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Temperature Controls Cold Hardening more Effectively than Photoperiod in Four Mediterranean Broadleaf Evergreen Species

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ABSTRACT

Forestry plantations with evergreen broadleaf species in Mediterranean climate sites usually perform poorly in the field. Holm oak (*Quercus ilex* ssp. *ballota* (Desf.) Samp.), cork oak (*Quercus suber* L.), wild olive tree (*Olea europaea* L. ssp. *europaea* var. *sylvestris*) and lentisk tree (*Pistacia lentiscus* L.) are extensively used for such plantations. In order to determine the environmental factor that induces cold hardening most effectively and the mechanisms that are involved in this process, seven month-old nursery seedlings were taken to three growth chambers during the hardening phase and submitted to: 22/17ºC (day/night) and decreasing photoperiod from 12 to 8 h (*Ph* chamber), 12 h photoperiod and decreasing temperature from 22/17ºC to 8/3ºC (*T* chamber); and progressive reductions in temperature and photoperiod (*PhT* chamber). The variation of morpho-physiological traits was assessed. Reducing the photoperiod by up to 8 h did not stop the growth in height and diameter. Air temperatures below 8ºC reduced substantially height growth, mainly in *Quercus* species, but not diameter growth. Reducing the temperature proved much more effective for cold hardening than reducing the photoperiod. Low temperatures induced seedlings to accumulate non-structural carbohydrates (soluble sugars in the leaves and roots, and starch in the roots), and to improve cold hardiness. Differences among species were observed in cold hardiness, with *Q. ilex* > *Q. suber* ≥ *P. lentiscus* ≥ *O. europaea*. The maximum level of cold hardiness achieved in this experiment by *Q. suber* and *Q. ilex* was reached when seedlings accumulated 775-800 h_8 (hours ≤ 8°C), 750 h_8 in *O. europaea* and 725 h_8 in *P. lentiscus*. Finally, measuring chlorophyll fluorescence (Fv/Fm) after a freezing test was useful in estimating the cold hardiness of these species, providing results in less than two days for plant quality purposes.

Keywords: chilling requirements, frost tolerance, *Quercus suber*, *Quercus ilex*, *Pistacia lentiscus*, *Olea europaea*

Abbreviations: $\mathbf{D_{GR}}$, absolute growth rate in stem diameter; **Fv/Fm**, variable-to-maximum chlorophyll fluorescence; $\mathbf{h_8}$, chilling-effective hours with air temperatures ≤ 8°C; $\mathbf{H_{GR}}$, absolute growth rate in height; **LT50**, temperature corresponding to 50% leaf damage; $\mathbf{SRDW_{VR}}$, the variation rate in shoot to root dry weight ratio; $\mathbf{TDW_{GR}}$, absolute growth rate in total dry weight

INTRODUCTION

Evergreen broadleaf species are used extensively for forestry plantations. Holm oak, the dominant sclerophyllous species in large parts of the Mediterranean basin, makes up more than 4.0 Mha of forests, and cork oak about 2.0 Mha (Ruiz de la Torre 2006). Wild olive tree and lentisk tree, like holm oak and cork oak, are very widespread in the Mediterranean Basin. The four species frequently coexist as part of forests and shrublands. Nevertheless, these species have a poor early out-planting performance when compared to other Mediterranean species, particularly when planted in sites with unfavourable climatic conditions (Baeza *et al*. 1991; Rey-Benayas 1998; Pausas *et al*. 2004), mainly as a result of summer drought. However, cold stress (Mitrakos 1980; Larcher 2000; Mollá *el at*. 2006) and other factors such as planting shock, frost, spring drought, grazing, etc., also affect negatively to seedling performance. Afforestation in Mediterranean climate is done during cold season, since spring planting could reduce root growth (Corchero-Torre *et al*. 2002) and could damage seedlings if a drought period occurs (Rose 1992). Autumn-planted seedlings can be threatened, however, by early frosts (Colombo *et al*. 2003; Mollá *et al*. 2006), especially if evergreen species are used (Larcher 2003; Varone and Gratani 2007).

Nursery practices, environmental conditions, and genetic factors affect the functional characteristics of seedlings and consequently their transplanting performance (Grossnickle and Folk 1993; Birchler *et al*. 1998). Nevertheless, the majority of research carried out to date on seedling quality and field performance has been conducted using conifer nursery stock, mainly in cold temperate and boreal forest species. Therefore, it needs to be verified and fine-tuned for broadleaved species (Colombo 2004). Walter Larcher (2000) conducted probably the most complete studies of low temperature effects on Mediterranean sclerophyllous species, but he did not deepen on the environmental control of frost resistance in these species.

Environmental factors at the nursery during the hardening phase such as chill temperatures, short photoperiods, irrigation and fertilisation can induce plant dormancy and improve plant hardiness (Colombo *et al*. 2003; Villar-Salvador *et al*. 2004; Fernández *et al*. 2007). Although irrigation and fertilisation increase hardiness, they do not always induce sufficient levels of hardiness in Mediterranean forest seedlings (Royo 1998; Villar-Salvador *et al*. 1999). On the other hand, temperature and daylength are important factors controlling growth, dormancy and stress conditioning in species from temperate climates (Greer and Warrington 1982; Taulavouri *et al*. 1997; Colombo *et al*. 2003; Turner and Mitchell 2003), even though the response can vary depending on the species and ecotype (Fernández and Pardos 1995; Boorse *et al*. 1998; Repo *et al*. 2000). Daylength and temperature can induce chemical changes in cell and thylakoid membranes, as well as elastic and osmotic adjustments, similar to those induced by moderate water stress (Folk *et al*. 1994; Karavatas and Manetas 1999; Valentini *et al*. 2000), lowering the cell freezing point (Sutinen *et al*. 2001).

Received: 31 July, 2008. Accepted: 23 October, 2008.

Original Research Paper

To avoid freezing damage after out-planting, it is possible to determine when seedlings are hardy enough to withstand autumn frosts (Colombo and Gellert 2002). Therefore, cold hardiness is operationally useful because it is relatively easy to monitor (Ritchie and Landis 2003). To measure the cold hardiness, many nursery managers record the number of chill hours, and assume adequate hardiness when 200 to 400 hours are reached, depending on the species and the geographic seed source (Lantz *et al*. 1996).

Temperature and photoperiod are two variables that can be modified in nurseries and greenhouses (Colombo 2004), but the few cold hardiness studies on the four species of this study examined simultaneous reductions in temperature and photoperiod (Fernández *et al*. 2005; Mollá *et al*. 2006). None of the studies describe the separate effect of a gradual decrease in temperature or the photoperiod on cold-hardening of holm oak, cork oak, wild olive tree or lentisk tree. Therefore, the major objectives of this study are to determine: i) the environmental factor that induces cold-hardening most effectively: a reduction in temperature or a reduction in the photoperiod; ii) the effects of these environmental factors during the hardening phase on plant growth, mineral nutrients content and non-structural carbohydrates; and iii) the chilling requirements for inducing cold hardiness and, consequently, determining when seedlings are ready for out-planting from the nursery. To achieve these objectives morphological and physiological attributes were assessed during hardening in growth chamber conditions.

MATERIALS AND METHODS

Young seedlings of holm oak, cork oak, wild olive tree and lentisk tree were first grown at the nursery of the Polytechnic School (Huelva, Spain, 37° 12′ N, 6° 54′ W) from March to September. Seedlings (1500 plants per species) were cultivated in the open air in a *Sphagnum* peat/older-peat/pine bark mix (1:1:1 v/v), with 300 cm^3 per plant, watered as needed and fertilised once a week with Peters Professional® 20-20-20. The containers (Super-leach® 300) were individual cells that can be interchangeable in separate tray systems. The size of the plants were within the normal range of those produced in Spanish nurseries, except for the wild olive tree that was at the higher end in terms of height (Navarro *et al*. 1998; Bañón *et al*. 2003). Cork oak averaged 35.9 ± 3.2 cm in height, holm oak 14.8 ± 0.8 cm, wild olive tree 71.4 ± 3.5 cm and lentisk tree 7.3 ± 0.7 cm. All the seeds were collected from natural forests in south-western Spain. The location and climate variables of the seed origins were: latitude 36° 00′ to 38° 25′ N; longitude 3° 21′ to 7° 32′ W; 660 to 750 mm average annual rainfall; 16.7 to 17.6°C mean annual temperature; 4.8 to 7.1 mean minimum temperature of the coldest month; and 30.9 to 34.2°C mean maximum temperature of the warmest month.

Three hundred seedlings per species were then randomly selected, divided into three equal parts and placed into three growth chambers with contrasting growing conditions. To acclimatise the seedlings to growth chamber conditions, all of them were left inside the growth chambers for three weeks, with a good water supply and under long daylength (from 16 to 14 h) and warm-temperatures (from 25/19 to 22/17°C, day/night). For the first week the growing conditions (daylength and temperature) were similar to those at the nursery. After the first three weeks, the experiment was subsequently carried out over a thirteen-week period. The *Ph* chamber was set at a constant temperature (22/17°C, day/night) and decreasing photoperiod from 12 to 8 h (-0.5 h/week from week 1 to 9, and a constant photoperiod of 8 h in the last four weeks). The *T* chamber was set at a constant photoperiod (12 h) and decreasing temperature from 22/17°C to 8/3°C (-2°C/week from week 1 to 8, and constant temperatures of 8/3°C in the last five weeks). And the *PhT* chamber was set at progressive reductions of photoperiod and temperature as described for *Ph* and *T* chambers for a thirteen-week period, as well. For operational reasons, the seedlings of the *PhT* chamber were selected from the nursery and acclimated two weeks before chambers *T* and *Ph*, so these were two weeks older than plants from the *PhT* chamber at the beginning of the experiment. The plants did not show any visual symptom of shoot growth when they were collected from the nursery. The photoperiod and daily temperatures employed in this study were chosen because these values are similar to those at the source locations of the seed origins from the beginning of autumn to midwinter. Temperatures between 0 and 10°C are expected to be effective to induce cold hardening and, for species adapted to warm climates, the higher values of this range are preferred (Royo 1998; Fernández *et al*. 2003, 2007). We therefore decided to calculate chilling-effective hours with air temperatures ≤ 8°C (h_8). The number of chill hours (h_8) in chambers *T* and *PhT* accumulated during the experiment were zero from day 1 to 28, 288 on day 47, 888 on day 72 and 1344 on day 91. There were no chill hours in chamber *Ph*.

Mean photosynthetic active radiation (PAR) was 400 µmol m^{-2} s^{-1} in the middle of the growth chambers, falling to 300 µmol m^{-2} s^{-1} at the edges. The trays were therefore rotated weekly. The plants were not rotated between chambers, but the results obtained indicated us that it was unlikely that they were significantly influenced by uncontrolled factors linked to a single chamber. Seedlings were well watered (every two days) and fertilised (once a week). A Compo® Universal liquid fertiliser (7-5-6 + micronutrients) was used, at a concentration of 2.5 cm^3/L (40 cm^3/plant of watering solution each time).

The following parameters were measured on four dates (days 1, 47, 72 and 91):

a) ***Morphology and growth*****.** On each harvest date, 6 seedlings per species and treatment were harvested. The following parameters were measured: height (*H*); the stem diameter two centimetres above the root collar (*D*); and leaf (LDW), stem (STDW) and root (RDW) dry weights oven-dried to constant weight at 75°C. Shoot dry weight (SDW = LDW+STDW), total dry weight (TDW), and the shoot-to-root dry weight ratio (SRDW) were calculated. The absolute growth rate in height (H_{GR}), in stem diameter (D_{GR}) and in total dry weight (TDW_{GR}), and the variation rate in SRDW ($SRDW_{VR}$) were also calculated. In addition, at the beginning of the experiment, ten seedlings were selected at random for each chamber (permanent sample). Their heights and stem diameters were measured on days 1, 18, 31, 47, 59, 72 and 91. The absolute growth and variation rates during the experiment were calculated as follows: $(x_{i+n} - x_i)/n$, where x_i and x_{i+n} were the values of a morphological attribute (*H*, *D*, TDW or SRDW) on days *i* and *i*+*n* respectively, and *n* was the number of days from day *i* to day *i*+*n*.

b) ***Mineral nutrient and carbohydrate content*****.** Once weighed, on each harvest date, the six seedlings were used to analyse macronutrients (N, P, K), soluble sugars (SS) and starch content in the shoot and root. The following analytical procedures were applied: elemental analysis for N (EA FLASH 1112 CHNS, CE Instruments Ltd., UK), flame photometry for K, hydro-alcoholic extraction and colorimetric titration with anthrone for SS, and acidic hydrolysis, followed by titration with anthrone for starch. A spectrophotometer (UVmini-1240, Shimadzu, Tokyo, Japan) was used for the analysis of P, SS and starch.

c) ***Whole-plant freezing test*** **(WPFT).** At each harvest, 2 seedlings per species and treatment were chosen as a control and another 5 were exposed to a slow temperature decrease (3°C/h), until the temperature reached the minimum point set for each freezing test (between -2 and -17°C, depending on the date). The control seedlings were used to test, and to correct if necessary, the effect of any uncontrolled factor apart from low temperature that could influence on the results. The roots were protected from low temperatures by surrounding them with Styrofoam. Root temperature, measured continuously with a thermometer, never dropped below -2°C during the coldest test. The temperature was maintained for three hours, after which it was increased by 5°C/h to room temperature. The plants were then transferred to a growth chamber at a temperature of 22/16°C (day/night), relative humidity of 70/90%, photoperiod of 16 h, and a PAR of 350 µmol m^{-2} s^{-1}. Two days later, the variable-to-maximum chlorophyll fluorescence (Fv/Fm ratio) was measured with a portable fluorometer (Fim 1500, ADC, London, UK). Two hours after the beginning of the light period, and after sufficient dark adaptation, a 5 s flash of actinic light with a flux of 1500 µmol m^{-2} s^{-1} and a peak wavelength of 650 nm was used. One month later, visual shoot damage was assessed based on the proportion of withered leaves. The temperature corresponding to 50% leaf damage (LT50) was calculated

Table 1 Visual assessment of frost damage at the beginning of the experiment (day 1), and at the third harvest (day 72) for the three growing conditions (*Ph*, *T* and *PhT* chambers). (h_8) = number of chill hours ≤ 8°C accumulated

Species	Day (h_8)	Treatment	Temperature of the freezing test (°C)				
			-4	-6	-8	-11	- 14
Q. suber	1 (0)	day 1	28 ± 7 a	59 ± 13 b	94 ± 5 c	100 ± 0 d	
	72 (888)	*Ph*	19 ± 8 a*	35 ± 12 a*^{o}	96 ± 3 b*	100 ± 0 c*	100 ± 0 c*
		T	0 ± 0 a^{o}	4 ± 2 b^{o}	9 ± 8 bc^{o}	24 ± 9 c^{o}	64 ± 11 d^{o}
		PhT	0 ± 0 a^{o}	3 ± 2 ab^{o}	10 ± 9 ab^{o}	26 ± 8 c^{o}	68 ± 9 d^{o}
Q. ilex	1 (0)	day 1	10 ± 8 a	15 ± 9 a	76 ± 11 b	100 ± 0 c	
	72 (888)	*Ph*	2 ± 2 a	17 ± 8 b*	52 ± 10 c*^{o}	90 ± 8 d*	100 ± 0 e*
		T	0 ± 0 a	2 ± 1 a^{o}	10 ± 9 ab^{o}	18 ± 9 b^{o}	54 ± 12 c^{o}
		PhT	0 ± 0 a^{o}	0 ± 0 a^{o}	7 ± 6 ab^{o}	20 ± 8 b^{o}	60 ± 9 c^{o}
P. lentiscus	1 (0)	day 1	24 ± 9 a	61 ± 11 b	100 ± 0 c	100 ± 0 c	
	72 (888)	*Ph*	16 ± 7 a*	51 ± 10 b*	96 ± 3 c*	100 ± 0 d*	100 ± 0 d*
		T	0 ± 0 a^{o}	4 ± 2 ab^{o}	10 ± 6 b^{o}	37 ± 11 c^{o}	84 ± 13 d^{o}
		PhT	0 ± 0 a^{o}	2 ± 2 a^{o}	14 ± 6 b^{o}	42 ± 9 c^{o}	83 ± 10 d^{o}
O. europaea	1 (0)	day 1	5 ± 3 a	50 ± 12 b	86 ± 11 c	100 ± 0 d	
	72 (888)	*Ph*	10 ± 6 a*	30 ± 8 b*	71 ± 9 c*	100 ± 0 d*	100 ± 0 d
		T	0 ± 0 a^{o}	2 ± 1 a^{o}	5 ± 2 ab^{o}	61 ± 12 c^{o}	100 ± 0 d
		PhT	0 ± 0 a^{o}	0 ± 0 a^{o}	8 ± 7 ab^{o}	66 ± 10 c^{o}	100 ± 0 d

Within each species: Letters in a row (^{a} to ^{c}) indicate significant differences among temperatures of the freezing tests ($p < 0.05$) on the same day under the same chamber. Asterisks (*) indicate significant differences between chambers ($p < 0.05$) under the same temperature test on day 72. Circles (^{o}) indicate significant differences between day 72 and day 1 ($p < 0.05$) under the same temperature test.
Blank boxes indicate the lack of freezing tests for these temperatures, and it was accepted that their values of frost damage were 100%.

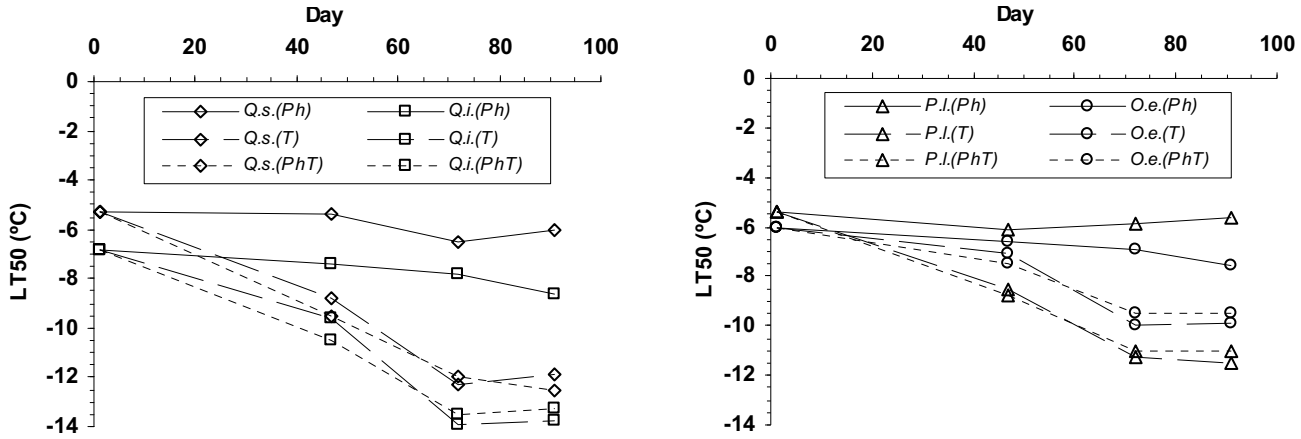

Fig. 1 Lethal temperature 50 (LT50, °C) acquired by the seedlings from the beginning to the end of the experiment, for all the species and chambers. *Q. suber* = ◇, *Q. ilex* = □, *P. lentiscus* = △, *O. europaea* = ○ (*Ph* chamber: continuous lines; *T* chamber: broken lines; *PhT* chamber: dotted lines). Each point is the mean of six seedlings per species and chamber.

by plotting visual damage against minimum temperature of the freezing tests.

d) *Data analysis.* One, two, or three way ANOVA was applied to the data (SPSS® 12.0, SPSS Inc.). Species, growing conditions (chambers) and harvest dates were taken as fixed factors. A repeated-measures ANOVA were used for *H* and *D* of the permanent sample seedlings. Data were checked for normality and homocedasticity. The Tukey HSD test (Tukey Honest Significant Difference) was used as a means of determining differences when these were significant ($p \leq 0.05$). Correlation analysis was used to examine the relationship between morphological and shoot damage parameters. Control-plant and tested-plant differences in the WPFT were analysed using one way ANOVA as well.

RESULTS

There were significant differences in cold hardiness ($p < 0.001$) between species, chambers and dates (**Table 1; Fig. 1**). Data from freezing tests at -2°C and at -17°C are not shown in this table because at -2°C there was no frost damage for any species, dates or chambers, and at -17°C the frost damage was 100% for all of them. Under chambers *T* and *PhT*, LT50 decreased from the beginning to the end of the experiment 7.0°C in *Q. suber*, 7.2°C in *Q. ilex*, 6.0ºC in *P. lentiscus* and 4.4°C in *O. europaea*, with *Quercus* sp. hardening significantly more than *P. lentiscus* and *O. europaea*. For all the species in these two chambers the differences in cold hardiness between dates were: days 72 and 91 > day 47 > day 1. Under chamber *Ph*, however, the change in LT50 (i.e. $\Delta LT50_{Ph}$) ranged from 0.5°C (*P. lentiscus*) to 1.7°C (*Q. ilex*), without significant differences between species or dates. When expressed in percentage, the differences in the variation of LT50 between *T* and *PhT* chambers in respect to *Ph* chamber (e.g.: 100 [$\Delta LT50_{Ph}$ / $\Delta LT50_{T}$]) were 17% for *Q. suber*, 23% for *Q. ilex*, 8% for *P. lentiscus*, and 36% for *O. europaea*. The seedlings did not noticeably react to cold conditions (i.e. ½ of ΔLT50) until they accumulated 300-370 h_8 (*Quercus*), 260 h_8 (*P. lentiscus*) and 420 h_8 (*O. europaea*). Maximum cold hardiness for this experiment was achieved when *Quercus* sp. accumulated 775-800 h_8, *P. lentiscus* 725 h_8, and *O. europaea* 750 h_8. These data were determined graphically by plotting LT50 against chill hour accumulated during the experiment.

There were significant differences ($p < 0.001$) in SS and root starch concentration between harvest dates. In the *T* and *PhT* chambers, significantly different from *Ph* chamber ($p < 0.001$), there was a significant increase in leaf and root SS concentrations and in root starch concentration between the second and third harvests. The evolution of leaf SS over the experiment is showed in **Fig. 2**. Similar evolution patterns were shown by root SS and root starch concentrations. Levels of root starch concentrations were significantly different between species, with *Q. ilex* (173 ± 8 mg g^{-1}) > *Q. suber* (95 ± 7 mg g^{-1}) > *O. europaea* and *P. lentiscus* (46 ± 9 mg g^{-1}) for all the harvests and chambers as a whole. Leaf starch did not differ significantly between species, dates or

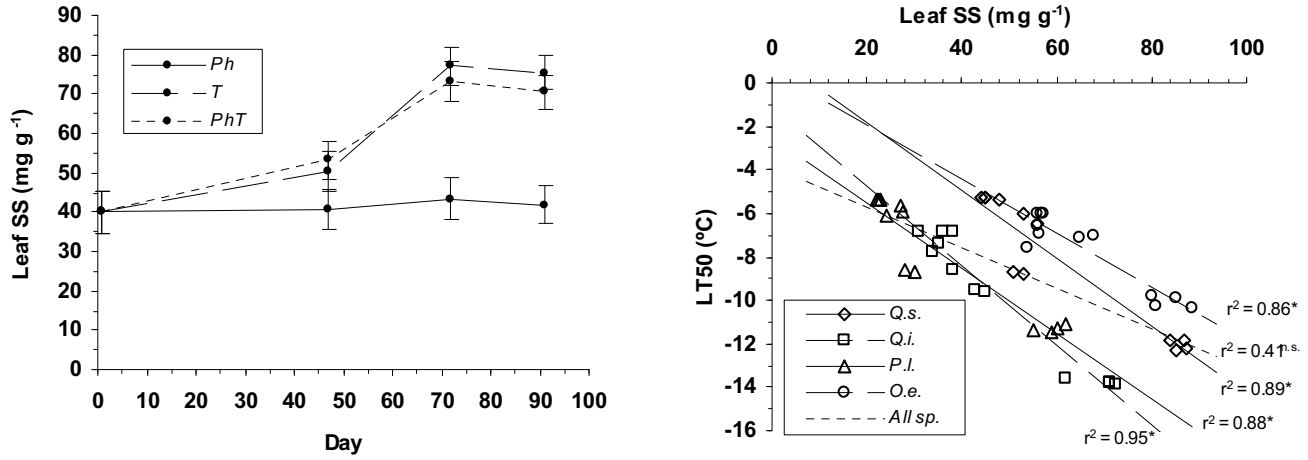

Fig. 2 (Left) Evolution pattern of leaf soluble sugars (SS) for the three chambers and all the species together. (Right) The relationship between leaf SS and lethal temperature 50 (LT50) for each species and for all the species together (*All sp.*). Each point is the mean of six seedlings per species. *Q. suber* = ◇, *Q. ilex* = □, *P. lentiscus* = △, *O. europaea* = ○. The significance level of the ANOVA for the linear regressions are showed: * = $p < 0.01$, $^{n.s.}$ = not significant.

chambers ($p \geq 0.245$), averaging 32.8 ± 3.5 mg g^{-1} for all the species, dates and chambers as a whole. In the *Ph* chamber, however, soluble sugars (SS) and root starch concentrations either decreased slightly or remained unchanged from the beginning to the end of the experiment. For all four species, there was a greater SS concentration in leaves than in roots, and leaf and root SS were significantly correlated ($SS_{root} = 0.337\ SS_{leaf} + 8.720$, $p = 0.035$, $r^2 = 0.40$, n = 48). Within each species, leaf SS concentration and frost tolerance (LT50) were highly correlated, but not if we consider all the species together (**Fig. 2**). Frost tolerance was also positively correlated with SS in roots (r^2 ranged from 0.70 for *Q. ilex* to 0.97 for *P. lentiscus*, $p \leq 0.05$, n = 12).

The significant relationship between visual shoot damage and Fv/Fm in the WPFT is illustrated in **Fig. 3**. Fv/Fm values of control plants ranged between 0.78 and 0.84. In general, frost temperatures damaged the plants (i.e. LT50 > 50%) if the Fv/Fm values were under 0.71. When values were above 0.80, the plants experienced little or no damage (LT50 < 50%). In the interval between 0.71 and 0.80, however, there was a large variation in the levels of damage predicted by Fv/Fm, being therefore unpredictable, at least for these species and by measuring chlorophyll fluorescence 48 h after the freezing test.

In general terms, the plants grew both in height (*H*) and stem diameter (*D*) over the course of the experiment (**Table 2**). Although *H* and *D* measurements were taken on seven dates and harvests were made on four dates, the results are well understood if the experiment is divided into two major periods of time: the first period from day 1 to 47 (the warmer period with longer photoperiods), and the second period from day 47 to 91 (with daily temperature ≤ 8°C at the *T* and *PhT* chambers, and with photoperiod ≤ 8 h at the *Ph* and *PhT* chambers). H_{GR} was significantly greater at the *Ph* chamber (0.94 ± 0.30 mm day^{-1}) than at the *T* and *PhT* chambers (0.32 ± 0.15 mm day^{-1} as a whole). For the three chambers as a whole, H_{GR} in *Quercus* sp. was larger in the first period than in the second one, while *P. lentiscus* and *O. europaea* showed the opposite pattern. Stems increased significantly in diameter throughout the experiment, with D_{GR} greater in the second period (7.3 ± 2.0 μm day^{-1}) than in the first one (3.3 ± 1.3 μm day^{-1}). There were not significant differences in D_{GR} between chambers or species. The significant interaction between species and chambers was due to a greater D_{GR} in *P. lentiscus* at the *Ph* chamber (11.7 ± 2.9 μm day^{-1}) than at the *T* and *PhT* chambers (2.6 ± 1.6 μm day^{-1}), while the other three species did not differ significantly between chambers.

TDW_{GR} did not vary significantly between chambers or dates, but it did between species (**Table 2**). *O. europaea* (32.1 ± 9.5 mg day^{-1}) showed a greater growth rate than the other three species (≤ 6.6 mg day^{-1}). The significant interaction between species and chambers was due to a greater TDW_{GR} at the *T* and *PhT* chambers than at the *Ph* chamber in *Q. suber*, *Q. ilex* and *O. europaea*. *P. lentiscus* showed, however, the opposite pattern. For the three chambers as a whole, *Q. ilex* grew significantly more in the first than in the second period, while *P. lentiscus* and *O. europaea* showed a greater TDW_{GR} in the second one.

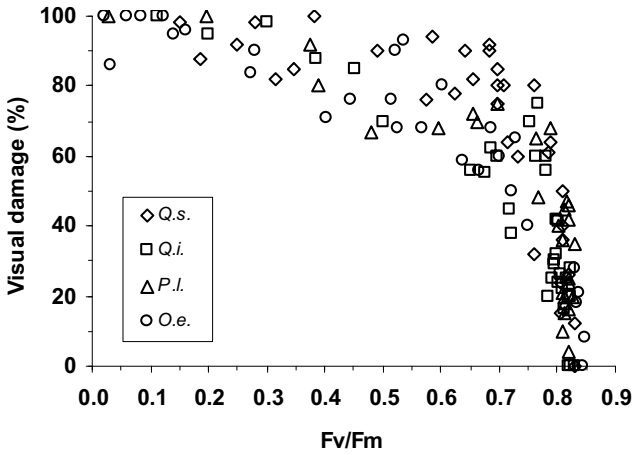

Fig. 3 The relationship between chlorophyll fluorescence (Fv/Fm ratio) two days after the freezing test and visual damage one month after the freezing test, for all the species, chambers and freezing tests. Each point is the mean value of 2 or 5 seedlings (control and tested plants respectively). *Q. suber* = ◇, *Q. ilex* = □, *P. lentiscus* = △, *O. europaea* = ○.

The variation rate in dry matter partitioning ($SRDW_{VR}$) differed between chambers, species and dates. The more relevant results to emphasize, however, could be the interactions species-chamber and species-period. *P. lentiscus* increased significantly in SRDW at the *Ph* chamber ($SRDW_{VR}$ = 32.4 10^{-3} day^{-1}) but not at the *T* and *PhT* chambers (0.6 10^{-3} day^{-1}); *Q. ilex* increased in SRDW at the *Ph* chamber but decreased it at the other two chambers; while *Q. suber* and *O. europaea* increased and decreased, respectively, the SRDW values in all three chamber conditions. With regard to species and period interaction, *Quercus* species increased in SRDW during the first period and decreased during the second one; *O. europaea* showed the opposite pattern than *Quercus* sp.; and *P. lentiscus* increased in SRDW during the two periods but significantly more during the second one.

Leaf, stem and root nutrient concentrations did not undergo great variations from the beginning to the end of the experiment. There were significant differences, however, between species for N and K, both in their leaves and roots ($0.030 > p > 0.001$). The plants did not show visual symp-

Table 2 Mean values (±SE) of growth rates in the two periods of the experiment (days 1-47 and days 47-91) for all the species and the three growing conditions (*Ph*, *T* and *PhT* chambers). $\mathbf{H_{GR}}$ = absolute growth rate in height; $\mathbf{D_{GR}}$ = absolute growth rate in diameter; $\mathbf{TDW_{GR}}$ = abslote growth rate in total dry weight; $\mathbf{SRDW_{VR}}$ = the variation rate in shoot-to-root dry weight ratio; *p*-value = significance level from ANOVA of growing parameters.

Species	Chamber	H_{GR} (mm day^{-1})		D_{GR} (μm day^{-1})		TDW_{GR} (mg day^{-1})		$SRDW_{VR}$ (day^{-1}) x10^{-3}	
		Day 1-47	Day 47-91	Day 1-47	Day 47-91	Day 1-47	Day 47-91	Day 1-47	Day 47-91
Q. suber	*Ph*	1.49 ± 0.21	0.32 ± 0.08	4.4 ± 1.5	5.6 ± 1.8	0.4 ± 2.7	1.4 ± 3.3	5.5 ± 1.5	-1.5 ± 1.2
	T	0.27 ± 0.04	0.11 ± 0.05	3.3 ± 1.6	3.9 ± 1.6	13.7 ± 6.2	10.7 ± 4.1	8.4 ± 3.1	-5.7 ± 2.2
	PhT	0.32 ± 0.04	0.13 ± 0.04	3.4 ± 1.5	3.9 ± 1.5	14.2 ± 5.8	10.5 ± 6.5	7.2 ± 3.5	-4.3 ± 2.7
Q. ilex	*Ph*	0.74 ± 0.18	0.51 ± 0.20	3.3 ± 1.7	7.9 ± 1.6	-0.1 ± 2.8	0.34 ± 1.7	10.1 ± 3.0	1.0 ± 3.2
	T	0.54 ± 0.07	0.14 ± 0.05	4.3 ± 1.4	8.3 ± 1.2	11.6 ± 5.2	-1.3 ± 3.6	-0.8 ± 2.1	-4.0 ± 2.1
	PhT	0.52 ± 0.06	0.13 ± 0.04	3.8 ± 1.5	8.0 ± 1.4	10.6 ± 4.5	0.3 ± 2.7	-0.8 ± 2.5	-4.2 ± 2.2
P. lentiscus	*Ph*	0.77 ± 0.14	2.19 ± 0.24	3.2 ± 1.3	18.3 ± 2.1	2.3 ± 2.5	15.8 ± 7.0	19.2 ± 9.8	41.6 ± 9.9
	T	0.13 ± 0.04	0.17 ± 0.03	2.1 ± 0.9	3.2 ± 1.1	0.7 ± 3.2	1.7 ± 2.1	-6.4 ± 4.7	7.7 ± 4.5
	PhT	0.11 ± 0.03	0.15 ± 0.04	2.1 ± 1.2	3.0 ± 1.1	0.7 ± 2.4	1.6 ± 2.8	-5.7 ± 5.5	8.3 ± 5.8
O. europaea	*Ph*	0.12 ± 0.10	1.24 ± 0.14	3.0 ± 1.2	6.1 ± 1.3	12.8 ± 6.5	22.9 ± 9.7	-18.5 ± 5.9	19.4 ± 6.8
	T	0.12 ± 0.05	0.12 ± 0.03	3.1 ± 0.8	2.9 ± 1.2	18.9 ± 7.4	46.2 ± 9.9	-19.3 ± 6.3	-1.9 ± 2.2
	PhT	0.12 ± 0.04	0.11 ± 0.04	3.0 ± 1.0	3.1 ± 1.0	17.5 ± 7.2	37.8 ± 9.0	-16.1 ± 7.5	-2.7 ± 2.9
	Species (S)	0.790		0.747		0.045		<0.001	
	Chamber (Ch)	0.008		0.248		0.397		0.002	
p-value	Period (P)	0.706		0.011		0.355		0.023	
	S x Ch	0.611		0.047		0.037		0.015	
	S x P	0.045		0.620		0.046		<0.001	
	Ch x P	0.403		0.301		0.919		0.190	
	S x Ch x P	0.382		0.639		0.793		0.515	

toms of nutrient deficiency and they were within the optimum range of N, P and K concentrations (Navarro *et al*. 1998; Castro-Díez *et al*. 2006). For instance, leaf N concentrations were 2.07 ± 0.10% for *Q. suber*, 1.84 ± 0.10% for *Q. ilex*, 2.64 ± 0.09% for *P. lentiscus* and 2.30 ± 0.11% for *O. europaea*. Differences between chambers were only significant for root K concentrations (p = 0.034), where concentrations under the *Ph* chamber (0.91 ± 0.05 %) were a bit higher than under the *T* chamber (0.80 ± 0.04 %). The K/N ratio did not differ significantly between chambers or harvest dates for all four species. Leaf and root P concentrations did not differ between chambers, as well. There were not significant correlations between nutrient concentrations and cold hardiness in this experiment.

DISCUSSION

The pattern of cold hardiness development revealed noticeable differences between species, ambient conditions and dates. Decreases in temperature (*T* and *PhT* chambers) increased significantly cold hardiness. A reduction in photoperiod up to 8 h can produce certain increases in cold hardiness in these species ($\Delta LT50_{ph} \leq 1.7$°C), but it scarcely influenced on cold hardening in *P. lentiscus*, and in *O. europaea*, the most sensitive of the four species, did not exceed 36% of the hardening brought on by low temperatures. Consequently, shorter days can have an important effect on hardening of deciduous species and conifers of mild-cold areas (Taulavuori *et al*. 1997; Hawkins and Shewan 2000; Colombo *et al*. 2003), but this did not happen, however, for the species used in our experiment or for other Mediterranean species (Nguyen *et al.* 1995). The maximum cold hardiness reached under the *T* and *PhT* chambers, and the chilling requirements to induce it, were similar to those obtained for these species under nursery conditions (Fernández *et al*. 2005), with a simultaneous and natural reduction in temperature and photoperiod. Larcher (2003) determined a maximum frost tolerance for *Q. ilex* saplings in the range -12 to -15°C, also similar to our results. Therefore, for our experiment it is possible that the accumulation of chill hours would be sufficient to induce the maximum level of tolerance in these species by itself, regardless of photoperiod.

The sharp increase in leaf SS concentration under treatments *T* and *PhT*, from the second to third harvests, under 8/3°C (day/night) air temperatures, indicates a period of accumulation, coupled with a cessation of height growth and probably a decrease in respiration, as an adaptive response to the cold (Ögren *et al*. 1997). Soluble sugars can act as osmotic-active solutes (Morgan 1984), increasing tolerance both to low temperatures and water stress. The rise in SS (leaf and root) and starch (only root) under *T* and *PhT* chambers indicates net storage and not translocation. The plants therefore experienced some physiological changes, and started the hardening process on the second harvest date under these ambient conditions when they had been exposed to temperatures below 8°C (h_8) for 288 hours. On the other hand, plants that experienced a reduction in the photoperiod (*Ph* treatment) did not reveal a net accumulation of non-structural carbohydrates (SS and starch). Although SS can be related to cold hardiness (Morin *et al*. 2007), each species may have a unique relationship of cold hardiness to SS. This could indicate that not only SS but other mechanisms (Larcher 2000), including lipids accumulation (Diamantoglou and Kull 1982) must be involved.

The ranking for cold hardiness in this experiment (*Q. ilex* > *Q. suber* ≥ *P. lentiscus* ≥ *O. europaea*) agrees with the results reported by other authors (Larcher 1981; Terradas 1999; Morin *et al*. 2007; Varone and Gratani 2007). The distribution of the four species in the Mediterranean basin, and mainly the altitudinal threshold they can reach, could explain these differences between species in frost tolerance. The four species can live together but according to Ruiz de la Torre (2006), *Q. ilex* principally lives between 200 and 1200 m altitude but can be found from 0 to 2000 m in South Spain, *Q. suber* can reach up to 1300 m but usually inhabit from 0 to 1000 m, and the other two species live in valleys and lowlands like *Q. suber*. Moreover, this matches with the distributions of the four species in Spain: *Q. ilex* usually spreads on forest sites with mean minimum temperatures of the coldest month between -4 and 10°C, *Q. suber* between -1 and 10°C, and *P. lentiscus* and *O. europaea* between 4 and 10°C (Rivas-Martínez 1987).

Chlorophyll fluorescence, a good indicator of photosystem II efficiency, has been used to estimate long-term responses to cold conditions and water stress, and has been used as an indicator of hardening and dormancy (Agati *et al*. 1996; Binder *et al*. 1997). Nevertheless, dormancy and stress hardiness are sometimes acquired and lost differently, which suggests that they are independent physiological processes (Hawkins and Shewan 2000). In the present study, Fv/Fm values revealed conclusive results after freezing. A sudden increase in shoot damage was noted for Fv/Fm values lower than 0.71, similar to the results of other authors working with Mediterranean species (Fernández *et al*. 2003; Aranda *et al*. 2005). Between 0.71 and 0.80, we found an unpredictable transition zone, and Fv/Fm values above 0.80 indicated little or no damage. The latter is related to the

discrimination capacity of Fv/Fm measurements to estimate the frost damage of seedlings (Fernández *et al*. 2003; González-Rodríguez *et al*. 2005).

Reducing the photoperiod from 12 to 8 hours did not stop H_{GR}, especially in *P. lentiscus* and *O. europaea*, but reducing the temperature from 22/17°C to 8/3°C had a substantial effect on height growth. A continuous increase in diameter, despite the reduction in temperature or photoperiod, is not surprising given that the temperature in the growth chamber never fell below 3°C. This reveals that, during cold periods, there can be cambial activity, even when apical meristem activity has stopped, and that cambial activity may not be dependent on daylength (Larcher 2003). In commercial nurseries, however, minimum winter temperatures usually drop below 0°C, which substantially reduces diameter growth (Fernández *et al*. 2005). The internal changes caused by modifications in the photoperiodic and temperature regimes are complex, and species-dependent (Nitsch 1957; Waisel and Fahn 1965). Moreover, the tendency to alternate between activity and rest ("growth in phases") of these evergreen species (Larcher 2003) make difficult to get solid conclusions about the relationship between shoot growth and cold hardiness in a 91-day experiment, because they could be influenced by the internal control of the growth.

Within each species, the values of TDW_{GR} during the experiment ran in parallel with H_{GR}, D_{GR} or both together. The SRDW ratio provides information on the balance between water transpiring and absorbing organs. This is very important during the post-plantation period, where nursery target values are ≤ 1.0 for oaks and ≤ 4.0 for wild olive tree for planting (Navarro *et al*. 1998). There are no standards for SRDW for lentisk tree. Because of the results obtained for H_{GR}, D_{GR} and $SRDW_{VR}$ during the hardening period under treatment *Ph*, it is not suitable for cold hardiness to reduce the photoperiod as a standard nursery practice unless this is accompanied by a reduction in temperature. Alternatively, it would be possible to reduce the temperature while maintaining longer photoperiods than those existing in normal environmental conditions. The limited response to reduced photoperiod differentiates these species from the reaction of those from higher latitudes and/or altitudes (Taulavuori *et al*. 1997; Colombo *et al*. 2003; Jensen and Deans 2004). Therefore, it can be inferred that the cold hardiness and the accumulation of non-structural carbohydrates induced by low temperatures could be related to a decrease in the apical meristem activity, but not in D_{GR} or TDW_{GR}, at least in the range of temperatures of this experiment. The two species that can inhabit colder sites, *Q. ilex* and *Q. suber*, were more dependent on photoperiod for height growth than *P. lentiscus* and *O. europaea*.

Leaf K concentration did not increase significantly, either as a result of lower temperatures or a shorter photoperiod, as can occur with other species of Mediterranean conifers (Fernández *et al*. 2003). N and P concentrations did not vary significantly during the experiment too. In the case of root K concentration, differences between treatments were too small to have any physiological relevance.

It can be concluded that moderate chilling (8-7°C, night temperature) marked the inflexion point for hardening induction in these four Mediterranean evergreen species in this experiment. Seedlings should not be lifted from the nursery until they have been subjected to 260-420 h_8, depending on the species. The maximum level of cold hardiness could be reached after about 775-800 h_8 in *Q. suber* and *Q. ilex*, 750 h_8 in *O. europaea* and 725 h_8 in *P. lentiscus*. Fv/Fm measurements, coupled with a freezing test, could be useful, quick and cheap methodology to evaluate plant cold hardiness. Nursery managers can use these chilling requirements to know where to locate the nurseries (e.g. coastal/inland, highlands/lowlands), where to cultivate the seedlings (e.g. outdoors/glasshouses), and when the plants can be outplanted without any risk of frost damage ("out-planting window of the nursery").

ACKNOWLEDGEMENTS

A substantial proportion of this research was supported by MCYT (I+D project AGL2002-02757). We would also like to thank the Doñana National Park and Viveros Poncelajara for the support that they have given in the collection of the seeds and seedling cultivation. Finally, we would like to thank Mrs Esther Martínez-Fernández for her help in proof-reading the text of this document.

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