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Distribution of pines in the Iberian Peninsula agrees with species differences in foliage frost tolerance, not with vulnerability to freezing-induced xylem embolism

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Abstract

Drought and frosts are major determinants of plant functioning and distribution. Both stresses can cause xylem embolism and foliage damage. The objective of this study was to analyze if the distribution of six common pine species along latitudinal and altitudinal gradients in Europe is related to their interspecific differences in frost tolerance and to the physiological mechanisms underlying species-specific frost tolerance. We also evaluate if frost tolerance depends on plant water status. We studied survival to a range of freezing temperatures in two-year-old plants and assessed the percentage loss of hydraulic conductivity (PLC) due xylem embolism formation and foliage damage determined by needle electrolyte leakage (EL) after a single frost cycle to -15 °C and over a range of predawn water potential ($\psi_{pd}$) values. Species experiencing cold winters in their range (P. nigra, P. sylvestris and P. uncinata) had the highest frost survival rates and lowest needle EL and soluble sugar concentration (SS). In contrast, the pines inhabiting mild or cool winter locations (especially P. halepensis and P. pinea and, to a lower extent, P. pinaster) had the lowest frost survival and highest needle EL and SS values. Freezing-induced PLC was very low and differences among species were not related to frost damage. Reduction in $\psi_{pd}$ decreased leaf frost damage in P. pinea and P. sylvestris, increased it in P. uncinata and had a neutral effect on the rest of the species. This study demonstrates that freezing temperatures are a major environmental driver for pine distribution and suggests that interspecific differences in leaf frost sensitivity rather than vulnerability to freezing-induced embolism or SS explain pine juvenile frost survival.
Drought and frosts are major drivers of plant evolution and distribution (Woodward and Williams 1987, Pockman and Sperry 1997, Choat et al. 2012). Both stress factors affect important plant physiological processes (Sakai and Larcher 1987, Mayr et al. 2006). Particularly, water transport and gas exchange are usually reduced due to frost-induced embolism and drought-induced stomatal closure (Sperry and Sullivan 1992, Davis et al. 1999, Willson and Jackson 2006). These physiological responses reduce the productive capacity and hydration of plants, might damage the plant, and eventually cause their death (McCulloh et al. 2011, McDowell 2011, Peguero-Pina et al. 2011). Most comparative studies on frost tolerance use foliage electrolyte leakage or decrease in fluorescence activity of photosystems to assess tissue damage (Strand and Öquist 1988, Climent et al. 2009), while few studies report plant survival (Hawkins et al. 2003), which is key for plant fitness. However, while simple and fast, electrolyte leakage or fluorescence of photosystems do not inform on the capacity of individuals to remain alive after a stress event.

The mechanism by which drought and frosts cause xylem embolism is different (Zimmermann, 1983), but in both cases the amount of embolism is expected to increase with declining xylem water potential ($\psi$) (Davis et al. 1999, Mayr et al. 2003). Drought-induced embolism occurs when low $\psi$ causes the air contained in air filled cells to be sucked through the pits of water-filled xylem conduits, forming bubbles that block the conduit (Zimmermann, 1983). Frost-induced embolism occurs when the bubbles formed after thawing of frozen water inside the xylem conduits expand and block xylem conduits (Robson et al, 1988; Langan et al, 1997).

Frosts can also damage plants by causing the loss of cell membrane functional integrity (Uemura et al. 2006), which mainly occurs in the most exposed organs. Freezing of apoplast causes the water to move outside the cell producing, large changes in protoplasm
volume, which can damage the cell membrane and hinder metabolism (Wesley-Smith et al. 2015, Sakai and Larcher 1987). Soluble sugars (SS) play an important role in plant frost tolerance. SS increase the stability of cell membranes to frost damage (Uemura and Steponkus 2003), may contribute to embolism repair (Zwieniecki and Hollbrook, 2009) and reduce the protoplasm osmotic potential, which increases cell desiccation tolerance and reduces the freezing point (Hinesley et al. 1992). Finally, plants use stored SS to support winter metabolism and early spring growth (Uscola et al. 2015). Consequently, many plants from the cold and temperate biomes increase their SS concentration during frost acclimation in the fall to a maximum level in midwinter, which is then reduced in spring and summer with cold deacclimation (Sakai and Larcher 1987, Martínez-Vilalta et al. 2016).

Distribution of pine species native to Europe appears segregated along latitudinal and altitudinal gradients in a predictable manner suggesting the existence of environmental factors that differentially influence the species. Some species, namely *P. halepensis* Mill., *P. pinaster* Ait., and *P. pinea* L., are concentrated in southern of Europe, particularly at Mediterranean climate sites with mild to cool winters and hot and dry summers (Figure S1). Other species, such as *Pinus nigra* J.F. Arnold, *Pinus sylvestris* L. and *Pinus uncinata* Raymond ex A. DC., are located in cold winter sites, either at high latitude (in central and northern Europe) or at high altitude (southern Europe including the Iberian Peninsula, Figure S1) (Richardson, 1998). In these high mountain locations rainfall can be higher, evapotranspiration is lower and thus summer drought is shorter and of moderate intensity relative to lower altitude sites. Intra- and inter-specific variability to drought-induced embolism is low in pines (Lamy et al. 2014; Martínez-Vilalta and Piñol 2002; Martínez-Vilalta et al. 2004, Martínez-Vilalta et al. 2009), which make differences in drought-induced embolism an unlikely explanation for differences in species distribution. We hypothesize that this latitudinal and altitudinal distribution pattern of European pines could be driven by species differences in cold tolerance.
The Mediterranean climate has a dry season concentrated in the summer but occasionally winters can also be dry. High-altitude locations in the Mediterranean basin such as the plateaus and mountain ranges of the Iberian Peninsula have cold winters. The combination of cold temperatures in winter and summer drought is an important determinant for plant life in these environments (Mitrakos, 1980). Besides, in dry winters years, plants at Mediterranean cold-winter sites can suffer drought stress (Peguero-Pina et al. 2011), which can promote the negative effects of frost-induced embolism (Willson and Jackson, 2006). This phenomenon has been related to pine die-back in high elevations in the Mediterranean mountains and boreal forests (Kullman 1991, Peguero-Pina et al. 2011). At the same time, drought stress can also increase frost tolerance in some plant organs (Medeiros and Pockman 2011, Villar-Salvador et al. 2013, Sperling et al. 2017). It is possible that drought-induced physiological acclimation responses such as changes in SS concentration, cell membrane stability or the reduction of cell osmotic potential (Serrano et al. 2005, Beck et al. 2007, Villar-Salvador et al. 2013) may also enhance cold tolerance. Numerous studies have addressed the effect of drought and frost independently on plant physiological performance (see Beck et al. 2004, Dobbertin et al. 2005), but relatively few studies have analyzed experimentally how drought affects frost tolerance of plants (Grossnickle et al. 1991, Medeiros and Pockman 2011).

The general objective of this study is to assess whether interspecific differences in frost tolerance are related to the distribution of the six-pine species native to the Iberian Peninsula and the physiological mechanisms underlying species differences in frost tolerance. These pine species (P. halepensis, P. pinea, P. pinaster, P. nigra, P. sylvestris and P. uncinata) are also common throughout Europe. Specifically, we asked: 1) Do pine species inhabiting cold sites have higher inherent cold tolerance than pines inhabiting mild or cool winter locations? 2) Are differences in frost tolerance explained by differences in frost-
induced embolism, needle frost tolerance or tissue SS? 3) What is the effect of drought stress on frost tolerance? To answer these questions, we conducted an experiment in which the frost survival, and the interaction between drought and frost on xylem embolism and needle damage was assessed in two-year-old plants. We used juveniles as early life stages are important bottlenecks in pine population dynamics (Castro 1999, Herrero et al. 2013).

Methods

1. Plant material.

Seeds of pine species were collected in populations of the Iberian Range (Eastern Spain), except for *P. uncinata*, whose seeds were collected in the Pyrenees (Table 1). Seeding was done in 2012 on different dates to synchronize seedling emergence. *Pinus halepensis* was seeded on January 30th, while *P. pinaster* and *P. pinea* were seeded 7 and 11 days later, respectively than *P. halepensis*. *Pinus nigra* subsp. *salzmannii* (hereafter *P. nigra*) and *P. uncinata* were seeded on February 15th while *P. sylvestris* was seeded on February 20th. Seedlings were cultivated in Plasnor trays (190/300-45, Plasnor, Spain) with 45 cavities of 300 ml. Growing medium was fertilized peat (White 420 F6 Kekkilä, Finland; pH 4.7, containing 0.8-1 kg/m³ of a slow release fertilizer NPK 16-10-20). Seedlings were initially grown in a greenhouse of the Centro Nacional de Recursos Genéticos Forestales “El Serranillo” (Guadalajara, Spain) to avoid frost damage. In mid May 2012, seedlings were moved outdoors and were kept well-watered, periodically fertilized and exposed to the natural seasonal changes in temperature, radiation and day length until January 2014.

2. Testing differences in seedling frost survival

Frost tolerance in the experiment was assessed by seedling survival, xylem embolism and needle damage. Survival was assessed after freezing batches of seedlings at the following
target temperatures: -5, -8, -12, -16, -20, -25 and -30 ºC. For each temperature, we used 10 seedlings per species, which were transplanted into styrofoam containers to isolate the roots. Plants were placed in a programmable freezing chamber (Dycometal, CCK, Spain) at the INIA (Madrid, Spain), and subjected to one frost cycle. Temperature was reduced from 5 ºC to 0 ºC in 1 h. Then, the temperature decreased at a ~3 ºC h\(^{-1}\) rate to the target temperature, which was maintained for 3 h. Finally, temperature was increased at a rate of 5 ºC h\(^{-1}\) up to 5 ºC, where temperature was maintained for 1 h. Then, seedlings were moved to a greenhouse and were well-watered to favor their recovery. After 2 months, we verified seedling status and we considered a seedling to be dead when it had not resumed growth and >95% of needles where brown.

3. Foliage electrolyte leakage and xylem embolism measurements

In early March 2014, we performed an experiment in which seedlings of all species were randomly distributed into four treatments: 1) Frost and well irrigated, 2) Frost with restricted watering, 3) Unfrozen and well irrigated and 4) Unfrozen with restricted watering. We used 14-18 seedlings per species and treatment. The sizes of shoot fractions of the seedlings used in the experiment are presented in Table 2. Restricted watering was achieved by stopping watering 7-10 days before physiological measurements. The remaining seedlings were irrigated every 2-3 days to keep them well-watered. For the frost treatments, seedlings were subjected to a single -15 ºC frost cycle using a programmable freezing chamber (A.S.L. Aralab International\textsuperscript{®}-CON-550-20, Madrid, Spain), using the same protocol explained in the previous section. Before frost exposure, we measured seedling predawn water potential ($\psi_{pd}$) using a Scholander Pressure Chamber (SKPM 1400, Skye Instruments, Llandrindod Wells, UK). In most cases, the $\psi_{pd}$ was measured on lateral twigs but in a few cases, we used needles attached to brachiblasts. Seedlings were covered with opaque plastic bags overnight to reduce
seedling transpiration before $\psi_{pd}$ measurements. $\psi_{pd}$ ranged between -0.23 to -1.68 MPa in P. nigra, P. sylvestris and P. uncinata, and -0.16 to -2.0 MPa in P. halepensis, P. pinaster and P. pinea.

We measured damage in secondary needles and xylem embolism after the -15 ºC frost to assess the importance of the interaction of frost and drought on species physiological performance. Needle frost damage was assessed by electrolyte leakage (EL) (Earnshaw 1993), while xylem frost-induced embolism was measured as the percentage loss in stem hydraulic conductivity (PLC) (Charrier et al. 2013). For needle EL measurements, secondary needles were cut in small pieces (fresh weight ~0.30 g) after the -15 ºC treatment, and washed twice in distilled water for 20 min and left in a vial with 20 ml of deionized water that was gently shaken (Orbital shaker, Selecta, Spain) on an illuminated bench under laboratory temperature (20-22 ºC). After 24 h, the electrical conductivity ($C_i$) of the water bathing the needles was measured with an electro conductivity meter (Crison® CM 35+, Spain). Then, the needles were autoclaved at 120 kg cm$^2$ for 10 min. After cooling the samples at room temperature, electrical conductivity was remeasured ($C_f$). EL was calculated as:

$EL = \left( \frac{C_i}{C_f} \times 100 \right)$

(Equation 1).

PLC was measured in stem segments 3 cm long excised from the lower part of the stem (immediately above the cotyledon insertion point). Working under water to prevent air entry into the tracheids, we cut the stem segments, removed the bark in the 3-4 mm extremes of the segments and then recut the stems to remove resin remains with a fresh razor blade under water. Water flow through the stem segments was measured using the Xyl’em Plus apparatus (Embolism Meter, INRA Licensed Instrumentec, France, Version 2.1, Cochard, 2002). Previously, the extremes of the stems segments were wrapped with Teflon tape before connection to the tubes to prevent lateral leaks. We calculated the PLC as:
PLC = \frac{K_i - K_m}{K_m} \times 100 \quad \text{(Equation 2)},

where \( K_i \) (mmol m s\(^{-1}\) MPa\(^{-1}\)) is the hydraulic conductivity of the segment after seedling freezing and before embolism removal and \( K_m \) was the maximum hydraulic conductivity, measured after removing embolism by immersing the stem segments in the same solution used for the flow measurements in a vacuum chamber (BR116, Selecta, Barcelona, Spain) for at least 12 h at a suction of 90 kPa. Both conductivities were calculated as the ratio between the flow of a 10 mmol KCl + 1 mmol of CaCl\(_2\) solution through the segment divided by the pressure gradient (pressure difference = 0.49 - 0.52 kPa). In all cases, the solution used for measurements was previously filtered with a 0.45 \( \mu \)m nylon syringe filter membrane (Filter-Lab, Barcelona, Spain) and degassed in the mentioned vacuum chamber for 24 h at a suction of 90 kPa.

4. Measurement of tracheid diameter

Tracheid diameter was measured only in the unfrozen, well-watered plants. Cross-sections 20 \( \mu \)m in thickness were cut from six individuals per species using a cryostat microtome (Microm HM 505 E, Ramsey, MN, USA). We used the same stem segments used for PLC. Cross-sections were bathed in bleach for 30 min, rinsed in tap water and finally stained with safranin (1 g in 50% ethanol). Tracheid diameter was measured using the ImageJ software on all the tracheids located in three randomly selected radial rows, from the pit to the outer border of the section. A minimum of 160 tracheids per individual was measured.

5. Soluble sugars determination.

Soluble sugars (SS) were determined only in unfrozen well-watered plants. Needles of six seedlings per species were stored frozen immediately after EL measurements. When SS were determined, needles were thawed and dried at 50 °C for 24 h. SS were extracted following the
methodology in Chow and Landhäusser (2004). Briefly, needles were ground in a ball mill (PM100, Retsch, Haan, Germany) and 50 mg of ground needles was used for SS extraction with 5 ml of ethanol 80% at 90 °C for 10 min. Then, the tubes were centrifuged at 2500 rpm for 5 min, and the supernatant was preserved. We repeated the extraction three times and the supernatant of the three extractions was combined and oven-dried at 50 °C until complete evaporation of the ethanol. Then, the residue was dissolved in 1 ml hot deionized water and filtered with 0.45 μm nylon syringe filter membrane (Filter-Lab, Barcelona, Spain).

We analyzed the concentration of main SS (see Table 2) using a High Performance Liquid Chromatography system (Agilent Technologies, 1100 series, Palo Alto, CA, USA) coupled to the refractive index detector (HPLC-RI) equipped with a quaternary pump, degasser, auto injector and HP-1047A RI detector. The chromatographic data were acquired using the ChemStation software. The samples were analyzed using a Supelcogel column Ca-59305U (30 cm x 7.8 mm) under isocratic condition with ultrapure water (Milli-Q). The injection volume was 20 μL and the flow rate was 0.5 mL min⁻¹. Column and detector temperature were 80 and 50 °C, respectively. Identification and quantification of sugars was determined by comparing the peak retention time and area of the samples with pure standard solutions of each sugar that were made by us in the laboratory.

6. Data analysis

To compare the frost survival of pine species, we compared the temperature at which survival is 50% (LT₅₀). We used a logistic model to fit survival data for each species against freezing temperatures (see Figure 1). LT₅₀ is the inflection point of the logistic model. We used a logistic model to fit survival data for each species against freezing temperatures (see Figure 1). For each LT₅₀ value, we calculated its 95% confidence intervals following Collet (1991).
A generalized linear model (GLM) was performed to test the effect of species (six levels), frost (two levels, unfrozen and -15 °C frost) and $\psi_{pd}$ on EL and PLC, testing all the interactions. As the Species × Frost × $\psi_{pd}$ was significant for EL, the least square means and standard errors were calculated at specific $\psi_{pd}$ values, -0.4, -1.2 and -1.5 MPa (see Figure 2).

A general linear mixed model (GLMM) was performed to compare the tracheid diameter among species. Data of tracheids in a row were nested within the individual, which was included as a random effect. Species differences in SS were analyzed using one-way ANOVA. Tukey’s Honest Significance Difference (HSD) test ($\alpha=0.05$) was used for multiple comparisons of mean values. All data was checked for residual normal distribution (Shapiro-Test) and variance homoscedasticity (Levene’s test). Statistical analyses were performed in R platform and Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA).

Results

Survival

In all species, fitted curves of survival against freezing temperatures were highly significant (P<0.001). Frost survival significantly differed among species (Figure 1) with a two-fold LT$_{50}$ variation between $P$. halepensis, the species with the lowest LT$_{50}$, and $P$. nigra, the species with the highest LT$_{50}$. $Pinus$ $nigra$, $P$. $sylvestris$ and $P$. $uncinata$ did not show any mortality until -15 °C, while at this temperature survival of $P$. halepensis and $P$. pinea was only around 20%. Four groups according to their LT$_{50}$ values and confidence intervals were distinguished: $P$. halepensis=$P$. pinea>$P$. pinaster>$P$. sylvestris=$P$. uncinata>$P$. nigra. The survival curve was similar for $Pinus$ $sylvestris$ and $P$. $uncinata$, and their LT50 values were slightly higher than those estimated for $P$. nigra. $Pinus$ pinaster had lower frost survival than $Pinus$ uncinata, $P$. sylvestris and $P$. nigra, but higher frost survival than the Mediterranean pines $P$. halepensis and $P$. pinea, which showed little frost survival differences between them.
Foliage electrolyte leakage

Across species, a single -15 °C frost increased foliage EL relative to unfrozen plants, particularly in *P. halepensis* and *P. pinea* (Figure 2). However, the effect of the frost on EL depended on seedling $\psi_{pd}$ and the species (Species × Frost × $\psi_{pd}$ interaction, Table 1). Thus, among the unfrozen seedlings, reduction in $\psi_{pd}$ increased EL in *P. nigra* and *P. sylvestris*, while EL remained low and was unaffected by $\psi_{pd}$ in the remaining species. Among the frozen plants, species differences in EL depended on seedling water status. Frozen *P. halepensis* seedlings had the highest EL values and reduction in $\psi_{pd}$ did not affect EL. Reduction in seedling $\psi_{pd}$ strongly decreased EL after frost in *P. pinea* and to a lesser extent in *P. sylvestris*. In contrast, reduction of seedling $\psi_{pd}$ increased foliage EL after frost in *P. uncinata*. Finally, reduction in $\psi_{pd}$ had no effect on EL after frost in *P. pinaster* and *P. nigra*.

Percentage loss in stem hydraulic conductivity (PLC) and tracheid diameter

Frost and $\psi_{pd}$ did not affect PLC (Table 1). However, *Pinus uncinata*, *P. nigra* and to a less extent *P. sylvestris*, tended to have slightly higher PLC values than the rest of the species (Table 2). Species differed in tracheid diameter (P<0.001; Table 2). *Pinus uncinata* had the narrowest tracheids followed by *P. halepensis*. In contrast *P. pinaster* and *P. pinea* had the widest tracheids while *P. nigra* and *P. sylvestris* had intermediate tracheid diameter. We did not find any significant correlation between PLC and $\psi_{pd}$ (*P. halepensis*: r=-0.17, p=0.35; *P. pinea*: r=0.064, p=0.72; *P. pinaster*: r=-0.06, p=0.73; *P. nigra*: r=0.18, p=0.33; *P. sylvestris*: r=0.16, p=0.36; *P. uncinata*: r=-0.33, p=0.05).

Soluble sugars
Four soluble sugars were detected in the foliage in the following concentration trend: glucose > fructose > galactose > raffinose. A complex of sucrose, maltose and lactose (SML) was also detected in all species. Raffinose occurred at a very low concentration, it was found in all species but *P. pinaster* and no species differences in raffinose were observed (Table 2). Galactose was only found in *P. pinaster* (7.51±3.9 mg g⁻¹). The total concentration of soluble sugars, SML and glucose and fructose showed significant differences among species (P<0.0001) *Pinus halepensis* and *P. pinaster* had overall the highest concentration while *P. nigra* and *P. sylvestris* had the lowest concentrations. Across species, total concentration of soluble sugar tended to increase with LT₅₀, but the correlation was not statistically significant (r=0.59, P=0.22).

**Discussion**

Differences in seedling frost survival are related to the natural distribution of pine species in Europe. Juveniles of the pine species studied showed remarkable differences in their ability to survive freezing temperatures. These differences in survival are largely consistent with their range in the Iberian Peninsula, suggesting that the distribution of studied pine species is in part controlled by low temperatures. Our frost survival results agree with the distribution of these species at European scale; but the use of one provenance per species restricted to the Iberian Peninsula requires some caution. *Pinus sylvestris* reaches very high latitudes and together with *P. uncinata* and *P. nigra* constitute the tree line in many mountains in the Iberian Peninsula and in other areas in southern Europe (Barbero et al. 1998; Strimbeck and Schaberg 2009). These pine species had LT₅₀ values < -21 °C. On the contrary, *P. halepensis* and *P. pinea* showed almost two-fold higher LT₅₀ values than the pines of the cold winter zones; a single -10 °C frost may cause 30% mortality in both species. The low frost tolerance of *P.
halepensis and P. pinea is consistent with their distribution in mild to cool-winter locations in southern Europe and indicate that strong frosts likely limit the colonization of these Mediterranean pines at higher latitude and altitude locations. Consistent with our findings, Climent et al. (2009) observed a negative relationship between the needle frost damage and the mean temperature of the coldest month of the seed source across several pine species. Similarly, comparing several provenances, Bachofen et al (2016) observed that P. halepensis needles were less frost tolerant than P. sylvestris and P. nigra needles.

An unexpected result was that P. nigra had a slightly higher frost tolerance than P. sylvestris and P. uncinata. Climent et al (2009) also observed that P. nigra secondary needles were less frost damaged than P. sylvestris needles. In the high mountains of southern Europe, P. nigra is distributed slightly below the P. sylvestris and clearly below P. uncinata altitudinal belts (Tapias et al. 2011), which would imply that P. nigra should be less frost tolerant than P. sylvestris and P. uncinata. This conflict between frost tolerance and the altitudinal distribution of P. nigra may be explained by provenances used in the study or species differences in competitive capacity at the coldest sites and/or the lower capacity of P. sylvestris and P. uncinata to withstand summer drought (Herrero et al. 2013, Matias et al. 2017, Tíscar et al. 2017). Drought usually increases at lower altitudes in the mountains of southern Europe (Barbero et al. 1998).

Frost tolerance was significantly higher in Pinus pinaster than in two species with which it frequently coexist, P. halepensis and P. pinea. Pinus pinaster has remarkable interpopulation differences in frost tolerance with the provenances of cold continental climates having higher frost tolerance than the mild winter sources (Corcuera et al. 2011). In this study, the seeds used to cultivate P. pinaster plants come from a cold winter site (Table S1), which may explain the observed high frost tolerance of this species. Similarly, we selected rather cold and continental provenances for P. halepensis and P. pinea and
provenances for *P. sylvestris* and *P. nigra* near the rear edge of their southern distribution in Europe, which might have contributed to reduce species differences in frost tolerance. Consequently, results in this study probably show a conservative measurement of the cold tolerance differences among studied species.

**Frost tolerance differences among pine species is explained by needle frost**

Our results provide insights into the mechanisms underlying the differences in frost survival among pines. Electrolyte leakage is a measure of cell membrane integrity after subjecting plants to stress (Earnshaw, 1993). We found that the two Mediterranean pines, *P. halepensis* and *P. pinea*, which showed the lowest frost survival presented the highest needle electrolyte leakage values after a frost of -15 °C (particularly at high \( \psi_{pd} \), Figure 2). This indicates that the low frost survival of the Mediterranean pines is mostly due to differences in the vulnerability of foliage to low temperature. We cannot exclude that differences in other organs, such as roots also contribute to the observed species differences in frost survival. Toca et al (2017) reported that root frost tolerance in *P. halepensis* and *P. pinea* is lower than in *P. pinaster* and much lower than in *P. nigra*.

Glucose and fructose were the most important sugars in the SS pool in all species, consistent with previous findings in other conifers (Hoch et al. 2003). In contrast, raffinose concentration remained low in all species, which contrasts with results in Strimbeck and Schaberg (2009), who reported high leaf concentrations of raffinose and that seasonal increase in raffinose was positively associated to higher frost tolerance in several conifer species. Similar to our findings, Hoch et al. (2003) observed very low levels of raffinose at the end of the winter in *P. sylvestris*. The concentration of SS in plant tissues increases during cold acclimation of perennial plants in temperate and cold biomes to a maximum in the middle of the winter, coinciding with the period of greatest frost tolerance (Chomba et al.
SS increase the fluidity and stability of the cellular membranes, which prevents tissue freezing damage (Sakai and Larcher 1987, Uemura and Steponkus 2003). Contrary to our expectation we did not find SS concentration to increase with frost tolerance across pine species. Rather, pines that live in mild or cool winter sites tended to have higher SS concentration than cold winter pines (Table 2). Most of the studies that have demonstrated a relationship between frost tolerance and SS are at the intraspecific scale (Hinesley et al. 1992, Ögren et al. 1997, Charrier et al. 2013). For example, Charrier et al. (2013) observed SS to increase with frost tolerance in 9 out of 11 tree species. In contrast to intraspecific comparisons, interspecific studies on the relation between frost tolerance and tissue SS are relatively scarce. Among Quercus species no clear relationship was observed between frost tolerance and tissue SS (Cavender-Bares et al. 2005, Morin et al. 2007). Overall, these results suggest that SS do not necessarily explain interspecific differences in frost tolerance. It is possible that compounds other than SS, such as specific proteins (Kontunen-Soppela et al. 2000), might be more important to explain frost tolerance across species.

Plants from cold climates tend to have higher respiration rates than their counterparts from mild climates (Mariko and Koizumi 1993, Reich et al. 1996, Heskel et al. 2016), which may lead to higher non-structural carbohydrate consumption under cool conditions. As the plants used in our study were cultivated in an open nursery, where the mean air temperature varied between 0 and 8 °C most of the winter, species differences in respiration and probably in photosynthesis may explain the lower SS concentration attained by the most frost tolerant species (P. uncinata, P. sylvestris and P. nigra) relative to frost intolerant ones (P. halepensis and P. pinea). Similarly, growth differences among species were large (Table S2), which may have affected the species SS differences.
In contrast to needle frost sensitivity differences, the very low PLC values and the lack of species differences in PLC after exposure to -15°C indicate that freezing induced xylem-embolism does not explain frost survival differences among pine species. Vulnerability to freezing-induced embolism is directly related to the diameter of xylem conduits and increases with decreasing xylem water potential (Davis et al. 1999, Pittermann and Sperry 2003, Sperry 1995). Xylem conduits in conifers are much narrower and consequently safer than angiosperm vessels (Pittermann and Sperry 2006; Cavender-Bares 2005). We observed that species differences in tracheid diameter were relative small, with average values ranging from 6.6 to 10.6 μm and unrelated to native PLC (Table 2). For frost-induced embolisms to occur in such narrow tracheids, xylem water potential must be < -2.0 MPa (Pittermann and Sperry 2006), which is significantly lower than both the lowest $\psi_{pd}$ to which the plants were subjected in our study and the $\psi_{pd}$ that these species commonly experience in the field during winter (Martinez-Ferri et al. 2004, Poyatos et al. 2008, Peguero-Pina et al. 2011).

A very interesting result of our study was that a moderate increase in drought stress ($\psi_{pd}$ up to -1.5 MPa) modulated the effect of frost on foliage damage. However, this effect differed among species (Figure 2) and, apparently, it was not associated with species ecology. Drought stress significantly reduced frost damage in P. sylvestris and, especially, in P. pinea. However, it slightly increased it in P. uncinata and had minor effects in the rest of the species. A similar reduction in frost damage in drought stressed P. sylvestris plants was reported by Sutinen et al. (1992). However, our results for P. sylvestris are not consistent with the interpretation by Peguero-Pina et al. (2011). They mentioned that defoliation in P. silvestris population at the southern edge of the species range during a dry winter, could be because drought-stressed trees were more predisposed to frost damage than relatively hydrated trees.
It is important to note that the EL values after freezing to -15 °C in *P. pinea* seedlings subjected to $\psi_{pd} < -1$ MPa were similar to the EL values of the most frost tolerant pine species at high $\psi_{pd}$. Similarly, Villar-Salvador et al. (2013) observed that *P. pinea* seedlings subjected to moderate drought stress cycles showed higher tolerance to frost than well-watered plants. The increase in frost tolerance when plants are subjected to moderate drought stress may explain that *P. pinea* can develop in sandy soils in the north of the Iberian Peninsula where the winters are colder than those experienced by *P. pinea* throughout much its range (Campelo et al. 2006). Increases in frost tolerance with drought can be due to activation of generic stress tolerance physiological mechanisms such as abscisic acid or changes in cell membrane composition in lipids and proteins induced by either frost or drought stress (Mäntylä et al. 1995, Pearce 2001, Shinozaki et al. 2003).

**Conclusions**

Our study evidences remarkable differences in the ability of juveniles of six common pines in Europe to survive to frosts, which were related to the winter climatic conditions within their Iberian range. This supports the idea that frost is an important filter for the distribution of the studied pine species in the Iberian Peninsula and likely in the rest of Europe. The Mediterranean pines, *P. halepensis* and *P. pinea* were the least frost tolerant species, while *P. sylvestris, P. uncinata* and *P. nigra*, which inhabit the coldest locations, were the most frost tolerant species. Interspecific differences in frost tolerance were due to differences in the frost tolerance of foliage, and not to different vulnerability to freezing-induced xylem embolism. Drought increased frost tolerance in *P. pinea* and *P. sylvestris*, but decreased it in *P. uncinata*. Species showing high frost survival had similar or lower concentration of soluble sugars than species having a low frost tolerance, indicating that other elements are more important than soluble sugars in determining differences in frost tolerance across pine species. Our results
suggest that the expected increase in winter temperatures associated to climate change will reduce the cold limitations of Mediterranean pines to colonize sites at higher latitude and altitude in Europe.

Acknowledgments

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Table 1. Model results of the effect of predawn water potential, frost, species and their interactions on the needle electrolyte leakage (EL) and percentage loss in stem hydraulic conductivity (PLC) in six pine species.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Needle EL</th>
<th></th>
<th></th>
<th>PLC</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>$\chi^2$</td>
<td>$p$-value</td>
<td>DF</td>
<td>F value</td>
<td>$p$-value</td>
</tr>
<tr>
<td>Predawn water potential ($\psi_{pd}$)</td>
<td>1</td>
<td>5.43</td>
<td>0.020</td>
<td>1</td>
<td>0.0119</td>
<td>0.91</td>
</tr>
<tr>
<td>Frost</td>
<td>1</td>
<td>168</td>
<td>&lt; 0.0001</td>
<td>1</td>
<td>0.881</td>
<td>0.35</td>
</tr>
<tr>
<td>Species</td>
<td>5</td>
<td>39.4</td>
<td>&lt; 0.0001</td>
<td>5</td>
<td>2.26</td>
<td>0.050</td>
</tr>
<tr>
<td>$\psi_{pd}$ × Frost</td>
<td>1</td>
<td>4.45</td>
<td>0.035</td>
<td>1</td>
<td>1.66</td>
<td>0.20</td>
</tr>
<tr>
<td>Frost × Species</td>
<td>5</td>
<td>56.0</td>
<td>&lt; 0.0001</td>
<td>5</td>
<td>0.615</td>
<td>0.69</td>
</tr>
<tr>
<td>$\psi_{pd}$ × Species</td>
<td>5</td>
<td>10.7</td>
<td>0.060</td>
<td>5</td>
<td>1.35</td>
<td>0.24</td>
</tr>
<tr>
<td>$\psi_{pd}$ × Frost × Species</td>
<td>5</td>
<td>25.2</td>
<td>&lt; 0.0001</td>
<td>5</td>
<td>0.693</td>
<td>0.63</td>
</tr>
</tbody>
</table>
Table 2. Percent loss in stem hydraulic conductivity (PLC) after a single -15 °C treatment, and tracheid diameters and concentration of different foliage soluble sugars of unfrozen, well-watered plants of six Iberian pine species. Data are mean ± SE. Same letter indicates not significant differences. SML: sucrose+maltose+lactose.

<table>
<thead>
<tr>
<th>Specie</th>
<th>PLC (%)</th>
<th>Tracheid diameter (µm)</th>
<th>Soluble sugars (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Raffinose</td>
</tr>
<tr>
<td><em>P. halepensis</em></td>
<td>15.0±1.24 c</td>
<td>7.6±0.44 bc</td>
<td>0.53±0.42</td>
</tr>
<tr>
<td><em>P. pinea</em></td>
<td>16.6±1.28 bc</td>
<td>10.5±0.3 a</td>
<td>0.31±0.49</td>
</tr>
<tr>
<td><em>P. pinaster</em></td>
<td>16.0±0.89 bc</td>
<td>10.6±0.63 a</td>
<td>Not detected</td>
</tr>
<tr>
<td><em>P. nigra</em></td>
<td>21.8±1.35 a</td>
<td>9.5±0.72 ab</td>
<td>0.61±0.66</td>
</tr>
<tr>
<td><em>P. sylvestris</em></td>
<td>20.0±1.22 ab</td>
<td>9.6±0.32 ab</td>
<td>0.80±0.57</td>
</tr>
<tr>
<td><em>P. uncinata</em></td>
<td>23.8±1.24 a</td>
<td>6.6±0.37 c</td>
<td>0.33±0.47</td>
</tr>
</tbody>
</table>
**Figure legends**

Fig. 1. Relation between survival and freezing temperatures among six pine species distributed in Europe. Data inserted in figure are mean temperature (ºC) values and the 95% confidence interval (in brackets). See Material and Methods for data fitting details.

Figure 2. Variation of foliage electrolyte leakage with seedling $\psi_{pd}$ in unfrozen (upper panel) and frozen seedlings (subjected to a single -15 ºC frost, lower panel) in six pine species. Data are least square means ± SE, estimated at -0.4, -1.0 and -1.5 MPa after ANCOVA.
Unfrozen plants

Electrolyte leakage (%)

-0.4 MPa
-1.0 MPa
-1.5 MPa

-15 °C frozen plants

P. halepensis  P. pinea  P. pinaster  P. nigra  P. sylvestris  P. uncinata
Supplementary material

Figure S1. Range and altitudinal segregation (inserted pictograph) of the natural stands of pine species native to Spain. Yellow symbols represent the species in the pictograph and the location of the seed sources of each species used to cultivate the seedlings. Source: Third National Forestry Inventory of the Spanish government http://www.mapama.gob.es/es/biodiversidad/servicios/banco-datos-naturaleza/informacion-disponible/ifn3.aspx

Table S1. Geographic location and climatic characteristics of the provenances where seeds of the study were collected. MAT= mean annual temperature, TCM= mean of the minimum temperatures of the coldest month. Provenance names follow nomenclature in Alía et al (2009)*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Provenance</th>
<th>Latitude (N)</th>
<th>Longitude (W)</th>
<th>Altitude (masl)</th>
<th>MAT (°C)</th>
<th>TCM (°C)</th>
<th>Annual rainfall (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. halepensis</em></td>
<td>Alcarria</td>
<td>40°24’52’’</td>
<td>2°24’33’’</td>
<td>860</td>
<td>12.6</td>
<td>-0.6</td>
<td>580</td>
</tr>
<tr>
<td><em>P. pinea</em></td>
<td>La Mancha</td>
<td>39°12’02’’</td>
<td>1°57’59’’</td>
<td>675</td>
<td>14.2</td>
<td>0.7</td>
<td>397</td>
</tr>
<tr>
<td><em>P. pinaster</em></td>
<td>Cuenca</td>
<td>39°38’44’’</td>
<td>1°13’52’’</td>
<td>1135</td>
<td>12</td>
<td>-1.5</td>
<td>540</td>
</tr>
<tr>
<td><em>P. nigra</em> subsp. <em>salzmannii</em></td>
<td>Sistema Ibérico Meridional</td>
<td>40°15’16’’</td>
<td>1°58’22’’</td>
<td>1515</td>
<td>10.4</td>
<td>-3.2</td>
<td>617</td>
</tr>
<tr>
<td><em>P. sylvestris</em></td>
<td>Montes Universales</td>
<td>40°28’09’’</td>
<td>1°38’42’’</td>
<td>1725</td>
<td>9.2</td>
<td>-4.8</td>
<td>894</td>
</tr>
<tr>
<td><em>P. uncinata</em></td>
<td>Pirineo Central</td>
<td>42°25’59’’</td>
<td>1°40’18’’</td>
<td>2050</td>
<td>5.3</td>
<td>-7.5</td>
<td>888</td>
</tr>
</tbody>
</table>

Table S2. Size of the shoot fractions of the saplings of the six Iberian pine species used in the study. Data are mean ± SE. Same letter indicates not significant differences (Posthoc test HSD Tukey).

<table>
<thead>
<tr>
<th>Species</th>
<th>Stem (g)</th>
<th>Needles (g)</th>
<th>Total needles (area cm$^2$)</th>
<th>Stem diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. halepensis</em></td>
<td>2.33±0.64 b</td>
<td>3.70±0.84 bc</td>
<td>308±66 ab</td>
<td>3.6±0.4 b</td>
</tr>
<tr>
<td><em>P. pinea</em></td>
<td>5.06±1.00 a</td>
<td>5.65±0.84 a</td>
<td>341±49 a</td>
<td>4.8±0.6 a</td>
</tr>
<tr>
<td><em>P. pinaster</em></td>
<td>1.86±0.38 bc</td>
<td>4.58±0.5 ab</td>
<td>259±19 b</td>
<td>3.8±0.4 b</td>
</tr>
<tr>
<td><em>P. nigra</em></td>
<td>1.69±0.45 bc</td>
<td>2.96±0.63 cd</td>
<td>182±31 c</td>
<td>3.6±0.3 b</td>
</tr>
<tr>
<td><em>P. sylvestris</em></td>
<td>1.84±0.35 bc</td>
<td>2.24±0.48 de</td>
<td>138±20 cd</td>
<td>3.6±0.2 b</td>
</tr>
<tr>
<td><em>P. uncinata</em></td>
<td>0.86±0.37 c</td>
<td>1.65±0.27 e</td>
<td>94±28 d</td>
<td>3.2±0.5 b</td>
</tr>
</tbody>
</table>