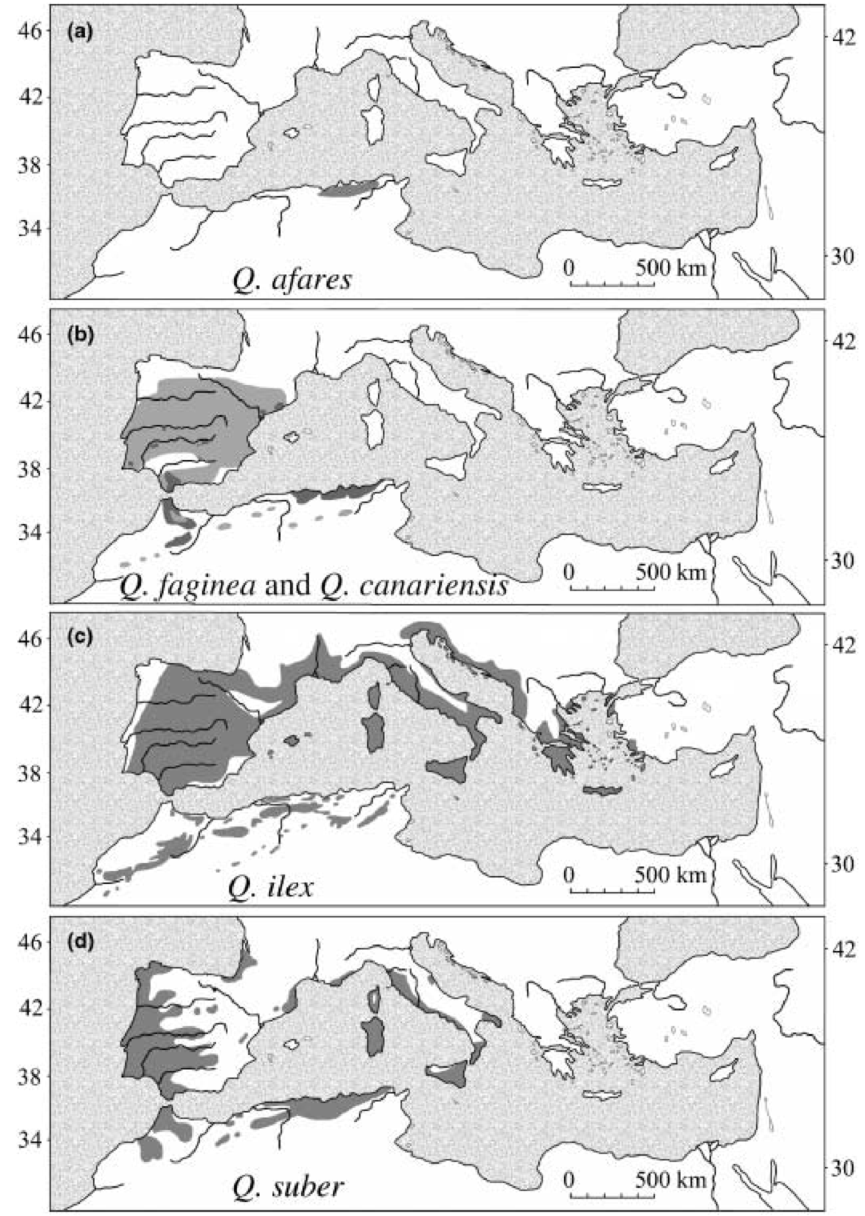

Fig. 1 Distribution maps of each of the four *Quercus* species examined in the common garden study. Maps are redrawn from Quézel & Médail (2003). In (b) distributions for both *Q. faginea* (light gray) and *Q. canariensis* (dark gray) are shown, as these are sometimes considered a single species.

and December, January and February are the coldest months. Based on meteorological records from 1971 to 2005, the annual minimum temperature typically experienced in the garden is −5°C, although minimum temperatures below −10°C are reached approximately every 11 yr. Freezing typically occurs up to 20 times per year, but most freezes only reach −1 or −2°C. Freezing events that include four consecutive days with minimum temperatures below −5°C occur every 5 yr.

We sampled shoots from six trees of each species in midsummer (July and early August) and in winter (December) of 2003 to determine differences among the species in their freeze–thaw-induced embolism and the viability of leaves after freezing at increasingly negative minimum temperatures. Sampling times are shown relative to minimum and maximum temperatures between July and December (Fig. 2). Note that during the December measurements, we measured the leaves and stems of the deciduous species just as they were starting to senesce. We were able to work largely with green leaves, but in *Q. afares*, some senescence had already occurred. As additional freezing events occurred subsequent to our measurement period, both deciduous species began to lose their leaves. By January, both species were almost bare.

Collection of samples

Whole shoots (approx. 50 cm long) were cut from the trees under water, from a sample of six trees per species growing in the common garden, and placed in plastic rose tubes (20 ml)

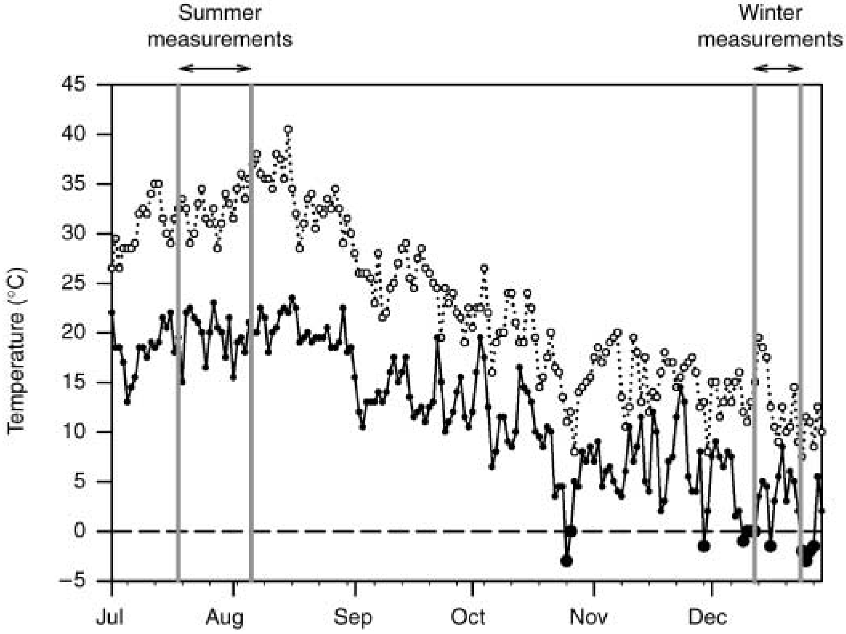

Fig. 2 Minimum and maximum daily temperatures in the common garden at CNRS in Montpellier, France, between July and December, 2003, measured at 1.5 m. Summer and winter sampling periods are indicated with vertical bars. Open circles, maximum temperatures; closed circles, minimum temperatures.

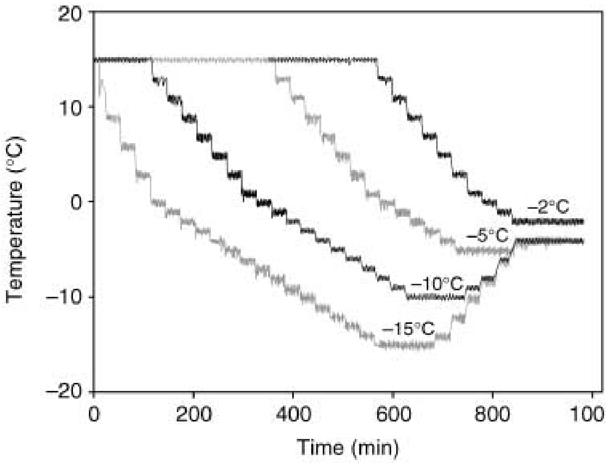

Fig. 3 Time course of temperature inside the freezer box during overnight freezing experiments for four minimum temperatures (–2, –5, –10 and –15°C). Curves are alternately shown in black or gray for clarity.

under water. A plastic cap formed a seal around the stem. Each day, we sampled individuals of all four species to prevent any possible bias from changing outdoor conditions. Shoots immersed in the water-filled tubes were then installed vertically into a freezer box at 15°C in the late afternoon each day, with leaves attached, allowing hydration to occur before controlled freezing.

Freezing and thawing procedure

A freezer box attached to a 23X Campbell data logger (Campbell Scientific Inc., Logan, UT, USA) was programmed to decline in 1°C intervals every 30 min to a designated minimum temperature (Fig. 3). Minimum temperatures were −2, −5, −10 and −15°C in summer (July/August) and −5 and −10°C in winter (December). The freezer was maintained at the minimum temperature for 2 h. If the minimum temperature was −5°C or below, the temperature was again increased at the same rate and held at −4°C until 08:00 hours. If the minimum temperature was −2°C, the freezer box was held at −2°C until 08:00 hours. The temperature at each interval in the freezing process was maintained with ±0.5°C, the minimum amplitude that was possible for the compressor in the freezer box. At 08:00 hours, all stems were removed and placed under illumination, emitted by a metal halide lamp with a circulating water bath on a glass tray below the lamp to remove excess heat. The leaves received cool light (*c.* 300 μmol photons m^{-2} s^{-1}) for 90 min before measurements of stem conductance. This allowed thawing to occur while leaves were transpiring so that slight tension was present in the xylem, simulating realistic outdoor thawing conditions. Water potential of an attached leaf was measured with a Scholander pressure bomb (PMS Instruments, Corvallis, OR, USA) before cutting the adjacent stem segment under water for hydraulic conductance measurement. Water potential values for leaves of all species were between −0.3 and −0.7 MPa in both winter and summer.

Exotherm measurements

Copper-constantin thermocouples (Omega, Stamford, CT, USA) were attached to the outside of the stems and on the upper surface of a leaf with a small strip of microporous tape, and temperatures were recorded at 1-min intervals throughout the chilling period using a 23X Campbell data logger. The appearance of exotherms allowed us to verify whether freezing occurred as the temperature dropped. Exotherms can often be detected as 1–3°C spikes in the temperature curve lasting for several minutes as heat is given off during the phase change of water from the liquid to the solid state. An additional thermocouple was placed in the center of the freezer box to monitor the air temperature inside and to serve as the reference temperature for the program. These data showed that the stem and leaf temperatures tracked the freezer box reference temperature very well and that the plants experienced homogeneous conditions. Exotherm data for all stems was not possible to obtain because of some loss of logged temperature data. In addition, we had difficulties observing exotherms in some cases. This may have been a result of poor thermocouple contact with the stem or because exotherms occurred gradually because of the slow cooling rate and were obscured by the temperature oscillations of the freezer. Alternatively, freezing may not have occurred due to supercooling. While we cannot rule out the last possibility, it is unlikely because the exotherms did not become easier to detect during the –15°C minimum temperature treatment. Leaves did not show noticeable exotherms, presumably because they do not have enough thermal mass. Lack of clear exotherms in the stems was perhaps exacerbated by the fact that bark was not removed in order to avoid injury and to maintain more natural freezing conditions. To avoid ambiguity in the exotherm data, we counted exotherms only if they showed a rise in temperature greater than 1°C. Hence, these values are likely to underestimate actual freezing temperatures in some cases. At high freezing temperatures (–2°C) imposed only during summer, xylem freezing does not appear to have occurred in all species. Summer exotherm temperatures for all species ranged from –2.0°C to –4.39°C (mean ± SE, 3.15 ± 0.15°C). In winter, exotherm temperatures ranged from –2.3°C to –6.2°C (mean ± SE, –4.1 ± 0.03°C) for all species.

Hydraulic conductance measurements

After experimental freezing was imposed, embolism was measured as the percent loss of hydraulic conductivity. Hydraulic conductivity was determined by measuring the flow rate of water under a known pressure gradient (Sperry *et al.*, 1988). Stem segments (*c.* 5 cm long) were cut under water and the ends recut with a fresh razor blade. Segments were immediately installed in a tubing apparatus for conductivity measurements. Conductivity was determined gravimetrically by measuring the flow rate of distilled and ultra-filtered (particle size < 0.2 μm) water, adjusted to pH 2.0 with sulfuric acid, through a stem segment under a known pressure gradient. All measured branch segments had diameters < 0.4 cm. Flow rate was determined by an electronic balance (±0.1 mg) connected to a computer. A thin film of mineral oil was used to minimize evaporation from the water reservoir located on the balance. Pressure differences across the stem segment were determined by measuring the vertical distance between the water levels in the supply reservoir and the balance. The hydraulic pressure head was maintained at approx. 2 kPa so as not to flush naturally formed embolisms from the stem segments. Before each measurement was taken we waited until the zero-pressure (background) flow was zero. Tubing was changed frequently to prevent fungal or algal growth.

Measurements were taken after steady state flow was reached (approx. 5–10 min). Following initial conductivity measurements, branches were flushed with water for 2 min at approx. 100 kPa using a mechanically operated syringe pump so that bubbles emerged immediately from the attached stem. We determined that this flushing protocol was sufficient to remove all emboli because higher pressures or longer flushing times did not increase the observed flow rate through the stem. The conductivity was then re-measured, and percent loss of conductivity (PLC) was calculated. In addition, conductivity was measured for approximately six to eight stem segments per species from shoots that did not undergo experimental freezing. These stems were subsequently flushed and re-measured to calculate PLC, providing a measure of native embolism. Sapwood area, calculated as cross-sectional area (without bark) minus the pith area, was used in the denominator for specific conductivity measurements. Leaf area was used as the denominator for calculations of leaf specific conductivity. All four species were measured on any given day.

Xylem vessel measurements

After conductivity measurements were completed, stems were removed from the conductivity apparatus and fixed in 70% alcohol. Transverse sections of 20 micrometers of the base of each stem were obtained with a sliding microtome and stained with safranin. The stem cross-sections were viewed under a microscope (BX60; Olympus, Hamburg, Germany) equipped with a digital camera (DP12; Olympus) and photographed. The images were recovered in Adobe Photoshop 6.0, and modifications were made that allowed us to differentiate the lumen of the xylem vessels (the vessel lumina were made white and the rest of the xylem appeared black). The images were imported into the program IDRISI32 (Clark Labs, Worcester, MA, USA) which calculates the pixel area of each vessel lumen. Diameters were calculated from lumen area assuming that they were perfect circles. The total cross-section of the stem was also calculated. Mean vessel diameter for each stem segment and grand means for each species were calculated. A hydraulically weighted mean diameter was also calculated as $2(\Sigma r^5/\Sigma r^4)$, where r is the radius of a conduit (Sperry *et al.*, 1994).

Foliar freezing damage

The decline in variable chlorophyll fluorescence has been shown to be a useful method for detecting chilling and freezing injury to leaf tissue and for assessing photosynthetic function, including the ability of photosystem II to shuttle electrons (Boorse *et al.*, 1998; Cavender-Bares *et al.*, 1999; Feild & Brodribb, 2001). The viability of leaves following exposure to overnight subfreezing temperatures was determined with chlorophyll fluorescence measurements using a pulse-amplitude modulated fluorometer (MINIPAM; Walz, Effieltrich, Germany). Dark adapted measurements of F_V/F_M (4000 µmol saturating pulse for 800 ms) were taken on two leaves of each shoot while still on the tree in the morning, using dark adaptation clips (Hansatech Instruments, Norfolk, UK) attached the evening before to ensure maximum reoxidation of photosystem II (PSII) reaction centers (Schreiber *et al.*, 1994). Spots where the dark adaptation clips were attached on the leaves were marked and the clips removed. Paired shoots, one for stem hydraulic conductance and one for chlorophyll fluorescence, were subsequently cut under water later in the day for experimental freezing, as described earlier. After exposure to illumination the following morning (as described earlier) shoots still in water-filled rose tubes were put in a dark cabinet for 6 h at 25°C. Dark adaptation clips were placed in the same location on the leaves as before. Post-freezing values of F_V/F_M were subsequently measured to determine the decline in F_V/F_M after freezing (Feild & Brodribb, 2001). Per cent decline in F_V/F_M was calculated relative to the maximal value that was generally close to 0.8. The same freezing protocol was used as described above (Fig. 3). Freezing was conducted at the same minimum temperatures as before, although several additional freezing experiments were conducted (at −7.5°C in July and −15°C in December) to increase the number of minimum temperatures tested for the quantum yield measurements.

Biochemical analyses of stems and leaves

Leaves and stems of each species were collected from trees of each species in late July (n = 5) and late December (n = 3) for biochemical analysis. All fractions were then dried at 60°C for 24 h and ground (Cyclotec 1093 Sample Mill; Tecator, Höganäs, Sweden). Concentrations of nitrogen, soluble sugars, starch and lipids were determined using near-infrared reflectance spectroscopy (see Joffre *et al.* (1992) and Damesin *et al.* (1997) for a description of the procedure). All samples were scanned with a NIRSystem 6500 spectrophotometer (NIRSystems Inc., Silver Springs, MD, USA). The spectral and wet chemical database used to build calibration equations comprises leaves and stems of 25 species, representing a wide diversity of Mediterranean woody species from Mediterranean areas of Portugal, Spain, France and Italy and includes part of the database of Meuret *et al.* (1993). Nitrogen content was determined with an elemental analyser (PE 2400 CHN; Perkin–Elmer, Norwalk, CT, USA) (van Soest & Robertson, 1985). Total lipids were obtained by weighing the residue extracted by a chloroform–methanol mixture (Allen, 1989). Starch and soluble sugar analysis was carried out following the method of Farrar (1993). The final calibration equations allowed the per cent concentration of biochemical constituents to be determined from the spectra, using partial least squares regression, with a standard error of prediction of 0.13% for nitrogen, 1.52% for lipids, 0.55% for soluble sugars, and 1.10% for starch.

Results

Loss of hydraulic conductivity in relation to minimum temperature

July measurements Native embolism in July did not differ significantly among the species and was between 20% and 35% (Fig. 4). All four species showed increasing loss of conductivity with decreasing minimum temperatures during July. *Q. ilex* showed the lowest sensitivity to freezing. At −2°C, evergreen species had less significant losses of conductivity than the deciduous species but at −5°C, *Q. suber* was almost completely embolized, as were the two deciduous species. Only *Q. ilex* maintained substantial xylem function (60% PLC). At −2 and −5°C, PLC showed a linear increase with species mean values of vessel diameter and hydraulically weighted vessel diameter (Fig. 5). However, at lower freezing temperatures (−10°C and −15°C), this relationship no longer held because stems of all species were nearly completely embolized.

December measurements Native embolism in December was highest for the two deciduous species, *Q. afares* and *Q. faginea* (both *c.* 70%). The evergreen species had lower native embolism but *Q. suber* (*c.* 50%) and *Q. ilex* (only 20%) were significantly different. The native PLC had the same rank order as mean hydraulically weighted vessel diameters (not shown). Subsequent freezing at −5°C only significantly increased embolism above the native value in *Q. ilex*. There was a trend that losses of conductivity due to freezing at −5°C were not as high in December as they had been in July. In December, at −10°C, all species were nearly completely embolized.

Photosynthetic function of leaves

July vs December measurements In July, dark-adapted F_V/F_M values of leaves were above or close to 0.8 for all species, indicating that leaves were functioning normally (Schreiber *et al.*, 1994). All species showed large and increasing losses of photosynthetic function after overnight freezing as minimum temperatures decreased. This is shown by the sharp increases in the per cent decline in dark-adapted F_V/F_M (Fig. 6). In

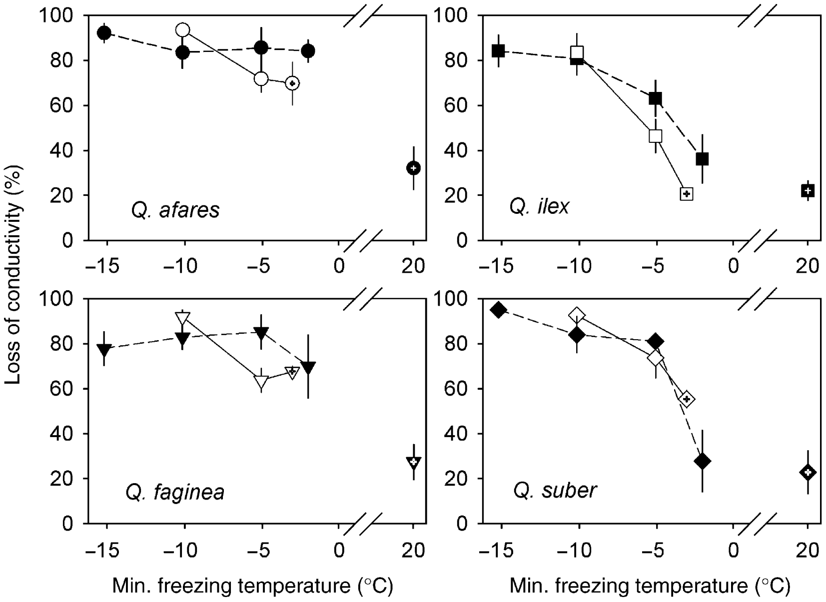

Fig. 4 The per cent loss of conductivity (PLC) ± SE in relation to minimum temperature for the four oak (*Quercus*) species. Closed symbols, July measurements; open symbols, December measurements. Symbols with crosses in the center indicate native PLC in July (shown at 20°C) and in December after a −3°C freeze had occurred.

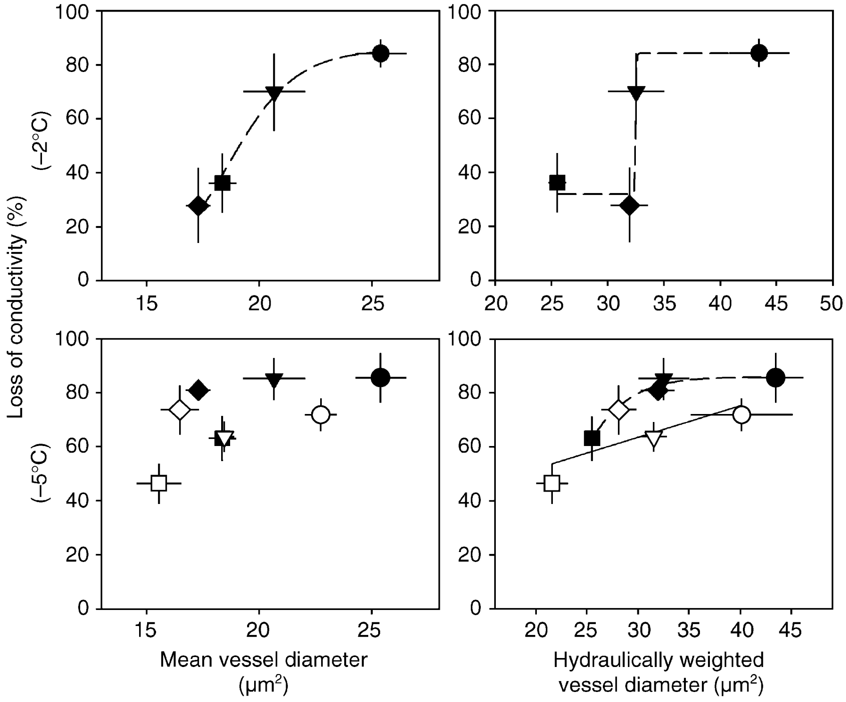

Fig. 5 Per cent loss of conductivity for July and December shown in relation to mean vessel diameter (μm) and hydraulically weighted vessel diameters (μm) for −2°C (upper panels), and −5°C (lower panels). Lower temperatures showed no trend with vessel diameter as stems of all *Quercus* species were nearly fully embolized. Closed symbols, July measurements; open symbols, December measurements.

December, despite the onset of cold temperatures and the commencement of senescence in *Q. faginea* and *Q. afares*, only *Q. afares* showed a lower dark-adapted F_V/F_M (*c.* 0.7) compared with summer (Fig. 6 inset). This may be a consequence of the fact that we avoided leaves showing advanced senescence. All species showed less severe losses of photosynthetic function after experimental freezing in December than in July. However, in *Q. afares*, this difference only occurred at −5°C and can be explained by the lower initial F_V/F_M before freezing. In this species, the raw F_V/F_M values after freezing treatments are not significantly different between summer and winter, indicating no apparent acclimatization of the photosynthetic apparatus to freezing temperatures. In the other species, however, there was a dramatic increase in the freezing tolerance of leaves in December relative to July. *Q. ilex* and *Q. faginea* showed almost no decline in F_V/F_M after exposure to −5°C. *Quercus*

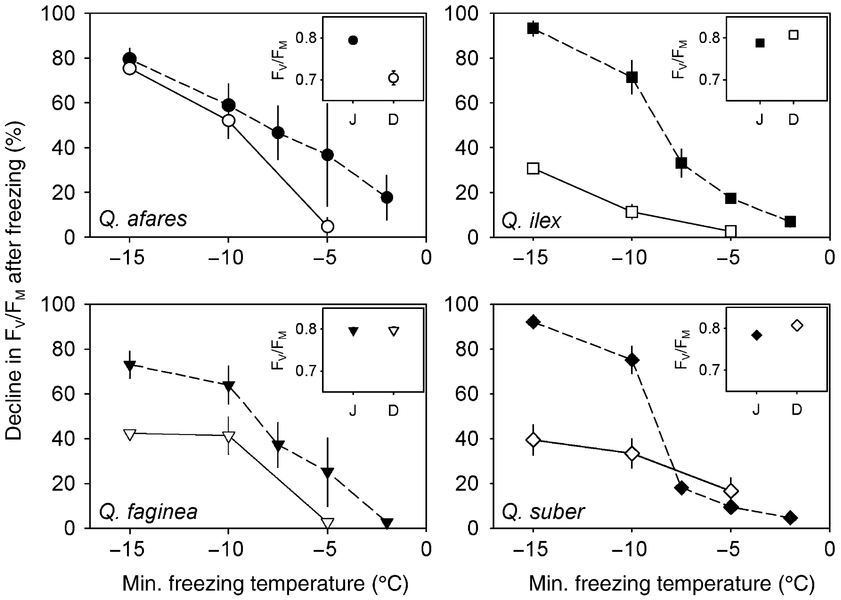

Fig. 6 The per cent decline in dark-adapted variable fluorescence, F_V/F_M, after freezing in relation to minimum temperature for the four oak (*Quercus*) species. Closed symbols, July measurements; open symbols, December measurements. Insets show the average *in situ* values of F_V/F_M before freezing for each species in July (J) and December (D).

suber showed a 20% decline in photosynthetic function in December after freezing at −5°C, which did not differ from the July value. However, as minimum freezing temperatures were dropped to −10°C and −15°C, *Q. suber* showed less than a 50% loss in F_V/F_M in December while in July these same temperatures caused almost complete loss of photosynthetic function.

Coordinated loss of leaf and xylem function There was curvilinear relationship between per cent loss of conductivity and the percent decline in F_V/F_M caused by overnight freezing, which varied by species and season (Fig. 7). In both summer and winter losses of leaf photosynthetic capacity coincided with loss of conductivity. In summer, the decline in F_V/F_M was rapid with increasing embolism. The relationship was much shallower in winter as a result of the cold acclimatization of leaves, such that high losses in conductivity occurred before comparable losses of F_V/F_M. The relationship was most shallow in the species with the longest leaf lifespan (*Q. ilex* and *Q. suber*, respectively). Leaf lifespan was correlated with the decline in F_V/F_M after freezing (shown in Fig. 8 for freezing at −10°C) as well as with freezing at −2°C temperatures (not shown) or with the native embolism in December (Fig. 8). Leaf lifespan was also correlated with leaf mass per area and average vessel diameter, indicating an overall coordination between leaf and xylem traits.

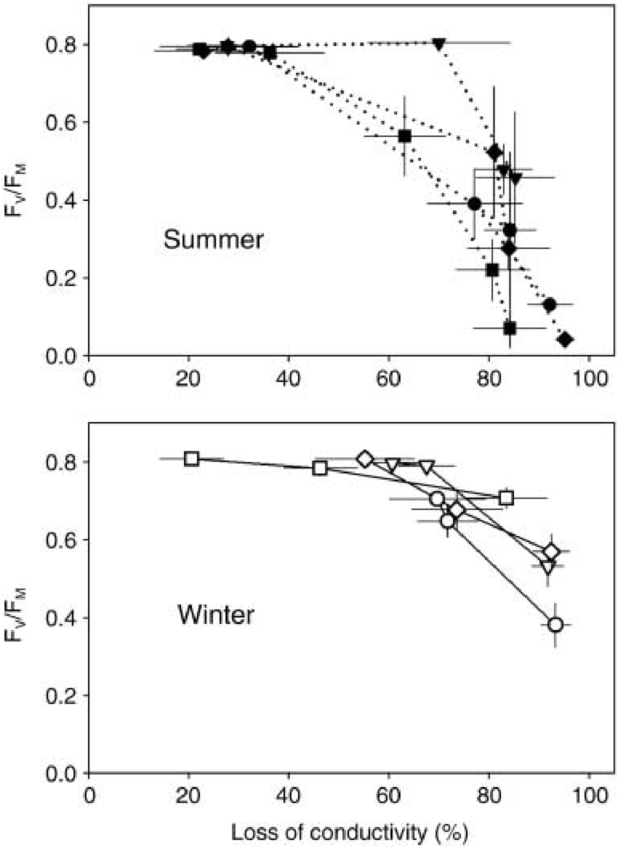

Fig. 7 Per cent loss of conductivity due to freezing in relation to F_V/F_M after freezing. Closed symbols indicate July measurements, open symbols indicate December measurements. Each *Quercus* species is indicated by a different-shaped symbol: circles, *Q. afares*; triangles, *Q. faginea*; squares, *Q. ilex*; diamonds, *Q. suber*.

Xylem vessel diameters

All species showed smaller mean vessel diameters in winter than in summer, giving rise to an overall significant effect of season on vessel diameter (Table 2). However, these differences were not significant within a species based on Scheffe's *post hoc*

Table 2 Mean vessel diameters, hydraulically weighted vessel diameters and exotherm temperatures for each of the four oak (*Quercus*) species for stems sampled in late July to early August (summer) and mid-December (winter)

	Quercus afares mean	SE	*Quercus faginea* mean	SE	*Quercus ilex* mean	SE	*Quercus suber* mean	SE
Mean vessel diameter (mm)								
Summer	25.40	1.12 a	20.66	1.38 b	18.37	0.59 b	17.29	0.53 b
Winter	22.73	0.70 a	18.47	0.41 ab	15.55	0.99 b	16.47	0.85 b
	ns		ns		*		ns	
Hydraulic vessel diameter (mm)								
Summer	43.46	2.66 a	32.52	2.48 b	25.52	0.74 b	31.93	1.61 b
Winter	40.13	4.98 a	31.53	1.27 ab	21.62	1.53 b	28.11	1.71 ab
	ns		ns		*		ns	
Exotherm temperature (°C)								
Summer	–2.95	0.09 a	–3.26	0.10 a	–3.21	0.22 a	–3.19	0.54 a
Winter	–4.27	0.24 a	–4.03	0.06 a	–4.02	0.17 a	–4.06	0.21 a
	**		*		ns		ns	

Anatomical measurements were made on a minimum of six stems per species. The comparison of species means based on Sheffe's *post hoc* tests is given to the right of the SEs with letters shown as different if $P < 0.05$. Results of *t*-tests comparing summer and winter values for each species are given below the means: ns, not significant; *$P < 0.05$; ** $P < 0.01$.

Table 3 Soluble sugars, starch, lipids and nitrogen (N) content of leaves and stems of each oak (*Quercus*) species collected in late July and mid-December

Species	Season	Soluble sugars Mean	SE	Starch Mean	SE	Lipids Mean	SE	%N Mean	SE
Leaves									
Quercus afares	Summer	13.14	0.07 ns	8.69	0.50 ns	18.07	0.34*	1.84	0.04**
	Winter	11.74	0.91	10.55	0.66	20.03	0.40	1.23	0.10
Quercus faginea	Summer	11.85	0.31 ns	6.10	0.21 ns	14.15	0.70 ns	1.67	0.05 ns
	Winter	11.00	0.27	6.11	0.69	14.57	0.31	2.07	0.08
*Quercus ilex*ns	Summer	9.75	0.28 ns	4.30	0.60 ns	10.40	0.67***	1.54	0.12ns
	Winter	11.55	0.31	5.28	0.19	14.41	0.54	1.55	0.07
Quercus suber	Summer	11.83	0.07 ns	6.45	0.61 ns	13.87	0.40*	1.55	0.03 ns
	Winter	11.91	0.19	5.72	0.31	16.21	0.16	1.86	0.06
Stems									
Quercus afares	Summer	5.98	0.08 !	9.68	0.27***	6.81	0.53*	0.55	0.04***
	Winter	7.08	0.24	13.84	0.35	9.31	0.29	0.78	0.02
Quercus faginea	Summer	6.03	0.49 ns	10.25	0.03***	7.61	0.71 ns	0.68	0.02 ns
	Winter	5.74	0.21	13.52	0.48	7.10	0.42	0.77	0.02
Quercus ilex	Summer	5.67	0.28**	6.76	0.64 !	6.87	0.98**	0.81	0.04 ns
	Winter	7.36	0.21	8.57	0.34	9.50	0.37	0.79	0.04
Quercus suber	Summer	5.58	0.22 ns	7.59	0.42**	6.88	0.30 ns	0.74	0.01 ns
	Winter	5.33	0.23	10.50	0.42	7.25	0.36	0.68	0.01

Significant differences between summer and winter means, based on Scheffe's *post hoc* tests, are shown by *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ or !$P < 0.08$; . $n = 3$ in July, $n = 6$ in December.

tests. The season effect was not significant for hydraulically weighted vessel diameter, because the number of large vessels stayed the same and hydraulic diameter weights large vessels more than small vessels. *Quercus afares* had significantly greater mean vessel diameters as well as hydraulically weighted vessel diameters than all of the other species based on the summer measurements. Winter measurements were similar in that *Q. afares* showed greater mean vessel diameters relative to the two evergreen species but there was not significant difference between the two deciduous species. Hydraulically weighted vessel diameters had the following rank order: *Q. afares* > *Q. faginea* > *Q. suber* > *Q. ilex*, although only *Q. afares* and *Q. ilex* differed significantly (Table 2).

Seasonal changes in nitrogen, soluble sugars, starch and lipids

The nitrogen content of leaves of *Q. afares* declined in December relative to July while the nitrogen content of stems increased (Table 3). Other species did not show significant

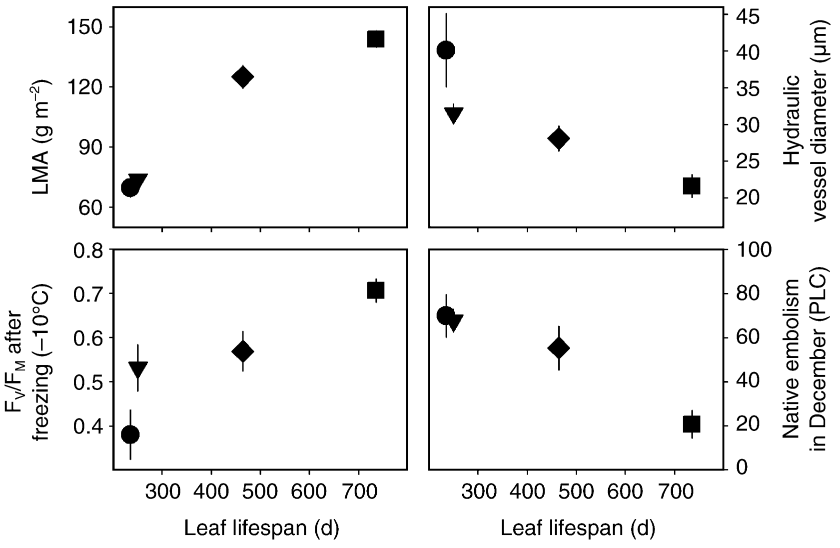

Fig. 8 Leaf mass per area (LMA), F_V/F_M after recovery from freezing at –10°C, hydraulically weighted vessel diameter, and native embolism in December (% loss of conductivity) after exposure to 3°C minimum outdoor temperatures in relation to leaf lifespan. The LMA was measured on sun leaves from the common garden, Centre d'Ecologie Fonctionelle et Evolutive, CNRS, Montpellier, France. Each *Quercus* species is indicated by a different-shaped symbol: circles, *Q. afares*; triangles, *Q. faginea*; squares, *Q. ilex*; diamonds, *Q. suber*.

changes in nitrogen. Starch content increased in stems of all species during winter, although this change was not significant in *Q. ilex*. Leaf starch content did not change significantly in any of the species. Soluble sugars increased only in stems of *Q. ilex* during winter, and none of the species showed differences in sugar content of leaves. Lipid concentrations increased in leaves of the two evergreen species and in *Q. afares*, although the increase in the *Q. ilex* was much higher (39%) than in *Q. suber* (17%) and *Q. afares* (10%). *Quercus ilex* also showed a significant increase in stem lipids, which did not occur in *Q. suber* and *Q. afares* but did in *Q. faginea* (Table 3).

Discussion

Leaf longevity and vessel diameter in relation to freezing sensitivity

In this study, leaf longevity, leaf mass per area (LMA) and mean vessel diameters of co-occurring oak species were correlated and predictive of the degree of cold acclimatization and freezing sensitivity in both leaves and xylem (Fig. 8). These relationships indicate coordination between stem and leaf traits, such that species with longer lived leaves (*Q. ilex* and *Q. suber*) show higher resistance to freezing. The deciduous species (*Q. faginea* and *Q. afares*) have less need to be protected against freezing since they become dormant in winter. Correspondingly, the oaks with longer life lifespans show greater acclimatization to cold temperatures than species with shorter leaf lifespans. Previous studies have documented cold acclimatization in photosynthetic function in evergreen Mediterranean oaks as a result of increases in antioxidant and carotenoid composition in the leaves (Garcia-Plazaola *et al.*, 1997, 1999).

Species with longer-lived leaves, particularly the true evergreen, *Q. ilex*, also showed greater resistance to freezing-induced xylem embolism. In Mediterranean Europe, where mild freezing conditions are common in winter, the maintenance of both photosynthetic and vascular function allows the evergreen species to continue photosynthesis and gas exchange throughout the winter, providing an advantage over their deciduous congeners. Studies of leaf economics show that evergreen species with longer-lived leaves have thicker leaves (higher LMA), higher leaf construction costs, and require a longer season for carbon gain to occur in order to repay these costs relative to their deciduous counterparts (Kikuzawa, 1995; Damesin *et al.*, 1998; Kikuzawa & Ackerly, 1999; Reich *et al.*, 1999). By contrast, deciduous species rely on faster carbon uptake rates during a shorter season to support lower construction costs. Because of their ability to achieve winter dormancy, deciduous species are generally thought to have an advantage in temperate climates with freezing winters where harsh conditions can be damaging to active tissues (Quézel & Médail, 2003). These contrasting leaf habits represent different strategies for maintaining carbon balance and suggest that evergreen and deciduous species are not equally competitive in Mediterranean ecosystems. Corresponding differences in vessel diameters (Fig. 8) indicate a trade-off in transport efficiency vs resistance to freezing-induced cavitation, predicting that species with shorter leaf lifespans and larger vessel diameters should inhabit soils with higher water and nutrient availability. A previous study found that larger vessel diameters in *Q. faginea* relative to *Q. ilex* accorded with their contrasting ecological distributions, such that *Q. faginea* dominates in sub-Mediterranean areas where soils are deeper and water availability is higher, while *Q. ilex* is more common in Mediterranean areas where soils are shallower (Villar-Salvador *et al.*, 1997).

Distributions of *Q. ilex* and *Q. suber* may also reflect their freezing resistance and carbon gain strategy, with *Q. ilex* able to occupy slightly colder regions than *Q. suber* as a result of its higher tolerance to freezing. Human history also plays a role in the distributions of these oaks, and the rapid expansion of *Q. ilex* over the last 6000 yr has been associated with the settlement of human populations (Barbero & Loisel, 1980). In addition, current ranges may reflect their refugial distributions during the Holocene (Carrion *et al*., 2000; Jalut *et al*., 2000) rather than their optimal distributions. Nevertheless, it can be hypothesized that the deciduous *Q. afares* and *Q. faginea*, which are currently restricted to northern Africa and southern Mediterranean regions (Fig. 1), are at a competitive disadvantage relative to evergreen congeners preventing northward expansion into temperate regions of Europe, where the deciduous habitat is thought to be more adaptive.

Dependence of embolism on vessel diameter

The freezing experiments demonstrated that loss of hydraulic conductivity in first-year stems increased with vessel diameter or hydraulically weighted vessel diameter at mild freezing temperatures (–2°C, –5°C) (Fig. 5). After experimental overnight freezing at –2°C in July, the two evergreen species, *Q. ilex* and *Q. suber*, which had the smallest diameter vessels, showed PLC values that were not significantly different from native embolism, and freezing exotherms were not observed. *Q. afares*, a deciduous species with the largest diameter vessels showed very high embolism (80% PLC) even at –2°C. The other deciduous species, *Q. faginea*, which had significantly narrower vessels (Table 2), experienced a PLC of *c.* 70% at –2°C. Among the four species, native embolism in December after a naturally occurring freezing event at –3°C was also dependent on vessel diameter. *Quercus ilex* (smallest vessel diameters) showed no increase in PLC after this event relative to July values of native embolism, *Q. suber* (intermediate vessel diameters) showed > 50% PLC, and the two deciduous species (largest vessel diameters) had a PLC of almost 70%. At lower minimum temperatures (–10°C, –15°C), embolism was very high in all species (80–100% loss of conductivity).

The relationship between freezing-induced xylem embolism and vessel diameter among these oaks is consistent with a number of studies showing that across diverse taxa, larger xylem conduits are more vulnerable to embolism by freezing than smaller conduits (Ewers, 1985; Sperry & Sullivan, 1992; Lo Gullo & Salleo, 1993; Davis *et al*., 1999; Feild & Brodribb, 2001; Pittermann & Sperry, 2003; Cavender-Bares, 2005; Tanedo & Tateno, 2005). The main explanation is that larger bubbles form in larger xylem conduits. Larger bubbles are more difficult to dissolve upon thawing and dissolution time increases approximately with the square of the initial bubble diameter (Ewers, 1985; Yang & Tyree, 1992). The tension during thawing and the timing of the onset of tension upon thawing influences whether dissolution or expansion occurs (Tyree & Zimmermann, 2002). At very mild freezing temperatures (–2°C), freezing apparently did not occur in *Q. ilex*, the evergreen species with the smallest diameter vessels, and exotherms were weak or unclear in *Q. suber*. As a result, loss of conductivity after exposure to –2°C was not significantly different from native PLC in July. Among the two deciduous Mediterranean oaks, *Q. afares* and *Q. faginea*, even mild freezing temperatures resulted in almost complete embolism (Fig. 4). Additional research is required to determine the causal mechanism, but it appears that the narrower vessels of the evergreen species permit greater supercooling than the deciduous ring-porous species. Supercooling refers to the cooling of a liquid below the freezing temperature that is expected based on the solute concentration. It can occur readily in very small volumes of water, where surface properties influence the free energy of water, particularly in the absence of nucleation particles or agents that initiate ice-crystal formation (Guy, 2003). Thus, differences in freezing temperature (Table 2) contribute to the correlation between vessel diameter and PLC at –2°C (Fig. 5, upper panels). LoGullo & Salleo (1993) hypothesized that larger diameter vessels froze before smaller ones, although they were referring to different vessels within the same stem (see later).

Dependence of embolism on minimum freezing temperatures

In addition to its dependence on vessel diameters, loss of conductivity after freezing was also dependent on minimum temperature, most apparent for the two evergreen Mediterranean oaks, *Q. ilex* and *Q. suber*. As explained above, at –2°C, these species showed no or only slight embolism, and clear exotherms were not observed. Freezing-induced embolism became apparent for all species at –5°C, when exotherms were also clearly visible. The degree of embolism increased with decreasing temperatures below –5°C (Fig. 4). Other studies have reported similar increases in embolism with declining temperatures (Pockman & Sperry, 1997; Pittermann & Sperry, 2003).

The water potential of ice declines approx. 1 MPa per °C (Hansen & Beck, 1988). Therefore, the tension in the xylem is likely to increase as temperatures decline. The increasing xylem tension that results as ice forms and temperatures continue to decline below the freezing point may give rise to the increasing embolism observed with decreasing minimum temperatures. Decrease in water potential with declining temperature should also cause a gradient in water potential between liquid water and ice. This gradient influences the way that water is redistributed during the freezing process (Lemoine *et al*., 1999; McCully *et al*., 2000; Ball *et al*., 2004). Water in the xylem may move into extracellular spaces in other tissues that freeze first or into other areas of the xylem more susceptible to freezing (Ball *et al*., 2002, 2004). Tension would increase even in vessels in which the xylem sap was not yet frozen, increasing their likelihood of embolism. A study by Kikuta & Richter (2003) using ultrasonic acoustic emissions indicates that embolism occurs during the process of freezing and increases as temperatures decline and with increasing xylem tension. Other evidence, however, suggests that cavitation only occurs during the process of thawing (Utsumi *et al.*, 1999). Whether winter embolism occurs during the process of freezing or only during thawing, the role of increased xylem tension with declining temperature and its effect on the redistribution of water may explain the dependence of xylem embolism on minimum temperature. This mechanism of embolism formation has not been emphasized in the literature as much as the role of xylem tension generated due to canopy transpiration during the thawing process.

In *Q. ilex*, the most freezing tolerant species, no significant cavitation was observed even after a −3°C natural freezing event in the garden in December. At −5°C, however, significant cavitation occurred in the experimental manipulations (*c.* 80% PLC), both during July and December. *Q. suber* was more susceptible to the natural −3°C freeze in the garden, showing a > 50% loss of conductivity. At −5°C, *Q. suber* showed nearly 80% losses of conductivity in both July and December.

LoGullo & Salleo (1993) demonstrated that within a given stem in *Q. ilex*, larger vessels were more likely to embolize than smaller vessels as a result of freezing, and that the number of embolized vessels increased with decreasing temperature. They suggested that freezing is not simultaneous in all xylem vessels and that different-sized vessels freeze at different temperatures. At lower temperatures, therefore, increasing numbers of vessels would freeze. At the same time, the water potential of the sap becomes increasing negative as the temperature drops. As a result, at lower minimum temperatures, not only are more vessels likely to freeze, but the tension in the xylem increases, as well as the gradient in water potential between liquid water and ice, potentially causing embolism to occur during the process of freezing.

Effects of cold acclimatization on xylem embolism

Winter acclimatization only slightly reduced vulnerability to freezing-induced embolism. Experimental freezing at −5°C during December resulted in a lower degree of embolism in *Q. faginea* and *Q. ilex* than in July, and was marginally lower in *Q. afares*. This decrease in vulnerability to freezing-induced embolism in winter relative to summer was not apparent for *Q. suber*. The decrease in vulnerability is associated with a decrease in vessel diameter (Table 2). Examined together, all species showed a significant decrease in average winter vessel diameters (Table 2), although this decrease was not necessarily significant for individual species. We are unable to say definitively whether the decline in average vessel diameter is caused by random sampling or by other factors. We suspect, however, that it results from continued growth of late wood in the late summer and fall. Late wood has smaller diameter vessels than early wood. Since the total cross-sectional area has a higher relative proportion of late wood in the winter than in the summer, the average vessel diameter should be lower in winter. A slight drop in the exotherm temperature was also observed in winter in all species (Table 2), although at −10°C, winter and summer embolism did not differ for any of the species.

Cold acclimatization of leaves

As expected, loss of photosynthetic function was dependent on minimum freezing temperature in all species (Fig. 6). Unlike xylem function, photosynthetic function in leaves showed a strong acclimatization response during winter in the two evergreen species, which protected the leaves even at −15°C. *Quercus faginea* also showed a significant acclimatization response, although it was not as dramatic, in part because loss of photosynthetic function due to experimental freezing at −10 and −15°C in summer was not as high as in the two evergreen species. *Quercus afares*, did not show any detectable acclimatization response during winter. Senescence had already commenced in this species at the time of measurement such that the leaves showed significant translocation of nitrogen to stems (Table 3) and native dark-adapted F_V/F_M values were significantly lower than in summer. None of the other species showed a decline in F_V/F_M in December. *Quercus ilex*, which showed the greatest sensitivity to freezing in July, showed the greatest tolerance of freezing during December, at minimum freezing temperatures of −10 and −15°C. *Q. ilex* and *Q. suber* differed in the sensitivity of their leaves to freezing at −5°C. At −5°C, both showed approximately equal declines in F_V/F_M in summer (20% and 10%, respectively). In winter, however, *Q. ilex* showed no decline in F_V/F_M at this temperature, while *Q. suber* showed a 17% decline in F_V/F_M. *Q. ilex* did not appear to suffer any leaf damage due to night-time freezing until temperatures reached −10°C. Even at −10°C, the decline in F_V/F_M was only about 10% in December compared with *c.* 30% in *Q. suber*. Earlier work showed that in January, 50% freezing injury of leaves in *Q. ilex* occurred at a slightly lower temperature (−15°C) than in *Q. suber* (−11°C) (Larcher, 1981). This difference in freezing sensitivity could be important in explaining the more northern distribution of *Q. ilex* relative to *Q. suber*.

Winter changes in N, soluble sugars, starch and lipids in leaves and stems

Biochemical changes in leaves during the onset of winter may help explain the processes going on during cold acclimatization including the increased tolerance to freezing in the evergreen species and senescence and onset of winter dormancy in the deciduous species. *Quercus afares* was the only species to show a significant loss of leaf N by December

(Table 3). This was accompanied by a significant increase in stem nitrogen, indicating that nitrogen had been translocated from leaves to stems. Nitrogen translocation helps to explain the significant winter decline in photosynthetic function in the leaves of *Q. afares*, as measured by dark-adapted F_V/F_M on attached leaves (Fig. 6), which did not occur in the evergreen species, and had not yet occurred by December in *Q. faginea*.

Sugar accumulation in living and nonliving tissues during winter in cold-hardy trees has long been observed and is thought to be important in cold acclimatization because it increases viscosity, which reduces ice crystal formation, helps stabilize membranes by binding to the free phosphate groups of membrane lipids, maintains respiration in living cells and allows cell metabolism to recover after freezing (Ashworth *et al.*, 1993; Schrader & Sauter, 2002; Guy, 2003; Wong *et al.*, 2003). An increase in sap osmolarity resulting from an increase in soluble sugars in the apoplast has also been shown to increase stem pressures and to play an important role in winter embolism repair (Améglio, 2004; Améglio *et al.*, 2001b; Cochard *et al.*, 2001; Ewers *et al.*, 2001). A winter increase in soluble sugars was apparent in both stems and leaves of *Q. ilex* (Table 3), although it was only significant in stems. Sugar accumulation in winter may contribute to the higher cold tolerance in *Q. ilex* relative to other species. It also suggests the possibility that *Q. ilex* can repair winter embolism through positive stem pressure as sap osmolarity increases (Améglio *et al.*, 2002; Améglio, 2004) as previously hypothesized (Lo Gullo & Salleo, 1993).

In contrast to soluble sugar content, starch content increased significantly in stems of all species except *Q. ilex*. Starch accumulation serves a storage function for carbohydrates but does not increase osmolarity of the apoplast or symplast. Lipid content increased in leaves of all species, although more significantly in the two evergreen species, particularly *Q. ilex*. Lipids may be important in maintaining fluidity of the plasma membrane. Deposition of lipids on the cell wall during cold acclimatization may also increase rigidity. The rigidity of the cell wall prevents cell contraction and collapse during freezing that could otherwise occur owing to the growth of extracellular ice crystals and protects against membrane damage (Burke *et al.*, 1976; Rajashekar & Lafta, 1996). Rigid cell walls may also be important in preventing nucleation of ice formation (Wisniewski & Ashworth, 1985; Ashworth *et al.*, 1993).

Coordination of leaf and stem function

The four congeners showed the same rank order of vulnerability to freezing in the leaves as in the xylem. For each species, F_V/F_M of leaves declined with increasing loss of stem conductivity after freezing (Fig. 7, upper panel). The slope of this relationship was shallower in winter (Fig. 7, lower panel), because cold acclimatization reduced vulnerability of leaf photosynthetic function more than vulnerability of xylem function. Individual species fell along different curves, with *Q. ilex*, the species with the longest leaf lifespan, showing the lowest losses of photosynthetic and xylem function and *Q. afares*, with the shortest leaf lifespan, showing the highest losses in function (Fig. 7, lower panel). We were surprised to find that in winter, leaves of *Q. ilex* were almost completely functional even while native embolism levels approached 80%. This discordance in short-term freezing responses between leaves and xylem further supports the hypothesis that *Q. ilex* is able to repair winter embolism. Otherwise, continued gas exchange in functional leaves supported by embolized vessels would lead to increasing xylem tension and could potentially cause runaway cavitation (Tyree *et al.*, 1993). The other three species, which showed greater losses in photosynthetic function, showed a more coordinated response of leaves and xylem to freezing.

In conclusion, these results show that susceptibility to freezing in both leaves and xylem should be considered with respect to minimum temperatures, and that leaf and hydraulic properties appear coordinated in Mediterranean oaks. Both the loss of photosynthetic function and loss of hydraulic conductivity increased with decreasing minimum temperatures. Greater loss of leaf and stem hydraulic function occurred in species with shorter-lived leaves and larger diameter vessels. Consequently, vulnerability of the photosynthetic apparatus and the vascular system to freezing were found to be coupled and linked to leaf longevity and vessel anatomy (Fig. 8). Cold acclimatization was also greater in evergreen species than deciduous species. Our results support the hypothesis that in Mediterranean regions where freezing is frequent but mild, evergreen oaks with narrow vessel diameters and freezing tolerant leaves can photosynthesize throughout the winter and outcompete deciduous oaks with large diameter vessels and freezing-sensitive leaves.

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