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Original article

Vulnerability to freeze stress of seedlings of *Quercus ilex* L.: an ecological interpretation

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Abstract – The vulnerability to freeze stress of seedlings of *Quercus ilex* L. was studied with the aim of defining the limits of the potential distribution area of this species in its northernmost habitat. In December 1996 seedlings were freeze stressed up to –8 °C for 3 d. Frost caused extensive functional damage to seedlings in terms of: a) leaf water status; b) root (K_r) and stem (K_s) hydraulic conductance; c) tissue disorder in the root (only nine seedlings out of 50 survived). In comparison with unstressed seedlings, K_r and K_s of freeze-stressed seedlings were reduced by 90 %. Root anatomy of freeze-stressed seedlings revealed that: a) cortex cells were dehydrated and had become separated from one another; b) the endodermis was oversuberized, thus isolating the stele from the cortex. Our conclusion was that *Q. ilex* is extremely vulnerable to freeze stress so that the distribution area of the species is restricted to zones with no frost events. (© Inra/Elsevier, Paris)

***Quercus ilex* L. / freeze stress / root and stem hydraulic conductance / water relations**

Résumé – La vulnérabilité au stress par congélation des semis de *Quercus ilex* L. : une interprétation écologique. La vulnérabilité au stress par congélation des semis de *Quercus ilex* L. a été étudiée avec l'objectif de définir les limites de l'extension géographique potentielle de cette espéce dans son habitat le plus septentrional. En décembre 1996 des semis ont subi le stress par congélation jusqu'à –8°C pour 3 j (*figure 1*). La gelée a provoqué des dommages remarquables aux plantes pour ce qui concerne : a) la condition hydrique des feuilles (*figure 3*) ; b) la conductivité hydraulique de la racine (K_r) et du fuste (K_s) (*figure 6*) ; c) un désordre du tissu dans le racine (*figure 2*) (seulement 9 plantes sur 50 ont survécu). En comparaison avec des plantes non stressées le K_r et le K_s de plantes stressées par congélation avaient été reduits du 90 % (*figure 7*). L'anatomie de la racine des plantes stressées a révélé que : a) les cellules du « cortex » avaient été déshydratées et écartées les unes des autres ; b) l'endoderme avait été excessivement liègifié isolant le stèle du « cortex ». Notre conclusion était donc que *Q. ilex* est tellement vulnérable au stress par congélation que l'aire de distribution de l'espèce est limitée à des zones qui ne sont pas touchées par les gelées. (© Inra/Elsevier, Paris)

***Quercus ilex* L. / stress par congélation / conductivité hydraulique de la racine et du fût / condition hydrique**

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1. INTRODUCTION

Plants exposed to freezing stress are subjected to dehydration as well as to mechanical damage [1, 15] due to ice forming in the extra/intracellular compartment [32]. A primary effect of freezing stress is xylem embolism [4, 5, 28, 33] caused by gaseous bubbles escaping from xylem sap during freezing [26] and expanding during subsequent thaw [24], thus pushing water out of xylem conduits and leaving them embolized.

In this regard, drought and freezing stress induce similar strains (xylem cavitation and embolism) although the onset of cavitation is different in the two cases [10]. Therefore, some morphological as well as functional features of plants that are related to drought resistance (e.g. low vulnerability to xylem cavitation, solute accumulation) might be also related to freezing resistance [3, 8, 20].

Mediterranean sclerophylls have been defined as a life form adapted to two distinct environmental stresses, i.e. summer drought and winter cold stress [13, 14], one of these stresses being better resisted than the other [7, 10, 19], depending on previous plant acclimation and adaptation. As a consequence, the typical distribution area of Mediterranean sclerophylls might be determined by their specific vulnerability to drought and/or cold stress.

A typical species in this regard is *Quercus ilex* L. (Holm oak) growing throughout the Mediterranean Basin at elevations which are higher at lower latitudes. As an example, *Q. ilex* grows in Sicily at an elevation of between 700 and 1 200 m [17] while in Venezia Giulia (northeastern Italy) this species grows at sea level. In both these Italian regions, *Q. ilex* may be exposed to drought stress due either to rainfall paucity in the summer (Sicily) or to the rather low water retention capacity of the Karstic soils in Venezia Giulia [27]. In the winter, minimum temperatures of –2 to –4 °C are recorded in both cases and in severe winters even up to –10 °C (in the northernmost distribution areas of the species).

A previous study [10] had provided evidence that Sicilian ecotypes of *Q. ilex* were sensitive both to summer drought and to winter cold stress. In fact, when plants were exposed to air temperatures of –2.5 °C for 3 h, a loss of hydraulic conductivity (PLC) of about 50 % was recorded in 1-year-old twigs of young *Q. ilex* plants, which was only partly recovered (PLC = 35 %) 24 h after the temperature had risen above 0 °C. Similar PLCs were recorded in *Q. ilex* plants deprived of water supply until their leaf water potential (Ψ_l) reached the turgor loss point (Ψ_{tlp}). Twenty-four hours after one irrigation corresponding to a rainfall of about 4 mm (a likely summer rainfall in Sicily), PLC was still about 30 %, thus suggesting that most of the damage to the vertical water conduction persisted. This was interpreted as a good explanation for the critical altitudinal borders of the distribution area of *Q. ilex* in Sicily (see above).

To the best of our knowledge, only a few studies exist in the literature on freeze resistance of *Q. ilex* roots. Larcher [6] reported that temperatures of –7 °C caused 50 % injury to root cambium and xylem, but it is not clear whether this lethal temperature referred to soil or air temperature.

Seasonal measurements of root hydraulic conductance (K_r) of *Q. ilex* seedlings (data not shown) indicated that a physiological decrease in this parameter occurs between November and January coinciding with the winter low temperatures. In the course of this study, an unusual frost was recorded in northeastern Italy at the end of December 1996. Air temperatures ranging, as usual, between +5 and +10 °C (*figure 1*), fell rapidly below 0 °C and reached values as low as

–8 °C which were maintained for 3 d with maximum temperatures of –6 °C.

At the end of February 1997, *Q. ilex* seedlings growing in the open were seen to be still alive although with some visible damage to their leaves, but most of them died in the spring.

The present study reports hydraulic measurements on roots and shoots as well as water relation parameters of leaves and root anatomy describing structural and functional damage suffered by *Q. ilex* seedlings exposed to rapidly developing freeze stress, with the aim of identifying some possible mechanisms of freeze resistance and providing an explanation for the typical distribution area of this species in its northernmost habitat.

2. MATERIALS AND METHODS

Experiments were conducted on 2-year-old potted seedlings of *Q. ilex* with total leaf surface area (A_l), height (h) and trunk diameter (Φ_T) reported in *table I*. Pots were conical in shape with a top diameter of 90 mm and height of 180 mm. All the seedlings studied had been grown in pots since seed germination in the Botanical Garden of University of Trieste (northeastern Italy) at about 100 m elevation. Seedlings were well irrigated with about 100 g water supplied every 2 d.

Two groups of 50 seedlings each were studied. One group of seedlings was located in the open so that it had been exposed to freeze stress (*figure 1*) while the other was grown in a greenhouse under natural light and at a temperature adjusted to range between +8 and +13 °C until the end of February. After this date, the temperature in the greenhouse was no longer controlled so that it varied between +12 and +22 °C. All the measurements were completed between the end of February and the end of April 1997.

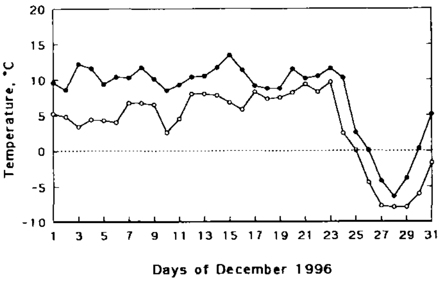

Figure 1. Maximum (solid circles) and minimum (open circles) air temperatures recorded in December 1996 in Trieste. Courtesy of Professor F. Stravisi, Institute of Oceanography, University of Trieste.

About 60 d after the frost event, the seedlings grown in the open showed no sprouting and some necrotic spots on their leaves.

2.1. Anatomical measurements

At least six roots from different freeze-stressed and unstressed seedlings were isolated from the soil under a gentle jet of water. Distal root segments about 6 mm long were excised and fixed with 4 % glutaraldehyde buffered at pH 7.8. Post-fixation of material with osmium

Table I. Height (h) above the soil of the *Quercus ilex* seedlings under study; Φ_T is the trunk diameter measured 4 mm above the soil; A_l is the average leaf surface area of the seedlings and A_r is the average surface area of all the roots with diameter within 2 mm as measured in the single seedlings. All values are given ± S.D. of the mean.

h ($m \times 10^{-2}$)	$\varnothing_T$ (mm)	A_l ($m^2 \times 10^{-3}$)	A_r ($m^2 \times 10^{-3}$)
17.0 ± 3.1	3.55 ± 1.14	8.37 ± 3.29	9.51 ± 5.68

tetroxide (1 % OsO_4) buffered at pH 7.8 was followed by repeated washing in distilled water. After dehydration in acetone, roots were first infiltrated with and then embedded in Spurr resin [25] and put into oven at 80 °C for 24 h for completing resin polymerization.

Cross sections 2–3 mm thick were cut using a microtome (LKB mod Ultrotome III) equipped with a diamond knife. They were stained with 0.1 % toluidine blue and observed under a light microscope. Ultrathin sections (0.7 mm thick) were also prepared for observation under electron microscope (Philips, EM 201).

Hand-cut cross sections of 1-year-old stems were also prepared and observed as fresh samples under light microscope.

2.2. Field measurements

To estimate the extent of the damage suffered by freeze-stressed seedlings, the diurnal time course of leaf conductance to water vapour (g_l), relative water content (RWC) and water potential (Ψ_l) were measured every 120 min between 0800 and 1800 hours. All these parameters were measured on eight leaves from four different seedlings growing both in the open and in the greenhouse.

The parameter g_l was measured on leaves still attached to the plant using a steady-state porometer (LiCor mod 1600). Each measurement was completed within about 30 s and the air relative humidity (r.h.) inside the chamber was kept near the ambient to reproduce external conditions. Ambient temperature and r.h. were also recorded at about 1 m from the leaves using a digital thermo-hygrometer (accuracy ± 1 °C and ± 1 %, respectively).

Ψ_l was measured using a pressure chamber [22] with a sheet of wet filter paper inside the chamber to minimize water loss during the measurements.

RWC was calculated by weighing leaves on a digital balance to obtain their fresh weight (FW). After Ψ_l recordings, leaves were resaturated with water to full turgor by immersing their petioles in water, covering the leaf blade with plastic film and leaving them in the dark overnight. Ψ_l was remeasured to check that it was higher than –0.05 MPa with no leaf oversaturation. Leaves were then reweighed to obtain their turgid weight (TW) and put into oven at 70 °C for 3 d to obtain their dry weight (DW). RWC was calculated as: RWC = (FW – DW/TW – DW) × 100.

2.3. Leaf water potential isotherms

In order to estimate the water status of leaves, five pressure–volume curves (P–V curves [21, 29]) were measured for both freeze-stressed and unstressed seedlings. This allowed the comparison of the leaf water potential at the turgor loss point (Ψ_{tlp}) as derived from P–V curves to Ψ_l as measured in the field. Also the osmotic potential at full turgor (π_0) was calculated so as to obtain information on the eventual solute accumulation in the leaves in response to freeze stress.

From the P–V curves, it was also possible to calculate the leaf apoplastic water fraction (W_A) as: W_A = (TW – DW) – W_0/ TW – DW where W_0 was the leaf symplastic water content at full turgor (corresponding to the x-axis intercept of the curve relating $1/P_B$ to W_e, where P_B is the chamber pressure and W_e is the weight of the water expressed from the leaf). Eventual changes in W_A measured in stressed seedlings would have suggested that cell rupture (increase in W_A) or xylem cavitation in the leaf veins (decrease in W_A) had taken place.

2.4. Hydraulic conductance of roots (K_r) and shoots (K_s)

Root hydraulic conductance (K_r) of five seedlings grown in the greenhouse (control seedlings) was measured using both the pressure chamber [2, 16, 23] and the high pressure flow meter (HPFM) recently described by Tyree et al. [30, 31].

In the case of the pressure chamber technique, seedlings were inserted into a pressure chamber larger than the standard model (internal diameter 120 mm, depth 210 mm). Plants were detopped at 40 mm above the soil and the flow (F) was measured at the trunk cut surface at different constant pressures. The pressure in the chamber was increased at a rate of 0.14 MPa min^{-1} up to 0.69 MPa. This pressure level was maintained constant for 40 min. During the first 10 min internal pressures were allowed to equilibrate, then F was measured every 2 min for 30 min by putting plastic capsules filled with sponge in contact with the stem cut surface and weighing them on a digital balance. The pressure was then decreased at a rate of 0.07 MPa min^{-1} and three decreasing pressure levels were applied, i.e. 0.52, 0.34 and 0.17 MPa. At each of the above pressures, F was measured using the same procedure as described above. At constant pressure, F was approximately stable (SD = ± 7 to 8 % of the mean), so measurements were quasi-steady state.

The measured F was plotted versus the applied pressure (P) and K_r was calculated from the slope of the straight line relating F to P.

The HPFM technique was used in the transient mode. The HPFM as described by Tyree et al. [31] and in a slightly changed version by Magnani et al. [11], consists of an apparatus allowing us to perfuse water into the base of a root system or a shoot while rapidly changing the applied pressure and simultaneously measuring the corresponding flow (transient mode). This procedure allows quite rapid measurements of F and P (of the order of seconds). Conductance of roots or stems was then measured from the slope of the linear regression of F to P.

After cleaning the pot's surface under a water stream, the pots were enclosed in plastic bags fitted tightly to the stem and immersed in water so that the stem could be excised under water at about 40 mm above the soil, thus preventing xylem embolization.

The pressure applied was increased continually from 0.03 to 0.42 MPa within 90 s. The HPFM was equipped to record F and the corresponding P every 3 s. From the slope of the linear region of the relation of F to P it was possible to calculate K_r.

During K_r measurements, the cut leafy stem remained in contact with water while enclosed in plastic film to prevent evaporation. The base of the stem was connected to the HPFM and perfused with distilled water filtered to 0.1 mm at a pressure of 0.3 MPa so as to allow leaves to reach full hydration. The pressure was then reduced to 0.03 MPa and maintained constant for 10 min. Three F measurements were performed in the transient mode, i.e. during continuous P changes. From the slope of the linear relation of F to P, the stem hydraulic conductance (K_s) was computed by linear regression of the data.

A spurious component of K measurements when using the HPFM might be that due to the expansion of the elastic parts of the instrument such as tubing or connections [31]. Therefore, additional transient measurements of F and P were performed with the connection to the samples closed with a solid plug. A linear relation of F to P with a minimal slope due to the intrinsic elasticity of the instrument was obtained which was subtracted from the slope of the straight line relating F to P as measured with the root system or the shoot connected to the HPFM.

After each experiment, total leaf surface area (A_l, one side only) of seedlings was measured using a leaf area meter (LiCor mod 3000-A).

The total root surface area (A_r) of the seedlings was estimated as follows: the soil was carefully removed from the root system under a gentle jet of water. The root system was then excised into segments with diameters within 2 mm and up to 50 mm in length. They were put into a glass box and covered with a white plastic sheet to keep them in a fixed position and obtain a more contrasted image of the roots. The box was placed on a scanner (Epson mod GT-9000) connected to a computer. A specialized software could read bit-map images and calculate the surface area of the roots. Root images were processed by the software and root surface area was obtained by multiplying the calculated area by π, assuming the root segments to be cylindrical in shape which is basically correct for short root segments.

K_r and K_s were both normalized by dividing them by A_l.K_r was also divided by A_r

Freeze-stressed seedlings were measured for K_r only using the HPFM because the resistance to flow of their roots in the basipetal direction was so high that it was not possible to use the pressure chamber in that pressures up to 1.38 MPa were unable to drive a measurable flow.

3. RESULTS

3.1. Root anatomy of freeze-stressed seedlings

Three increasing levels of damage to root cortex parenchyma were identified:

1) cortex dehydration as indicated by shrinkage of cells with sinuous walls (*figure 2b*); 2) cell 'unsticking', i.e. cells no longer connected to the neighbouring ones so that the cortex appeared as quite spongy (*figure 2c*); 3) more pronounced cell shrinkage with reduction in cortex thickness and multilayer endodermis (*figure 2d*). In case 3), TEM observations showed that many cortex cells were dead. In all the roots of freeze-stressed seedlings the endodermis showed no cells with permeable tangential walls as usually found externally to the root xylem bundles whenever endodermal cells are completely suberized.

Stems of stressed seedlings showed no visible mechanical damage to living cells but numerous xylem conduits appeared filled with solid particles of unknown nature, probably deriving on conduit wall degradation.

3.2. Field measurements

Between March and April 1997, when g_l, RWC and Ψ_l were measured, air temperatures were somewhat higher than usual and ranged between +8 and +16 °C in the field and between +12 and +22 °C in the greenhouse. At the same time, r.h. was only between 27 and 40 % in the field and somewhat higher in the greenhouse (30–52 %).

In comparison with leaves of unstressed seedlings with RWC around 95 % and Ψ_l higher than –1 MPa (*figure 3*, solid cir-

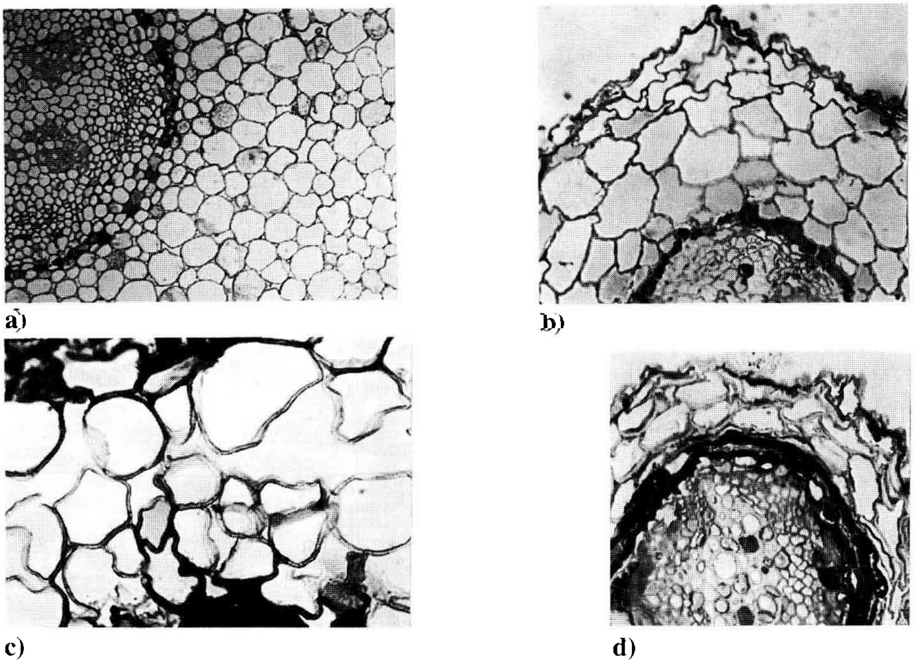

Figure 2. Cross sections of primary roots of *Quercus ilex* seedlings. a) Roots from unstressed seedlings: note the normal cortex cells and the unilayered endodermis; b) root cortex with shrunken cells; c) cortex cells unsticked from one another; d) more pronounced cell shrinkage with dead cortex cells and multilayer oversuberized endodermis.

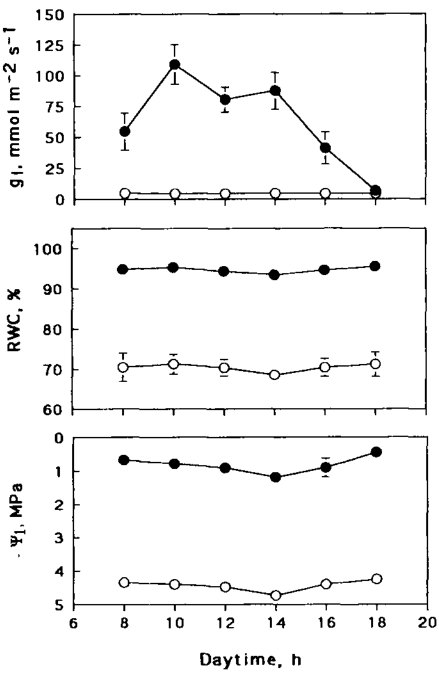

Figure 3. Diurnal time course of leaf conductance to water vapour (g_l), relative water content (RWC) and water potential (Ψ_l) measured in March 1997 in unstressed (solid circles) and freeze-stressed (open circles) seedlings. Vertical bars are S.D. of the mean (n = 8) unless eclipsed by the symbol.

cles), the leaves of freeze-stressed seedlings were dehydrated in that their RWC was only about 70 % and Ψ_l between –4.3 and –4.8 MPa, i.e. well below their turgor loss point (Ψ_{tlp} was –2.85 MPa, *table II*).

Accordingly, g_l was at merely cuticular values (g_l was about 7 mmol $s^{-1}m^{-2}$ versus over 100 mmol s^{-1} m^{-2} as recorded in unstressed seedlings at 1000 hours).

The leaf apoplastic water fraction (W_A, *table II*) was significantly lower in freeze-stressed seedlings than in unstressed ones (0.44 versus 0.65, respectively), i.e. W_A was reduced by one third. This suggests that freeze stress might have caused xylem embolism in the leaf veins or in the mechanic tissues surrounding the vascular bundles [24].

The more negative leaf osmotic potential at full turgor (π_0, *table II*) as measured in stressed seedlings with respect to control ones (–2.31 versus –2.07 MPa, respectively with a reduction of about 10 %), was probably too little to represent an osmoregulatory response to freezing stress.

3.3. Hydraulic conductance of roots and shoots

The relation of F to P measured in the root system of unstressed seedlings using the pressure chamber (*figure 4*, solid squares) was linear, at least at applied pressures between 0.17 and 0.72 MPa.

The HPFM allowed measurement of F at lower P values. Up to applied pressures of about 0.2 MPa, the relation of F to P was non-linear (*figure 4*, solid circles). Beyond this P value, F increased with P linearly with a good correlation coefficient (r^2 = 0.996). The intercept with the

Table II. Leaf water potential at the turgor loss point (Ψ_{tlp}), osmotic potential at full turgor (π_0) both derived on the leaf water potential isotherms. W_A is the leaf apoplastic water fraction. All values are given ± S.D. of the mean.

	$-\Psi_{tlp}$ (MPa)	$-\Pi_0$ (MPa)	W_A
Unstressed	2.85 ± 0.34	2.07 ± 0.07	0.65 ± 0.11
Freeze stressed	2.76 ± 0.04	2.31 ± 0.12	0.44 ± 0.01

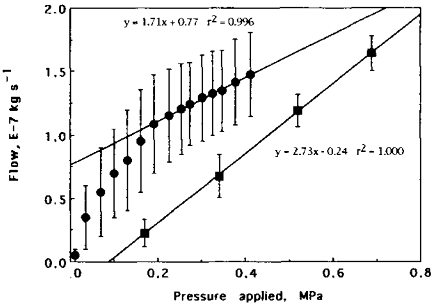

Figure 4. Water flow rate versus applied pressure recorded in roots of unstressed seedlings, using the pressure chamber (squares) and the high pressure flow meter (circles). Vertical bars are S.D. of the mean ($n = 5$).

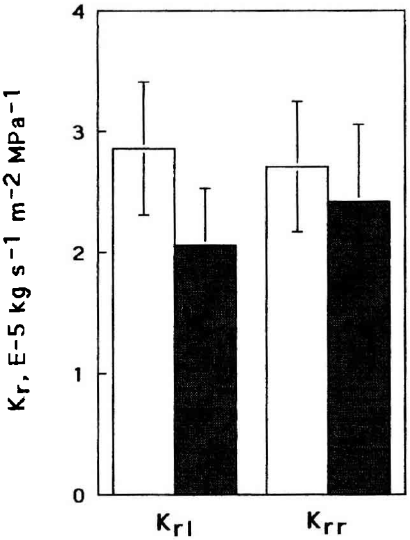

Figure 5. Root hydraulic conductance (K_r) normalized for the leaf (K_{rl}) and root (K_{rr}) unit surface area. K_{rl} and K_{rr} were measured using the high pressure flow meter (white columns) and the pressure chamber (dark columns). Vertical bars are S.D. of the mean ($n = 5$).

y-axis of the linear region of the relation of F to P was as far from the y-axis origin as at about 0.75×10^{-7} kg s^{-1}.

The slopes of the linear regression of F to P as recorded in the root systems of unstressed seedlings using the pressure chamber (in the quasi-steady-state mode) and the HPFM (in the transient mode) allowed computation of their respective K_r.

The seedlings of *Q. ilex* under study were fairly homogeneous in their dimensions (height and trunk diameter, *table I*) but leaf and root surface areas were rather different in different seedlings as indicated by the SDs of the means of A_l and A_r which were 40 and 60 % of the mean, respectively (*table I*). Therefore, it was decided to normalize K_r data by dividing them by total leaf (A_l) and root (A_r) surface areas, thus obtaining K_{rl} and K_{rr}, i.e. K_r referred to the A_l or the A_r unit surface area (*figure 5*).

It can be noted that K_{rl} and K_{rr} were quite similar to each other, irrespective of the instrument (pressure chamber or HPFM) and the mode of measurement (quasi-steady-state or transient mode) used. In other words, the two techniques yielded similar results and K_r ranged between 2.5 and 3.5×10^{-5} kg s^{-1} m^{-2} MPa^{-1} if referred to the A_l unit surface area and between 2.0 and 2.5 kg s^{-1} m^{-2} MPa^{-1} if referred to the A_r unit surface area.

Since it was not possible to compare the two methods in stressed seedlings unless applying very high air pressures in the pressure chamber, the comparison of K_r between freeze-stressed and unstressed seedlings reported in *figure 6* refers to measurements performed using only the HPFM. Here, the relations of F to P are reported, as recorded in roots and shoots of unstressed seedlings (solid circles and squares, respectively) and of freeze-stressed ones (open circles and squares, respectively). It can be noted that: 1) K_r and K_s were not significantly different from each other both in control (solid sym-

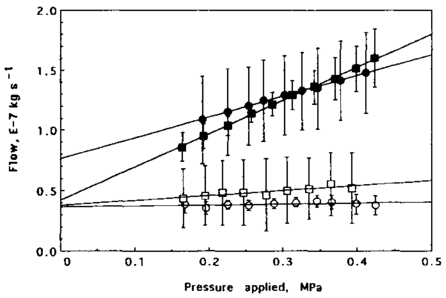

Figure 6. Water flow rate versus applied pressure as measured using the high pressure flow meter in shoots (squares) and roots (circles). Solid symbols refer to unstressed seedlings. Open symbols refer to freeze-stressed seedlings. Vertical bars are the S.D. of the mean ($n = 5$).

bols) and in stressed seedlings (open symbols). However, the slope of the linear regression of F to P as measured in roots and shoots of freeze-stressed seedlings was minimal. This suggests that both roots and stems of freeze-stressed seedlings had suffered extensive damage to their water conducting system.

When K_r and K_s were both normalized for A_l (*figure 7*), it appeared that K_{rl} and K_{sl} of freeze-stressed seedlings were only about 0.4×10^{-5} kg s^{-1} m^{-2} MPa^{-1} versus 3.0 to 3.5×10^{-5} kg s^{-1} m^{-2} MPa^{-1} as recorded in control seedlings. This means that the loss of hydraulic conductance suffered by roots and shoots of stressed seedlings was about 90 %.

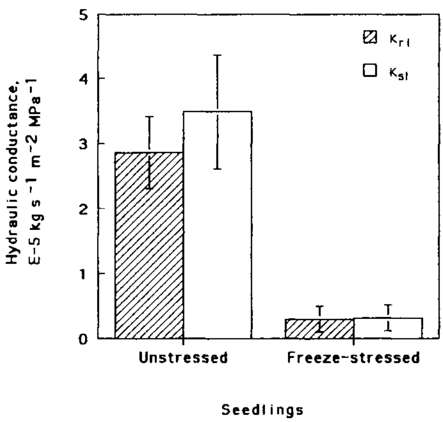

Figure 7. Hydraulic conductance of shoots (K_{sl}) and roots (K_{rl}) normalized for the leaf unit surface area, as measured in unstressed and freeze-stressed seedlings. Vertical bars are S.D. of the mean ($n = 5$).

4. DISCUSSION

The freeze stress suffered by seedlings of *Q. ilex* (*figure 1*) was extremely severe in two senses: 1) temperature dropped from +10 °C to below 0 °C in only 2 d without any previous acclimation of plants and 2) maximum.temperatures remained below 0 °C for 3 d (*figure 1*) which is rather an unusual occurrence in the coastal regions of northeastern Italy where the species grows.

Nonetheless, freeze-stressed seedlings appeared to be still alive 2 months after the frost event although leaf stomata remained closed for most of the time (*figure 3*). At the end of April 1997, however, only nine freeze-stressed seedlings out 50 were still surviving.

The major effect of freeze stress on *Q. ilex* seedlings was an extensive damage to their water conducting system, accompanied by tissue disorder in the roots.

A drop in leaf RWC of about 30 % as that measured in leaves of stressed seedlings (*figure 3*) is per se not so drastic and, in fact, many Mediterranean species undergo similar decreases in leaf RWC without any damage to plants (e.g. *Olea oleaster* [9]). The measured drop in W_A (from 0.65 to 0.44, *table II*), however, suggests that part of the leaf veins and/or mechanical tissues surrounding the vascular bundles in the leaves were embolized. If this was the case, leaves were no longer receiving water from the roots and stomata closed. Since *Q. ilex* has sclerophyllous leaves with thick cuticle and epidermal cells with thick walls, stomatal closure preserved leaves from further water loss and RWC remained relatively high (70 %).

The loss in the relative leaf symplasmic water content was probably of about one third (i.e. from 35 % in control leaves to about 25 % in those of stressed seedlings with RWC reduced to 70 %). The leaf water potential isotherms showed that a symplasmic water loss of this order of magnitude would cause a drop in Ψ_l to about –3.5 MPa. In our opinion, the much more negative Ψ_l (–4.5 MPa) as measured in leaves of stressed seedlings was probably underestimated because of the embolism of the minor veins which increased the resistance to flow within the leaf blade, thus requiring more pressure to drive a flow through the petiole cut surface.

Roots of freeze-stressed seedlings appeared to have suffered dehydration as well as mechanical damage (*figure 2*). Most cortex cells were apparently shrunk and in some cases 'unsticked' from one another. We advance the hypothesis that endodermal cells underwent oversuberization [12] with the likely effect of isolating the stele from the cortex, thus preventing hydraulic continuity between roots and soil so that in absence of new root primordia, roots will die.

If this was the case, the combined effects of root cortex dehydration and endodermis oversuberization, made measurements of K_r with the pressure chamber impossible, unless sufficiently high air pressures were applied to force a water flow through the air-filled cortex and the impermeable endodermis.

The two methods used for measuring K_r of control seedlings, i.e. the pressure chamber and the HPFM were shown to measure the same quantity in that they gave similar K_r values although the operational modes were different (measuring F at constant P or quasi-stready-state mode or measuring dynamic F while changing P or transient mode, respectively). In both cases, K_r (*figure 5*) was between 2 and 3×10^{-5} kg s^{-1} m^{-2} MPa^{-1} in the unstressed seedlings whether normalized for leaf (K_{rl}) or root (K_{rr}) unit surface area.

Initial non-linear relation of F to P has been interpreted by Tyree et al. [31] as due to elastic flow, i.e. to flow due to the elastic expansion of the entire system (HPFM plus plant). It has to be taken into account that the intrinsic elasticity of the HPFM components, i.e. F values at increasing P as measured with the flow outlet closed off (see above), had already been subtracted from the F values as recorded at the same P with the HPFM connected to the root system. Therefore, the y-axis intercept of the straight line relating F to P might be due to the elasticity of the root tissues. In particular, two explanations not necessarily alternative to each other can be advanced: 1) when roots are perfused with water under pressure in the apical direction, xylem tissues would tend to swell and/or native emboli would be compressed and/or dissolved. Once xylem conduits reach their maximum elastic expansion and are completely filled with water, the relation of F to P becomes linear; 2) if the root tissues had some water saturation deficit, F would increase with P following a saturation curve. Once all tissues are completely saturated (or completely infiltrated) with water, the relation of F to P becomes linear. In case 2) the y-axis intercept would represent the tissue water capacity. Taking into account that control seedlings were grown in a greenhouse and were well irrigated, we have no reason to suspect xylem cavitation to have occurred in their roots. In other words, we feel that the non-linear relation of F to P recorded up to applied pressures of 0.2 MPa should be the result of the elastic swelling of root vascular and non-vascular tissues. If this was the case, the intercept with the y-axis of the relation of F to P would equal (1/e) dP/dt where e is the modulus of elasticity of root tissues and dP/dt is the time derivative of P [31]. Although the interpretation of the y-axis intercept of the F to P relation has to be considered as only tentative, this could be an interesting starting point for further studies of changes in root tissue elasticity as related to adaptation to changes in soil water content.

When the relation of F to P was measured in freeze-stressed seedlings (*figure 6*), it appeared that the slopes of the linear regression of F to P were extremely low both in their roots and shoots which corresponded to K_r and K_s values of about 0.4×10^{-5} kg s^{-1} m^{-2} MPa^{-1} versus 3.0 to 3.5×10^{-5} kg s^{-1} m^{-2} MPa^{-1} as recorded in control seedlings, i.e. about seven-fold less. The intercepts with the y-axis of the linear regressions of F to P measured in shoots of freeze-stressed and unstressed seedlings were approximately coincident to one another (*figure 6*). Again, no xylem cavitation could be suspected in stems of control plants. Therefore, the coincidence of these intercepts was likely to be due to the bulk elasticity of stems and not to emboli persisting in the wood. The higher y-axis intercept measured in roots of control seedlings in comparison with that in stressed roots can be explained in terms of the high elasticity of the tissues of healthy roots. Stressed roots, on the contrary, were shown to be highly dehydrated with flaccid cell walls (*figure 2*). This had the likely effect of decreasing the overall elasticity of their tissues.

The total leaf (A_l) and root (A_r) surface areas of the seedlings were, on average, the same (*table I*) so that K_r and K_s, when normalized for A_l or A_r (K_{rl} and K_{rr}, *figure 7*) were not significantly different from each other. This is not surprising because seedlings growing in pots under equal environmental conditions are likely to produce similar amounts of biomass.

The severe freeze stress suffered by *Q. ilex* seedlings caused high mortality (about 82 %). The drop in the stem and root hydraulic conductances as measured 2 months after the frost event, might well be due to xylem cavitation. Nonetheless, it is likely that phenomena other than cavitation concurred to cause the observed high mortality. It is still unclear whether the HPFM technique allows the accurate measurement of embolism-induced K_r decrease. Air bubbles are likely to shrink under pressure, thus water filling cavitated conduits. In our opinion, the extremely high hydraulic resistance recorded in stems was probably due to solid particles plugging xylem conduits. In turn, tissue disorder in the root cortex strongly decreased root permeability to water.

The only possibility for seedlings to survive freeze stress was to produce new roots, thus re-establishing hydraulic continuity with the soil.

In conclusion, the high vulnerability of *Q. ilex* to freeze stress explains why this species is confined to the coastal ranges of northern Italy. Even when the species is found at somewhat higher elevations [7, 18], Holm oak communities are represented by shrub forms only growing on the south-facing slopes of mountains.

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