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Species ecology determines the role of nitrogen nutrition in the frost tolerance of pine seedlings

Andrei Toca^{1,3}, Juan A. Oliet², Pedro Villar-Salvador¹, Judit Maroto^{1,2},

5 Douglass F. Jacobs^{3,4}

¹Forest Ecology and Restoration Group, Departamento de Ciencias de la Vida, Apdo.
20, Universidad de Alcalá, E-28805, Alcalá de Henares, Madrid, Spain

²E.T.S. Ingenieros de Montes, Forestal y del Medio Natural, Departamento de Sistemas y
Recursos Naturales, Universidad Politécnica de Madrid, 28040 Madrid, Spain

10 ³Hardwood Tree Improvement and Regeneration Center, Department of Forestry and
Natural Resources, Purdue University, West Lafayette, IN 47907, USA

⁴Corresponding author: djacobs@purdue.edu, Phone +765.494.3608, +Fax
765.496.9461

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20 **Abstract:** Frost determines the evolution and distribution of plants in temperate and
cold regions. Several environmental factors can influence frost acclimation of woody
plants but the magnitude and direction of the effect of nitrogen (N) availability is
controversial. We studied the effect of N availability on root and shoot frost tolerance in
mid-fall and in winter in seedlings of four pines of contrasting ecology: *Pinus nigra*, *P.*
25 *pinaster*, *P. pinea* and *P. halepensis*. Needle and root N and soluble sugar concentration,
and timing of cessation of shoot elongation were measured to assess the physiological
mechanisms underlying frost acclimation. N was supplied at high and low rates only
during the pre-hardening period and at a moderate N rate during hardening in the fall.
Shoot frost tolerance increased over winter while root frost tolerance did not change in
30 any species. Pre-hardening N availability affected the frost tolerance of both roots and
shoots, although the effect was species-specific: high N reduced the overall root and
shoot frost tolerance in *P. pinea* and *P. halepensis*, increased the frost tolerance in *P.*
nigra, but had no effect in *P. pinaster*. N supply in the fall consistently increased frost
tolerance in all species. Differences in frost tolerance among species and N treatments
35 were not explained by variations in organ N or soluble carbohydrate concentration, nor
by timing of cessation of shoot elongation; although the most frost tolerant species
ceased elongation earlier than the least frost tolerant species. Despite the close
phylogenetic relatedness of the studied species, the effect of N availability on seedling
frost tolerance differed among species, indicating that species ecology (especially frost
40 acclimation physiology and timing of N supply) drives the effect of N availability on
frost tolerance of pine species.

Keywords: cessation of shoot elongation, cold hardiness, soluble carbohydrates, fall
fertilization, *Pinus*, root frost tolerance, species ecology.

45 **Introduction**

Low temperature is a key environmental factor determining the evolution and distribution of plants (Grace 1987, Hawkins et al. 2014). Frost can damage plants through xylem embolism and the formation of extracellular ice, which causes cell dehydration and disruption of cell membranes (Zwiazek et al. 2001, Willson and Jackson 2006, Charrier et al. 2015). Woody plants have remarkable differences in frost tolerance, which are frequently related to the minimum temperatures within their distribution range (Kreyling et al. 2014). At a plant scale, organs also differ in frost tolerance, with aboveground vegetative parts having greater frost tolerance than roots (Bigras et al. 2001, Charrier et al. 2015). Plants have developed specific adaptations to low temperatures (Levitt 1980, Larcher 2005). Perennial plants from cold and temperate biomes undergo a complex cold acclimation process during fall in which they experience deep reversible physiological changes to survive the cold season (Bigras et al. 2001, Charrier et al. 2015). Consequently, plant frost tolerance increases through the fall to a maximum in mid-winter and releases in spring to a minimum tolerance in early summer. However, decoupling of cold plant acclimation with timing of climate events has increased in the last decades. This phenomenon increases the chance of frost damage to plants due to unusual fall and spring frosts events, which are expected to increase with climate change (Augspurger 2013). At the same time, the reduction of the snow cover and earlier snow melting can increase the risk of root frost damage due to lower soil and root insulation (Groffman et al. 2001, Schaberg et al. 2008).

Cessation of shoot elongation is a requisite for frost acclimation (Greer et al. 2000, Repo et al. 2000, Heredia-Guerrero et al. 2014), triggering the accumulation of nutrients and the synthesis of metabolites involved in frost tolerance (Charrier et al. 2015). Specifically, soluble carbohydrates (SC) progressively accumulate in plant

70 tissues during cold acclimation (Schulze et al. 2005, Martínez-Vilalta et al. 2016). *In*
vitro addition of sucrose enhances structural stability of the cell membrane to frost
damage and increases cytoplasm osmotic potential, which stabilizes intracellular
structures and lowers the freezing point (Wolfe and Bryant 2001, Uemura and
Steponkus 2003, Charrier et al. 2015). These cellular changes explain the reported
75 positive relationship between frost tolerance and SC concentrations in plants (Ogren et
al. 1997, Tinus et al. 2000, Kreyling et al. 2012). Lipid concentration and composition
also varies during frost acclimation, reducing the protein to lipid ratio and increasing the
degree of unsaturation of the hydrocarbon chain in membranes. These changes increase
membrane fluidity and also reduce their disintegration when plant tissues freeze
80 (Schulze et al. 2005). Finally, antifreeze proteins and several amino acids are involved
in the frost tolerance of plants (Griffith and Yaish 2004), inhibiting the growth of ice
crystals (Atıcı and Nalbantoğlu 2003) and contributing to osmotic adjustment (Zwiazek
et al. 2001).

Photoperiod and temperature trigger and determine the intensity and speed of
85 frost acclimation in woody plants (Welling et al. 2002, Charrier et al. 2015). While both
temperature and photoperiod control shoot frost tolerance, temperature seems to be the
main control of root frost tolerance (Fernández et al. 2008, Ryppö et al. 2008).

Nutrient availability, especially of nitrogen (N), can also affect plant frost tolerance
(Taulavuori et al. 2014, Charrier et al. 2015). However, some studies indicate that roots
90 do not harden in response to environmental variations such as temperature, even in cold
climates (Tinus et al. 2000; Schaberg et al. 2008). In contrast to temperature and
photoperiod, however, no clear conclusions have been developed regarding the
relationship between frost tolerance and N availability (see Taulavuori et al., 2014).
Because frost tolerance of roots and shoots responds differently to environmental

95 stimuli such as temperature and photoperiod, N may have variable effects on the frost tolerance of plant organs.

Nitrogen deposition increases soil N and is a major environmental issue associated with global change (Rennenberg and Gessler 1999). Therefore, knowledge of the relationship between N and frost tolerance is important for assessing the potential
100 responses of forest species to N deposition. Some studies have reported that high N availability reduces frost tolerance (Hellergren 1981, Schaberg et al. 2002, Villar-Salvador et al. 2013, Heredia-Guerrero et al. 2014), associated with a lower tissue SC concentration (Andivia et al. 2012) and delayed cessation of shoot elongation in fall (Hawkins et al., 1995; Heredia-Guerrero et al. 2014). In contrast, other evidences
105 suggest that plant frost tolerance increases with N availability in trees from temperate climates (DeHayes et al. 1989, Andivia et al. 2012, Taulavuori et al. 2014). N-rich osmolytes such as amino acids and cryoprotection proteins are expected to increase with soil N availability during frost acclimation (Lähdesmäki and Pietiläinen 1988; Griffith and Yaish 2004, Berrocal-Lobo et al. 2011). This may explain why N supply in fall
110 during cold hardening increases frost tolerance in plants from cold and temperate ecosystems (DeHayes et al. 1989, Andivia et al. 2012).

Contradictory results among studies on the effect of N on tree frost tolerance could be related to differences in N availability and application timing (Oliet et al. 2013) and to environmental conditions during the experiment (Heredia-Guerrero et al.
115 2014). However, variation among studies could also be attributable to functional differences among species. Particularly, physiology of dormancy as related to species ecology could determine the effects of N on frost tolerance during acclimation. Frost acclimation in trees adapted to mild winters is mainly controlled by temperature, while photoperiod plays a secondary role (Nguyen et al. 1995). These trees maintain growth

120 during fall as long as temperature is not limiting and mild temperature spells in winter
can quickly reduce frost tolerance (Charrier et al. 2015). High N availability, especially
during the hardening period in fall, may hinder frost tolerance in these tree species
(Puertolas et al., 2005). In contrast, frost acclimation in tree species from cold winter
climates is usually under tight environmental control. Frost acclimation and cessation of
125 shoot elongation begins early in the fall with photoperiod triggering frost acclimation
(Charrier et al. 2015). These species usually maintain high frost tolerance during the
cold season irrespective of winter conditions (Repo 1992). We expect that high N
availability during the pre-hardening and hardening periods will enhance frost tolerance
in these tree species. Therefore, to better understand the relationship between frost
130 tolerance of tree species and N nutrition, and help to disentangle the underlying
physiological mechanisms, comparative experiments where species are grown under the
same environmental conditions are needed.

The objective of this investigation was to analyze the role of plant ecology in
determining the effect of N on frost tolerance. We selected four pine species that have
135 contrasting ecological characteristics and differ in their frost acclimation physiology
that were cultivated under the same environmental conditions. We compared frost
tolerance of roots and shoots of seedlings during cold hardening in mid-fall and in
winter. We also evaluated tissue N and SC concentration in needles and roots, and
cessation of shoot elongation to assess the physiological mechanisms underlying frost
140 acclimation. Differences in N and SC concentration in organs and growing cessation
were achieved by supplying N at high and low rates during the pre-hardening period
(growing season) and N loading during cold hardening in fall.

The selected pine species are widespread in southern European regions
characterized by Mediterranean climates. *Pinus halepensis* Mill. and *Pinus pinea* L. are

145 thermophilic species that inhabit mild winter regions and have weak environmental
control of dormancy. During winter, these species have high photosynthetic activity, can
reactivate growth during mild spells (Puértolas 2005, Climent et al. 2011) and do not set
buds in autumn during the seedling and early sapling stage (Mutke et al. 2012). *Pinus*
pinaster Ait. is a mesophilic species that thrives mostly in mid-elevation mountains in
150 Mediterranean-climate locations, while *Pinus nigra* J.F. Arnold is a high mountain,
psychrophilic pine (Barbero et al. 1998). The last two species, especially *P. nigra* are
usually exposed to intense and frequent frost events, set buds before dormancy in winter
mediated by a strong endodormancy, and develop secondary needles earlier in their
ontogeny than *P. halepensis* and *P. pinea* (Kreyling et al. 2012, Lafuente Laguna et al.
155 2013, Peñuelas et al. 2013). Some of these species coexist in the limits of their
altitudinal range. We hypothesized that 1) the effect of N availability on frost tolerance
during acclimation will be mediated by species ecology: while pine species inhabiting
mild winter regions will have reduced frost tolerance under high N availability, those
from cold winter regions will be positively influenced; 2) while timing of nutrient
160 application (i.e., pre-hardening *versus* fall hardening) will affect frost tolerance of
seedlings, the magnitude of this effect will depend on species; and 3) frost tolerance of
roots will be less affected by N availability and timing of supply than shoots, because
roots experience less cold acclimation in response to environmental shifts.

The findings of this study can shed light on the mechanisms of frost tolerance in
165 response to N availability, which up to date are far from being clarified for forest
species (Taulavuori et al. 2014). This could help to better understand the effects of
global change drivers such as N deposition on plant functioning, as well as to improve
the cultivation of nursery seedlings.

Materials and methods

Plant material and experimental design

Seeds of the four-pine species were collected in the Southern part of the Iberian range (eastern Iberian Peninsula (Table S1, in Supplementary Material, Alía et al., 175 2009). Seeds were sown on February 2012 at the Centro Nacional de Recursos Genéticos Forestales “El Serranillo” (Central Spain, 40°40’N, 3°10’W, 650 m a.s.l.) into plastic trays (Plasnor® 190/300-45, Legazpi, Spain). These trays have 45 cells of 300 ml, and cultivation density is 283 plants·m⁻². Growing medium was *Sphagnum* peat moss pH=4.7, enriched with a 16-10-20 NPK slow release fertilizer at a rate of 0.9 180 kg·m⁻³ (Kekkila® White 420 F6, Finland). Trays were kept in an unheated greenhouse during germination and emergence phases to avoid late spring frost damage. On May 17, 2012 seedlings were transferred to the School of Forestry at the Technological University of Madrid (40°27’N; 3°43’W, 664 m a.s.l.) where the plants remained outdoors for the rest of the experiment. Plants were assigned to three fertilization 185 treatments: 1) Pre-hardening high and 2) low fertilization, where each plant was supplied with 150 and 20 mg N, respectively, during the seedling active growth period before fall cold hardening, from May 22 to September 19, 2012; 3) Fall fertilization, where each plant was supplied with 60 mg N during the seedling active growth period from May 22 to September 19 2012 plus 40 mg N·plant⁻¹ during the fall, from 190 September 26 to November 8, 2012. Fertilizer was applied by hand at a weekly constant rate using a water-soluble fertilizer. Fall fertilization extra supply was applied as ammonium nitrate, while for the rest of treatments the fertilizer was 20N–20P₂O₅–20K₂O (Scotts Co., Marysville, OH, USA) with N sources being ammonium nitrate (10%) and urea (10%). On every fertilization date, each seedling received 55 ml of the

195 fertilization solution. After fertilization, seedlings were watered for 5 min to remove the
fertilizer remaining on the needles. Supplemental irrigation was applied to field capacity
based on gravimetric methods (Timmer and Armstrong 1987). Each fertilization
treatment had three trays, resulting in a total of nine trays per species. Trays were
200 completely randomized in space and their position was rotated every 15 days to
minimize edge effects. Experimental design was a two factorial, with species (four
levels) and fertilization treatments (three levels) as main effects. Seedlings were grown
under full sun except from June 20 to September 15, where plants grew under a shading
with a 20 % light transmission to reduce evapotranspiration.

205 *Frost tolerance measurements*

Freezing tests were carried out on two dates: in mid-November 2012, when
seedlings were still frost acclimating and in late-January 2013, when plants are fully
frost-hardened (Climent et al, 2009; Pardos et al. 2014). The temperature was measured
every 10 minutes during the acclimation period using temperature probes placed
210 adjacent to the experiment (Figure S1, in Supplementary Material). Accumulated
chilling hours (air temperature ≤ 8 °C calculated from September 1) in the nursery were
130 h in mid-November and 1367 h in January. Temperature frequently dropped below
0 °C before the freezing test in January, reaching a minimum temperature of -4.9 °C,
while in November frosts were only registered in three days (Figure S1, in
215 Supplementary Material). The average temperature the 20 days before the freezing tests
was 10.0 °C in autumn and 5.9 °C in winter (Figure S1, in Supplementary Material).
Frost tolerance was assessed on intact plants and freezing separately the roots and the
shoots. Six seedlings per species, treatment and plant fraction (two seedlings per tray,
72 seedlings per plant fraction) were randomly selected on each date and subjected to an

220 8-h frost cycle in freezing chambers with a programmable temperature controller (ASL-
Snijders International® CON-550-20, Madrid, Spain and Dycometal® CCK81,
Barcelona, Spain, for the November and January tests, respectively). Temperature was
reduced from 5 °C to a target temperature where plants remained for 4 h. Then the
temperature was progressively raised until 5 °C. The target temperatures varied
225 depending on the date and plant fraction (roots and shoots) (Table 1) and were chosen
based on previous results obtained in other studies with these species (Puértolas et al.,
2005; Villar-Salvador et al., 2013; Kreyling et al., 2012; Climent et al., 2009). Cooling
and warming rates in a frost cycle ranged between 2 and 8 °C h⁻¹ in order to simulate
natural frost cycle and to keep the freezing test duration to 8 h. The exception was for
230 the target temperature of -23 °C, where the cooling rate was 13 °C h⁻¹ while warming
rates were 18 °C h⁻¹. Frost tolerance of shoots was assessed by inserting the trays in a
polystyrene box that isolated the plugs from the frost. A thermocouple was placed inside
the insulation cover and attached to the surface of the plug to record its temperature,
which was on average 6.5 °C higher than the air inside the freezing chamber for the -8
235 °C freezing test and 12.5 °C higher for the remaining target temperatures. To assess the
frost tolerance of roots, seedlings were placed in a modified cultivation tray in which
the wall of the cells was replaced by a 2-mm plastic mesh that allowed the plug to be
fully frozen. To prevent root desiccation, the plants were watered the day before and the
plug was wrapped in aluminum foil during freezing tests. Shoots were insulated from
240 frost by the same polystyrene cover used to isolate the root system.

After freezing tests, seedlings were transferred to a 60% light transmission
greenhouse and kept watered at field capacity for two months. The average temperature
in the greenhouse was 17 °C during the two months following November frost tests and
19.5 °C for the two months after the January frost tests. After the two months, shoot

245 damage was assessed visually by two independent observers as the percentage of
withered needles. This value (visual damage index, VDI) is strongly correlated with
other frost tolerance indicators (Andivia et al. 2011) and was considered as an estimator
of shoot or root frost tolerance based on previous studies that show that root and shoot
damage have an effect on the whole seedling (Carles et al. 2011).

250

Growth and nitrogen and soluble carbohydrates concentration

From August 21, 2012, prior the hardening stage, to January 3, 2013, seedling
height was measured weekly on five randomly chosen seedlings per tray (15 plants per
fertilization treatment and species, 180 seedlings in total). Measurements were carried
255 out on the same plants throughout the study period. Shoot height was measured from a
set point 5 mm below the cotyledon insertion scar to the apex of the bud in *P. nigra*, and
to the tip of the shoot in the remaining species, which do not set a bud during the first
years of life. The Julian day of cessation of shoot elongation was determined when
weekly shoot elongation was lower than 0.6 cm (Heredia et al., 2014).

260 To determine seedling mass and N and SC concentration during hardening, 15
seedlings per species and treatment (five seedlings per tray) were randomly harvested at
both freezing test dates and frozen to -20 °C until processing. Once defrosted, shoots
were cut 5 mm below the cotyledon insertion point and separated into needles and
stems, and roots were carefully washed from the growing medium with tap water.

265 Samples were rinsed in distilled water for 3 min to avoid contamination. Then leaves,
stems and roots were dried at 60 °C for 48 h and weighed to assess their mass. To
analyze organ N and SC concentrations, the organs of the five seedlings of the same tray
were composited and ground in a ball mill (PM 100, Retsch, Haan, Germany).

SC were analyzed in leaves and roots using a high-performance liquid
270 chromatograph (HPLC). Grounded leaf samples of 50 mg were extracted twice in 80%
aqueous ethanol (v/v) at 80 °C for 90 min. Samples were centrifuged at 13000 g for 5
min and the supernatant was completely evaporated and resolubilized in 5 ml distilled
water and boiled for 5 min. The resulting solution was filtered through a 0.45 µm nylon-
syringe and 20 µl was injected to HPLC using an Agilent 1100 Series (Agilent
275 Technologies, Palo Alto, CA) equipped with a refractive index detector. A carbohydrate
column (SupelcogelTMCa, 30 × 0.78 cm, Supelco, Bellefonte, PA, US) was used for the
analysis. SC peaks were detected by refractive index and were identified and quantified
by comparison with retention times of fructose, glucose and sucrose standards (Heredia
et al. 2014). Determination of leaf and root N concentration was done by the standard
280 Kjeldahl method using an auto-analyzer (CFA SAN++, Skalar, Breda, The
Netherlands).

Data analysis

For all analyses, P-values were computed using F-tests as well as a
285 randomization protocol that generated null distributions of variables and interactions by
randomly reshuffling these data across species 1000 times (Monte Carlo method,
Crowley 1992). For each of these subsamples we obtained the F-value of a two-way
ANOVA analysis with species and treatments as main factors including interactions.
The resulting 1000 F-values were compared with the F-value (ANOVA) from the
290 observed data. The P-value is the proportion of all data arrangements resulting in a test
statistic at least as extreme in magnitude as the F-value from the observed data. P-values
thus generated are expected to be more robust against potential biases in data (Crowley
1992). The differences between means were identified using Fisher's least significant

difference (LSD) test. All analyses were performed in R software (R Foundation for
295 Statistical Computing, Vienna, AT).

Results

Differences in root frost damage

Species differed in VDI after root freezing at -10 °C both in November and
300 January (Table 1). *Pinus halepensis* and *P. pinea* had the highest VDI values, with no
significant differences between them ($30.6\% \pm 6.7$ in November and $36.2\% \pm 9.3$ in
January for *P. halepensis* and 26.0 ± 5.7 and $33.5\% \pm 9.4$, respectively for *P. pinea*). In
contrast, *P. nigra* had the lowest VDI values ($7.6\% \pm 1.5$ and $2.6\% \pm 0.6$ in November
and January, respectively), while *P. pinaster* had intermediate VDI values ($12.4\% \pm 1.5$
305 and $20.9\% \pm 3.4$ in November and January, respectively) between *P. nigra* and the other
pine species (Figure 1).

Fertilization significantly affected VDI after root freezing, but differences
among treatments depended on species in November (interaction Species \times
Fertilization, Table 1). By this date, the highest VDI occurred for the high pre-hardening
310 fertilized *P. halepensis* and *P. pinea* seedlings, while the opposite was observed for *P.*
nigra, with the low pre-hardening fertilized seedlings having the highest VDI. In
addition, fall-fertilized seedlings presented similar or slightly lower VDI values than
those from low fertilized plants and in most cases, lower VDI values than those from
high fertilized plants (Figure 1a).

315 In January, fall-fertilized seedlings showed the lowest root VDI values ($7.0\% \pm$
 1.5), while high and low pre-hardening fertilized seedlings showed the highest VDI
(Figure 1b). Among species, *P. pinaster* VDI responses to fertilization were the less

plastic at both dates. On average, root VDI remained similar between November and January ($18.5\% \pm 2.3$ and $23.4\% \pm 3.7$, respectively).

320

Differences in shoot frost damage

Species differed in VDI after shoot freezing at -8°C in November, with overall VDI values of $50.0\% \pm 8.3$, $36.0\% \pm 5.2$, $17\% \pm 2.9$ and $4.0\% \pm 0.7$ for *P. halepensis*, *P. pinea*, *P. pinaster* and *P. nigra*, respectively. However, a strong Species \times Fertilization interaction was observed (Table 1). Low fertilization resulted in the highest VDI values in all species except for *P. pinaster*, which showed the highest VDI values in both high and low fertilized plants. Fall fertilized seedlings had the lowest VDI in *P. halepensis* and *P. pinaster*, while no difference between fall and high fertilization was found in the rest of species. In contrast, high fertilization led to intermediate VDI values between high and fall fertilized plants in *P. halepensis* (Figure 2a).

After the -15°C frost in November, VDI differed among species but not among fertilization treatments (Table 1). While *P. halepensis* and *P. pinea* showed almost complete damage (overall mean for both species: $98.7\% \pm 0.7$) irrespective of fertilization treatment, *P. nigra* had the lowest VDI values ($17.7\% \pm 4.2$ across fertilization treatments). Finally, *P. pinaster* had intermediate VDI values ($61.8\% \pm 6.1$) between *P. nigra* and the other pine species.

In January, after a -15°C frost VDI significantly differed among species (Table 1), with *P. halepensis* and *P. pinea* showing the highest values ($59.3\% \pm 8.8$ and $41.3\% \pm 5.2$, respectively) followed by *P. pinaster* ($17.8\% \pm 2.0$) and finally *P. nigra*, which had the lowest VDI value ($3.4\% \pm 0.5$). We found a marginally significant Species \times Fertilization interaction ($P=0.065$), explained by a maximum VDI of high fertilized *P.*

340

halepensis and *P. Pinea* seedlings, that did not occur in *P. nigra* and in *P. pinaster* (Figure 2b). On average, shoot VDI after -15°C frost decreased strongly from November to January (69.4 ± 4.3 and 29.7 ± 3.6 , respectively).

345 In January, all seedlings of *P. halepensis*, *P. pinea* and *P. pinaster* died after the -23°C frost test. In contrast, most *P. nigra* seedlings survived with fall fertilized and pre-hardening high fertilized seedlings having the lowest VDI values, without significant differences between them ($9.3\% \pm 2.4$ and $16.8\% \pm 5.5$, respectively), while low fertilization the highest ($32.5\% \pm 7.7$) (Species \times Fertilization interaction, Table 1).

350

Cessation of shoot elongation

On average, *P. nigra* and *P. pinaster* seedlings ceased shoot elongation 17 days earlier than *P. halepensis* and *P. pinea* ($F=14.9$, $P<0.001$), while no significant difference existed between the species within the two previous groups (Figure 3).

355 However, a significant Species \times Fertilization interaction occurred on cessation of shoot elongation ($F=7.8$, $P<0.01$). The effect of N fertilization on the cessation of shoot elongation presented a similar pattern in *P. halepensis*, *P. pinea* and *P. nigra*, with high and fall fertilization delaying the cessation of shoot elongation by 20 days on average relative to low fertilized plants (Figure 4). In contrast, fall fertilized *P. pinaster* plants
360 stopped shoot elongation earlier than high and low fertilized plants, which showed no differences between them.

Nitrogen and soluble carbohydrates concentration

Needle N concentration was highest among the fall and high fertilized seedlings
365 and lowest in the low fertilized plants in all species and in both dates. However, in both

freezing dates, the fertilization effect on needle N concentration was mediated by an interaction between Species and Fertilization, ($F=3.4$, $P<0.008$). In November, fall fertilized *P. halepensis* seedlings showed higher needle N concentration than high fertilized seedlings (Figure 4a) while in January, needles of high fertilized *P. pinea* seedlings had higher N concentration than the fall fertilization treatment (Figure 4b). Root N concentration was lower than needle N concentration but differences among species and fertilization treatments followed an overall similar pattern as described for needle N (interaction Species \times Fertilization at both dates, $F=3.4$, $P<0.007$; Figure 4c and 4d), especially in *P. pinaster* and *P. nigra*.

Irrespective of fertilization treatment, N concentration differed among species in November. By this date, species ranking in both needle and root N concentration was *P. nigra* > *P. halepensis* > *P. pinea* = *P. pinaster* (Figure 4). N concentration decreased in both needles and roots from November to January in all species and differences in organ N among species were small in January.

Needle and root SC concentration varied significantly among species in November ($F= 19.6$ $P<0.001$ for roots; $F= 57.7$ $P<0.001$ for needles). *Pinus pinaster* and *P. nigra* needles showed the highest and lowest needles SC concentration respectively, while *P. halepensis* and *P. pinea* had intermediate values between the former species with no significant differences between them (Figure 5a). In contrast, SC concentration in roots was higher in *P. nigra* and *P. pinea* than in *P. pinaster* and *P. halepensis*, while no significant differences existed between the species within the two previous groups (Figure 5b). On the same date, high and fall N fertilization had similar but higher needle SC concentration than the low N fertilization ($F=8.0$, $P<0.01$). Root SC concentration in November was highest in the high fertilized plants followed by fall

390 fertilized seedlings, while low fertilized seedlings showed the lowest SC concentration
($F=19.9$, $P<0.001$). No interaction between Species and Fertilization occurred for SC.

In January, SC concentration was affected by a significant Species \times
Fertilization interaction in both shoots and roots ($F=3.4$, $P=0.015$ and $F=7$, $P<0.001$,
respectively). In both fractions, high fertilized *P. nigra* seedlings had higher SC
395 concentration than low and fall fertilized seedlings, which showed no difference
between them (Figure 6). In contrast, fall fertilized plants and low fertilized plants had
in almost all cases the highest and lowest SC concentration, respectively, in *P. pinea* and
P. halepensis. Finally, *P. pinaster* root and needle SC concentrations were not affected
by N fertilization (Figure 6). Root and shoot SC significantly decreased between
400 November and January ($F=4.5$, $P=0.007$ and $F=7.1$, $P<0.001$, respectively).

Discussion

Despite the close phylogenetic relatedness of the studied species, N availability
differentially affected seedling frost tolerance among them. These differences conform
405 with knowledge of their ecology and frost acclimation physiology. We found three
patterns in the frost tolerance responses to N availability during the pre-hardening
period: 1) High N increased frost tolerance in *P. nigra*, the psychrophilic species that
lives in cold winter locations and has strong endodormant control of growth and cold
acclimation (Peñuelas et al. 2013); 2) High N hindered frost tolerance in the
410 thermophilic species, *P. halepensis* and *P. pinea*, the species inhabiting the mild winter
sites and having an eco-dormant growth and cold acclimation physiology (Navarro
Cerrillo et al. 2013, Puértolas et al. 2013); and 3) N fertilization had little effect on the
frost tolerance of the mesophilic species *P. pinaster*. This result supports our first
hypothesis and indicates that species ecology and dormancy physiology determine the

415 effect of N availability on plant frost tolerance. A major finding of our study was that
despite strong species differences in the frost response to N availability during pre-
hardening, N supply in the fall during the hardening period consistently increased the
frost tolerance in all species. This result does not support our hypothesis that the timing
of nutrient application will have a varying effect among species with contrasting
420 ecology. In contrast to findings linking frost tolerance to cessation of shoot elongation
in the fall (Repo et al. 2000, Heredia-Guerrero et al. 2014), SC concentration (Greer et
al., 2000; Tinus et al., 2000; Villar-Salvador et al. 2013), and organ N concentration
(DeHayes et al., 1989, Rikala and Repo, 1997), we found these attributes to be largely
unrelated to frost tolerance differences across species and N treatments. It is possible
425 that the differences among species may be the result of different strategies in using N
for frost tolerance and mechanisms of growth control (Charrier et al. 2013). We discuss
these species differences in frost tolerance strategies below.

Root and shoot frost tolerance

Shoot and root frost acclimation physiology showed notable differences. First,
430 shoot frost tolerance increased in all species between mid-fall and early-winter as
indicated by lower VDI values in January than in November after the -15°C freezing
tests. This result agrees with the well-known fall freezing acclimation patterns observed
in the aboveground tissues of temperate- and cold-climate trees (Tinus et al. 2000,
Bigras et al. 2001, Pardos et al. 2014). In contrast to shoots and consistent with our third
435 hypothesis, frost tolerance of roots remained unchanged between studied dates in all
species (Figure 1). A similar lack of root cold acclimation through the fall was found for
P. halepensis and *Pinus radiata* (Tinus et al. 2000) and *Juglans regia* (Charrier, Poirier,
et al. 2013). In contrast to our findings, significant fall root cold hardening was reported
for *Pseudotsuga menziesii* (Tinus et al. 2000). Soil insulates the roots from frost under

440 natural conditions, which could explain the low or lack of cold acclimation in some woody species. In climates where snow remains on soil for an extended period, however, the reduction of snow cover and/or earlier snow melting increases the risk of root damage by frost (Groffman et al. 2001, Schaberg et al. 2008).

Second, shoot and root frost tolerance responded similarly to N availability
445 during pre-hardening in the psychrophilic and mesophilic species (*P. nigra* and *P. pinaster*, respectively) and the response changed little between the studied dates. N availability during pre-hardening also affected frost tolerance in the thermophilic pines (*P. halepensis* and *P. pinea*) but unlike the psychrophilic pines, the response of frost tolerance to N availability diverged between organs and dates. While the roots exhibited
450 lowest frost tolerance in mid-fall and in winter with high rate of pre-hardening N fertilization, shoots had the opposite response in November, showing the lowest frost tolerance with the low rate of pre-hardening N fertilization. However, in January shoots had the same response to N as roots. The different effect of N availability on shoot frost tolerance in the thermophilic species through the cold season likely was the result of
455 distinct physiological processes, which importance varied along the cold acclimation process. We suggest that shoot frost tolerance was lowest in low fertilized seedlings in November, likely due to low N-rich cryoprotectant metabolites (Andivia et al. 2012). This hypothesis is supported by the fact that needle N concentration in November in the low fertilized plants was in most cases close to 10-13 mg g⁻¹ (Figure 4), which is
460 considered deficient for these species (deficient threshold values for studied species range between 11 to 15 mg g⁻¹ (Oliet et al. 2006, Lafuente Laguna et al. 2013, Navarro Cerrillo et al. 2013, Peñuelas et al. 2013, Puértolas et al. 2013). In contrast to results in November, the lower frost tolerance of high N fertilized seedlings in January of thermophilic species might be explained by cold dehardening compared to the other N

465 treatments (Fløistad and Kohmann 2004), thereby reducing their frost tolerance. The
earlier cold dehardening in high N fertilized seedlings could be due to the increase in
temperatures registered in mid-January (Figure S1, in Supplementary Material). In the
cryophilic species, *P. nigra* and to a lesser extent in *P. pinaster*, frost tolerance in both
shoots and roots was lowest for the low N pre-hardening treatment during both fall and
470 winter, which also supports the N deficiency hypotheses. Contrary to shoots, the effect
of N availability on root frost tolerance remained similar among species over the cold
season in our study, probably because roots do not experience cold hardening in these
species (Tinus et al. 2000, Kreyling et al. 2012).

Fernández et al (2017) showed no differences among our studied species in
475 vulnerability to stem xylem freezing-induced embolism. Thus, we presume that
differences in stem xylem freezing-induced embolism at interspecific level due to
changes in N availability are unlikely and that the differences in frost sensitivity in
response to N availability are due to differences in foliage and root frost sensitivity.

Cessation of shoot elongation

480 Cessation of shoot elongation is essential for frost acclimation to occur (Greer et
al. 2000). The idea that high-N fertilized plants are more vulnerable to frost lies on
observations that high N availability delays the cessation of shoot elongation in fall
(Heredia-Guerrero et al., 2014; Rikala and Repo, 1997). Consistent with this idea, an
increase in N availability delayed the cessation of shoot elongation in our study.
485 However, we did not find a clear connection between cessation of shoot elongation and
frost tolerance. Several results support this assertion. First, low N availability during
pre-hardening advanced the cessation of shoot elongation 20 days in three out of four
species, but low N seedlings had the lowest shoot frost tolerance in November (Figure

2). Second, fall fertilization strongly delayed growth cessation in most species but these
490 seedlings showed the highest frost tolerance in mid fall. Third, *P. nigra* and *P. pinaster*
showed similar timing of cessation of growth elongation but *P. nigra* was more cold-
tolerant than *P. pinaster*. A similar lack of relationship between frost tolerance and
growth cessation was found in *Pseudotsuga menziesii* (Hawkins et al., 1995) and *Pinus*
sylvestris (Rikala and Repo 1997). Thus, our results illustrate that cessation of shoot
495 elongation may trigger frost acclimation but does not affect the intensity of frost
acclimation. Consequently, species with late cessation of shoot elongation such as *P.*
pinea and *P. halepensis* incur a higher risk of freezing damage due to early fall frost
than species that cease elongation earlier in the fall, but without additional cascading
effects. We believe that the benefit of high N availability during fall may override
500 differences in timing of growth cessation once it has occurred (mid-fall frost tolerance
measurements were conducted after shoot growth cessation of all treatments and
species), and reinforces the idea that N supplied during hardening is preferentially
invested in frost tolerance N-rich metabolites.

Soluble carbohydrates

505 The general consensus in scientific literature is that SC are involved in plant
frost tolerance and an increase in frost tolerance is positively related to SC at a within-
species level (Greer et al. 2000, Tinus et al. 2000, Morin et al. 2007, Kreyling et al.
2012). However, we did not find a clear link between frost tolerance across N
fertilization treatments and SC. In our study, needle SC concentration was lower in the
510 psychrophilic (most frost tolerant) species *P. nigra*, than in the thermophilic (least frost
tolerant) species. Within species, frost tolerance followed a very different pattern
compared to SC concentrations under the influence of N treatments. Furthermore, SC

concentration in November was higher than in January; however, shoot frost tolerance increased over the same period. The lack of studies that compare the effect of soluble carbohydrates on frost tolerance across species complicates the clarification of this relationship. To our knowledge, this relationship has only been addressed in two interspecific studies (Morin et al. 2007, Charrier et al. 2013); however, the results are not conclusive, especially when broadleaves and conifers are compared under the same environmental conditions. Nevertheless, some studies show that a clear cause/effect relationship does not exist between soluble carbohydrates concentration and frost tolerance (Zhang et al. 2003, Andivia et al. 2011). In agreement with these studies, our results show a similar lack of relationship between soluble carbohydrates concentration and frost tolerance in the tested pine species, reinforcing the idea that other metabolites than soluble sugars such as antifreeze proteins and membrane stabilization metabolites are more important for cold hardening of pines species (see discussion below).

As for the shoot, root SC concentration was unrelated to frost tolerance. Roots of pine species invest most SC in growth during winter (Hansen and Beck 1994) and so it is likely that SC are involved relatively little in frost tolerance; rather, variation in concentration might reflect physiological activities such as storage, remobilization, and fine root growth over winter.

Nitrogen concentration in needles and roots

The role of tissue N in the frost tolerance of plants is controversial (Villar-Salvador et al. 2015). While some studies have shown positive links between frost tolerance and tissue N (Rikala and Repo 1997, Bigras et al. 2001, Andivia et al. 2011) others have shown the reverse trend (Hawkins et al. 1995, Fløistad and Kohmann 2004, Villar-Salvador et al. 2013). In our study, we have found evidence supporting a positive relationship between both variables. For instance, low-N fertilized plants, which showed

the lowest tissue N concentration (Figure 4), had overall lower shoot frost tolerance in November than the high-N fertilized seedlings. Many plants invest available N during
540 cold hardening in antifreeze proteins and dehydrins (Kontunen-Soppela et al. 2000, Atıcı and Nalbantoğlu 2003, Griffith and Yaish 2004) and N was found to be critical for repairing root xylem frost-induced embolism in several species (Ewers et al. 2001). Other results of our study, however, do not support a link between frost tolerance and tissue N. For instance, tissue N concentration in November was greatest in the highest
545 and lowest frost tolerant species, *P. nigra* and *P. halepensis*, respectively. Similarly, within species, seedlings showing similar N concentrations varied significantly in their frost tolerance. Specifically, fall fertilized seedlings had higher frost tolerance than pre-hardening high-N fertilized plants, particularly in thermophilic species. Taken together, our results indicate that timing of N supply is critical for frost tolerance and that the
550 variation in frost tolerance between treatments and species are not driven by differences in tissue N concentrations *per se*, but likely by how N is allocated to major plant functions. It is possible that N taken up during the period of cold hardening and cessation of shoot elongation is mainly allocated to N-rich compounds involved in frost tolerance (Andivia et al. 2012), while N supplied during the pre-hardening season is
555 primarily allocated to growth at the expense of frost tolerance and this trade-off may differ among species. This suggestion could explain why some studies have reported that high fertilization applied at pre-hardening reduced frost tolerance of thermophilic species (Puérolas et al. 2005; Villar-Salvador et al. 2013). Future comparative studies of the timing and magnitude of N availability should be designed to: 1) address whether
560 species differ in allocation to N-metabolites involved in frost tolerance; and 2) identify specifically which N-metabolites are involved in these processes.

Conclusions

Four *Pinus* species of close phylogenetic relationship, yet with distinct
565 ecophysiology of cold hardening, showed variable frost tolerance response to N
availability at pre-hardening when cultivated under the same environmental conditions.
In contrast, N availability during hardening clearly increased frost tolerance of all four
species, highlighting the importance of timing of N availability for development of frost
resistance of seedlings. Our results can help to design specific fertilization regimes for
570 plant production, with emphasis on the fall fertilization treatment due to its increase of
frost tolerance and N reserves. Differences in frost tolerance among species and levels
of N availability were not explained by gradients of soluble carbohydrate concentrations
nor by timing of growth cessation. Additionally, frost resistance dynamics during
hardening varied between roots and shoots. Shoots and roots were sensitive to N
575 availability, but while shoots increased in frost tolerance over winter and (in the
thermophilic species) can alter the relationships between N and frost tolerance, roots
remained unchanged along the hardening period. These contrasting effects of N on the
frost tolerance of roots and shoots among pine species may be a result of physiological
differences among species and the frost acclimation strategies of specific organs.

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Table 1: Statistical results of the effect of species and fertilization treatments on visual damage index values after root and shoot freezing tests at different target temperatures in November and January. Data are F-values and P-values in brackets.

	Root		Shoot			
	November	January	November		January	
	-10 °C	-10 °C	-8 °C	-15 °C	-15 °C	-23 °C
Species	9.1 (<0.001)	6.2 (0.001)	32.3 (<0.001)	118 (<0.001)	30.1 (<0.001)	549 (<0.001)
Treatment	5.7 (0.005)	7.0 (0.002)	13.5 (<0.001)	0.6 (0.45)	6.7 (0.002)	4.2 (0.02)
Spp. x Treat	2.9 (0.015)	1.8 (0.11)	6.3 (<0.001)	0.2 (0.023)	2.0 (0.065)	4.1 (0.002)

Figure captions

Figure 1: Visual damage index (VDI) after root freezing at -10 °C in November (a) and January (b) in one-year old seedlings of four pine species that were cultivated with three nitrogen fertilization treatments (pre-hardening low and high N, and fall fertilization). Data are means \pm 1 SE. Mean values not sharing common letters are significantly different.

Figure 2: Visual damage index (VDI) after shoot freezing at -8 °C in November (a) and -15°C January (b) in one-year old seedlings of four pine species that were cultivated with three N fertilization treatments (pre-hardening low and high N, and fall fertilization). Data are means \pm 1 SE. Mean values not sharing common letters are significantly different.

Figure 3: Julian day of cessation of shoot elongation in one-year old seedlings of four pine species that were cultivated with three N fertilization regimes (pre-hardening low and high N, and fall fertilization). Data are means \pm 1 SE. Mean values not sharing common letters are significantly different.

Figure 4: N concentration (Mg/g) in one-year old seedlings needles and roots of four pine species that were cultivated with three N fertilization regimes (pre-hardening low and high N, and fall fertilization) in November (a,b) and January (c,d). Data are means \pm 1 SE. Mean values not sharing common letters are significantly different.

Figure 5: Species (left figures) and N fertilization treatment (right figures) differences in soluble carbohydrates (SC; Glucose+fructose+sucrose) concentration in needles (upper row) and roots (lower row) in one-year old seedlings of four pine species sampled in November. Data are means \pm 1 SE. Mean values not sharing common letters are significantly different.

Figure 6: Soluble carbohydrate (SC; Glucose+fructose+sucrose) concentration in needles (a) and roots (b) of one-year old seedlings sampled in January in four pine species that were cultivated with three N fertilization regimes (pre-hardening low and high N, and fall fertilization). Data are means \pm 1 SE. Mean values not sharing common letters are significantly different.

Figure S1: Temperature (°C) progression in the nursery from September 2012 to the end of January 2013. The arrows indicate the date of the freezing test.

Figure 1

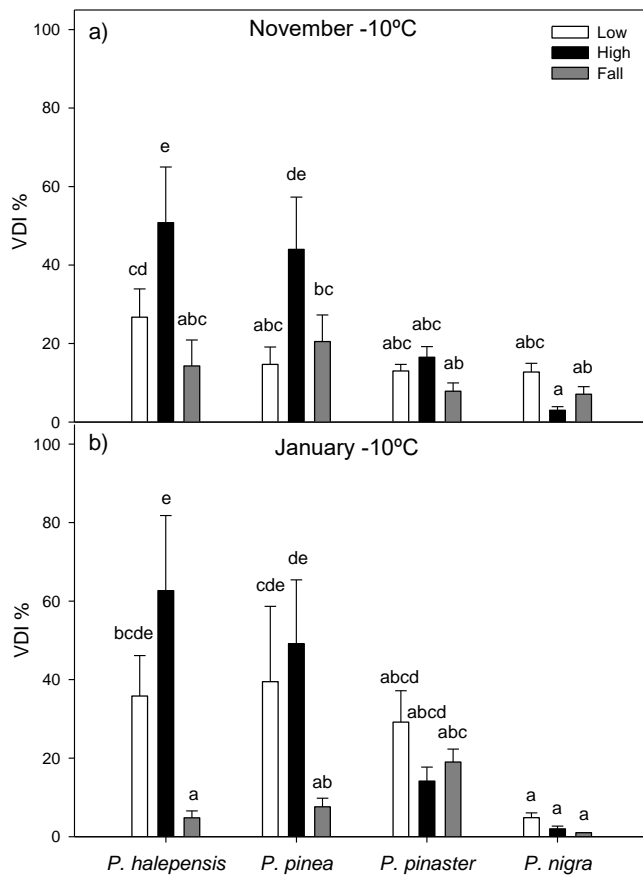


Figure 2

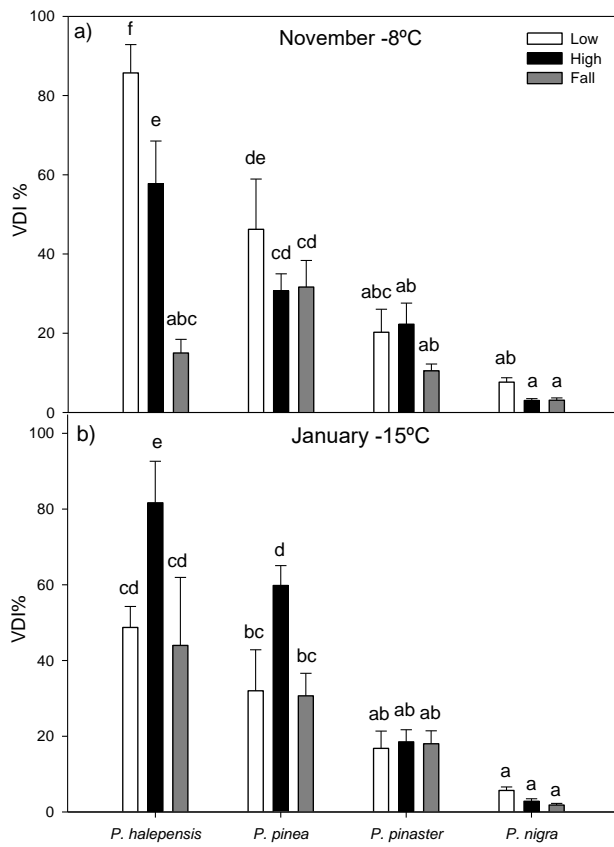


Figure 3

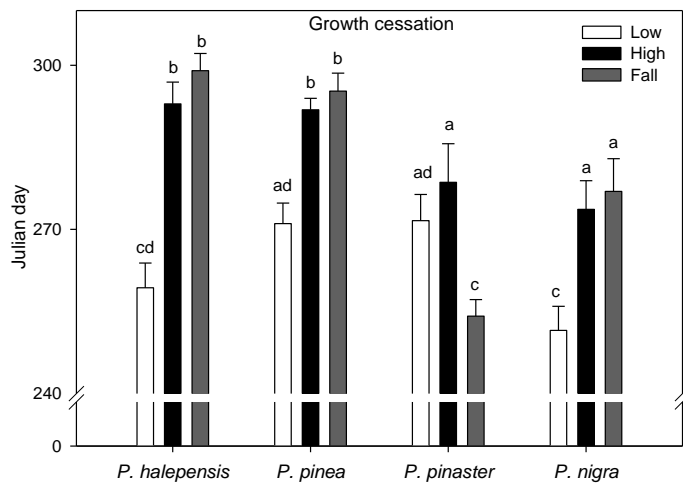


Figure 4

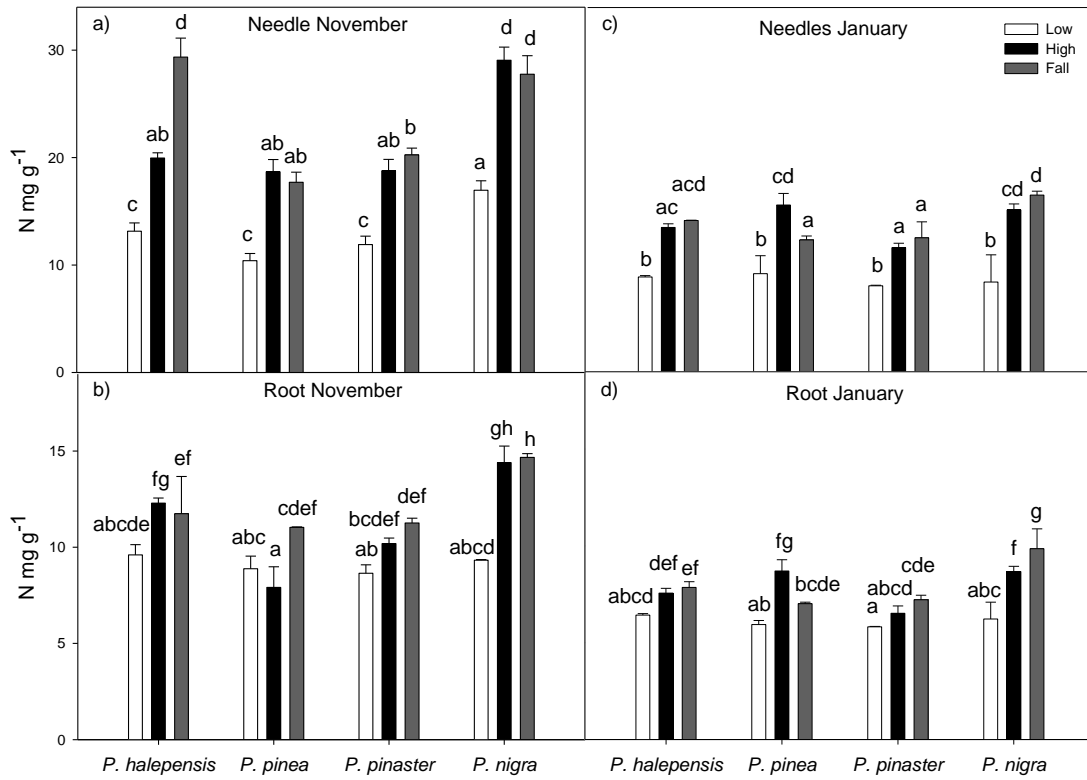


Figure 5

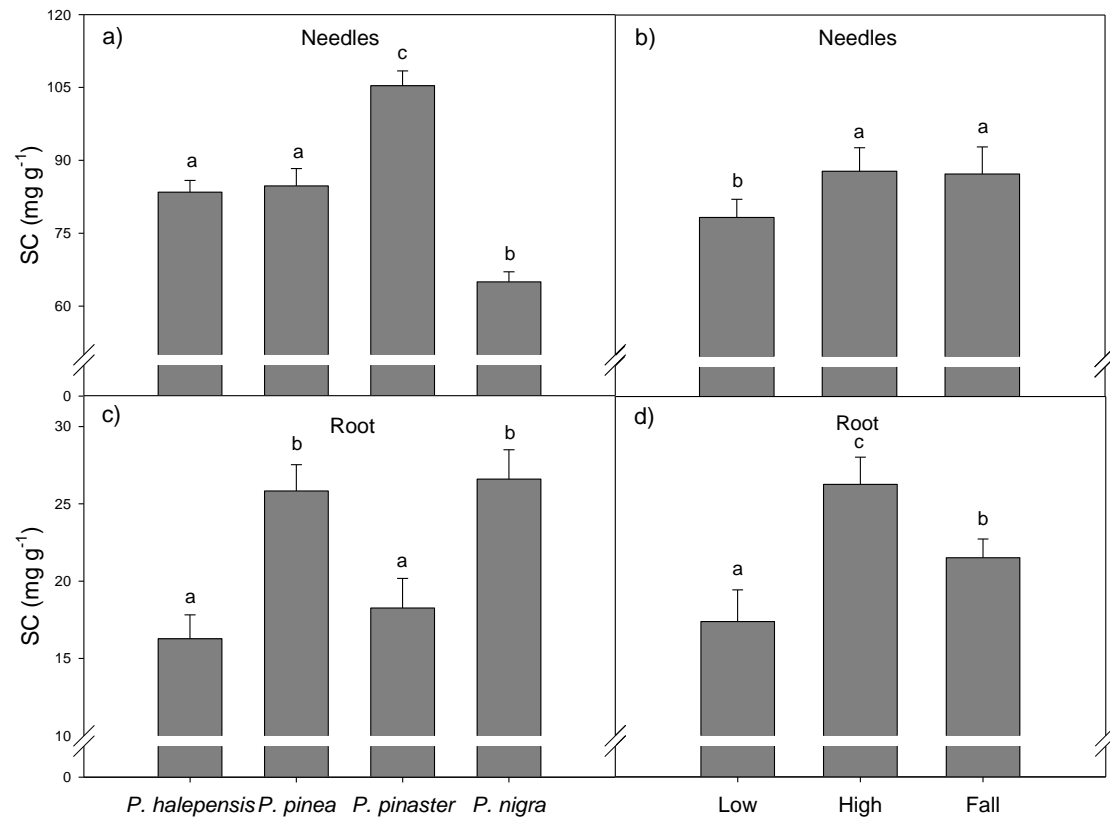
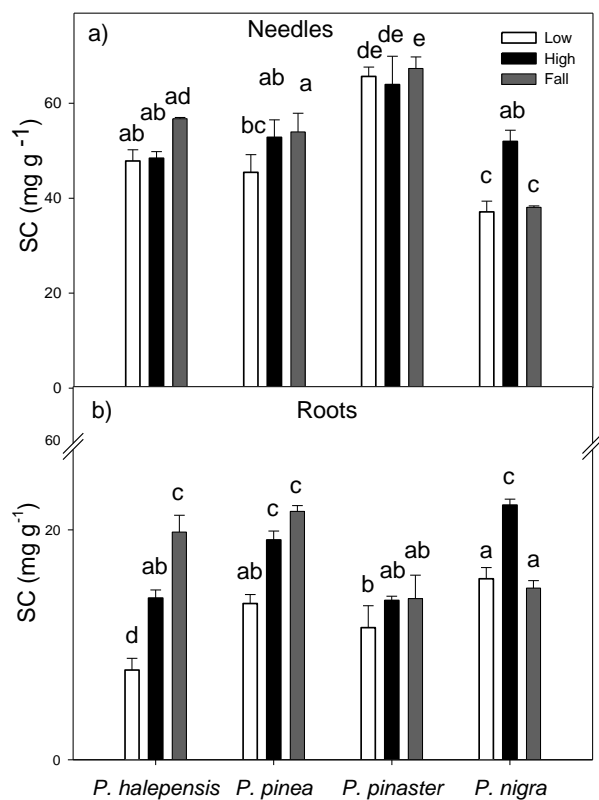


Figure 6



Supplementary material

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

Species	Provenance	Latitude (N)	Longitude (W)	Altitude (masl)	MAT (°C)	TCM (°C)	Annual rainfall (mm)
<i>P. halepensis</i>	Alcarria, ES07	40°24'52''	2°24'33''	860	12.6	-0.6	580
<i>P. pinea</i>	La Mancha, ES03	39°12'02''	1°57'59''	675	14.2	0.7	397
<i>P. pinaster</i>	Cuenca, ES12	39°38'44''	1°13'52''	1135	12	-1.5	540
<i>P. nigra</i> subsp. <i>salzmanii</i>	Sistema Ibérico Meridional, ES07a	40°15'16''	1°58'22''	1515	10.4	-3.2	894

Figure S1

