



Research paper

Nitrogen nutrition and drought hardening exert opposite effects on the stress tolerance of *Pinus pinea* L. seedlings

Pedro Villar-Salvador^{1,3,4}, Juan L. Peñuelas¹ and Douglass F. Jacobs²

¹Centro Nacional de Recursos Genéticos Forestales 'El Serranillo', Ministerio de Agricultura, Alimentación y Medio Ambiente, Apdo. 249, 19080 Guadalajara, Spain; ²Department of Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center, Purdue University, West Lafayette, IN 47907-2061, USA; ³Present address: Forest Ecology and Restoration Group, Departamento de Ciencias de la Vida, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid, Spain; ⁴Corresponding author (pedro.villar@uah.es)

Received May 30, 2012; accepted December 12, 2012; published online January 30, 2013; handling Editor João Pereira

Functional attributes determine the survival and growth of planted seedlings in reforestation projects. Nitrogen (N) and water are important resources in the cultivation of forest species, which have a strong effect on plant functional traits. We analyzed the influence of N nutrition on drought acclimation of *Pinus pinea* L. seedlings. Specifically, we addressed if high N fertilization reduces drought and frost tolerance of seedlings and whether drought hardening reverses the effect of high N fertilization on stress tolerance. Seedlings were grown under two N fertilization regimes (6 and 100 mg N per plant) and subjected to three drought-hardening levels (well-watered, moderate and strong hardening). Water relations, gas exchange, frost damage, N concentration and growth at the end of the drought-hardening period, and survival and growth of seedlings under controlled xeric and mesic outplanting conditions were measured. Relative to low-N plants, high-N plants were larger, had higher stomatal conductance (27%), residual transpiration (11%) and new root growth capacity and closed stomata at higher water potential. However, high N fertilization also increased frost damage (24%) and decreased plasmalemma stability to dehydration (9%). Drought hardening reversed to a great extent the reduction in stress tolerance caused by high N fertilization as it decreased frost damage, stomatal conductance and residual transpiration by 21, 31 and 24%, respectively, and increased plasmalemma stability to dehydration (8%). Drought hardening increased tissue non-structural carbohydrates and N concentration, especially in high-fertilized plants. Frost damage was positively related to the stability of plasmalemma to dehydration ($r = 0.92$) and both traits were negatively related to the concentration of reducing soluble sugars. No differences existed between moderate and strong drought-hardening treatments. Neither N nutrition nor drought hardening had any clear effect on seedling performance under xeric outplanting conditions. However, fertilization increased growth under mesic conditions, whereas drought hardening decreased growth. We conclude that drought hardening and N fertilization applied under typical container nursery operational conditions exert opposite effects on the physiological stress tolerance of *P. pinea* seedlings. While drought hardening increases overall stress tolerance, N nutrition reduces it and yet has no effect on the drought acclimation capacity of seedlings.

Keywords: carbohydrates, drought tolerance, frost damage, Mediterranean, plasmalemma stability, residual transpiration, root growth capacity, stomatal conductance, survival.

Introduction

Water stress and frost are major limiting factors for plant life in Mediterranean continental climate areas (Larcher 1981, Castro

et al. 2004). Seedlings planted in forest projects in these areas must therefore be cold resistant and able to withstand drought. Water stress applied in the nursery can enhance drought and

frost tolerance of plants before planting (Blake et al. 1979, Vilagrosa et al. 2003, Villar-Salvador et al. 2004a, Beikircher et al. 2010). Specifically, seedlings of Mediterranean woodland species are capable of osmotic adjustment, reduced transpiration, and enhanced dehydration tolerance and photochemical efficiency in response to drought hardening during nursery cultivation (Vilagrosa et al. 2003, Villar-Salvador et al. 2004a, Bañon et al. 2006). These changes can potentially enhance plant performance under dry conditions (Arnott et al. 1993, Bañon et al. 2006). van den Driessche (1991a) observed that drought hardening improved seedling outplanting survival in two boreal conifer species through a reduction in the shoot/root mass ratio.

Soil nutrient availability induces large changes in plant functional attributes, which affect the water and carbon economy of plants (van den Driessche 1992, Salifu and Timmer 2003). In particular, nitrogen (N) can affect the cold and drought tolerance, yet there is no clear consensus on the magnitude or direction of its effect. Low tissue N concentration may hinder either cold or drought hardening (Saneoka et al. 2004, Islam et al. 2009, Andivia et al. 2011) but high N may increase frost susceptibility by reducing either seedling frost tolerance or accelerating cold dehardening and delaying cold acclimation (Hellergren 1981, Zhu et al. 2001). In some species, high N reduces the capacity to maintain turgor pressure at low water potential (Morgan 1986, Tan and Hogan 1995) and increases vulnerability to xylem cavitation (Harvey and van den Driessche 1997). Similarly, high N fertilization can cause pronounced morphological imbalances that may adversely affect the water economy of the plant (Lloret et al. 1999), potentially hindering outplanting performance on dry sites (Trubat et al. 2011). In contrast, plants with high tissue N content support greater new root and shoot growth after outplanting through nutrient remobilization (Salifu and Timmer 2003), which may be particularly important for plants growing on poor soils and dry sites (Oliet et al. 2009, Salifu et al. 2009, Villar-Salvador et al. 2012).

When frost and drought both impair seedling establishment, such as in Mediterranean continental areas, performance of forest plantations would likely be improved if planted seedlings exhibited some of the functional attributes induced by high fertilization rates, yet without potential negative impacts. This might be achieved by growing plants at high N fertilization rates during the entire cultivation period to promote nutrient loading but subjecting them to moderate water stress during the final weeks of cultivation to stop growth and stimulate drought resistance. In this study, we examined the interaction of N nutrition and drought hardening on several physiological and morphological attributes relevant for stress resistance and establishment of the Mediterranean pine *Pinus pinea* L. (stone pine). Earlier studies on the combined effect of water stress and nutrient availability have been chiefly performed in herbaceous crop plants (Morgan 1986, Ghashghaie and Saugier 1989). In con-

trast, studies on woody forest species are less common and have not shown any clear trend regarding drought tolerance of woody species. Some studies have reported synergy (DaMatta et al. 2002), antagonism (Walters and Reich 1989) or no interaction (Correia et al. 1989, Kleiner et al. 1992) between N availability and drought hardening on drought acclimation of plants. *Pinus pinea* is a drought-resistant Mediterranean tree that commonly thrives on sandy soils, which are very nutrient poor and have low water-holding capacity. Silvicultural treatments such as improvement of soil fertility or planting of seedlings inoculated with mycorrhiza increase outplanting survival and growth of *P. pinea* seedlings on plantation sites (Parladé et al. 2004, Larchevêque et al. 2006). This pine is frequently planted for ecological restoration and the high economic value of its pinyon nuts. Specifically, we assessed whether (i) high N availability reduces drought and frost tolerance of stone pine seedlings, and if this occurs, whether drought hardening can reverse the effect of high N fertilization, and (ii) drought hardening and N fertilization improve outplanting survival under drought conditions. To fulfil these objectives, seedlings were grown under two contrasting N fertilization regimes and subjected to three drought-hardening levels. Water relations, gas exchange, frost tolerance, N and non-structural carbohydrate concentration and growth were measured. Finally, we assessed the survival and growth of seedlings outplanted under controlled xeric and mesic conditions.

Material and methods

Plant material and experimental design

The experiment was conducted in the nursery of the Centro Nacional de Recursos Genéticos Forestales 'El Serranillo' (MAGRAMA), which is located in Guadalajara (the center of the Iberian Peninsula). A seed lot of *P. pinea* from La Mancha provenance was used to cultivate 2400 plants. Seeds were sown in early April 1997 in 48 Forest Pot300® (Nuevos Sistemas de Cultivo S.L., Girona, Spain) trays (each tray has 50 cavities of 300 ml). The growing medium was a 80 : 20 (v : v) mixture of blonde peat and vermiculite. Two N fertilization regimes were differentiated: high (N+) and low (N-). In the former, half of the plants (1200 seedlings, 24 trays) received a total of 100 mg N seedling⁻¹ during the entire cultivation period. In the latter, the remaining half of the plants received 6 mg N seedling⁻¹. The high N fertilization rate applied in our study is slightly higher than the rate typically used to cultivate stone pine in Spanish nurseries (Domínguez-Lerena et al. 2006), but it is within the range of N fertilization rate recommended to cultivate high-quality seedlings in several Mediterranean forest trees (Villar-Salvador et al. 2004b, Oliet et al. 2009). Nitrogen was supplied using NH₄NO₃ fertilizer (33.5% N richness, Fertiberia, Madrid, Spain). Plants from both fertilization treatments received 20 and 40 mg per plant of P and K, respectively,

using $K_2P_2O_5$ fertilizer (Inafol O11, Sipcam-Inagra, Valencia, Spain) and a mixture of micronutrients (Kanieltra, Hydro Agri, Oslo, Norway). Fertilization began on 1 June, 1 month after seedling emergence, and was implemented once per week in two steps. First, all plants were supplied with the N-, P, K and micronutrient fertilization solution by overhead sprinkling fertirrigation. Then, the N+ plants individually received the remaining N that was applied manually with a syringe. Until 15 August, each fertilization treatment received 70% of their total N. The remaining 30% was applied over 4 days along the drought-hardening period.

Drought hardening was imposed from mid-August to mid-November 1997. Three drought treatments were differentiated: well-watered (W), moderate (M) and strong (S) drought. W plants were irrigated every 1–3 days and M and S plants were watered when container weight was reduced to 43–45 and 47–50%, respectively, of their maximum weight at saturation. Six treatments resulted from the drought hardening \times N fertilization combination: high N with well-watered (WN+), moderate (MN+) and strong drought stress (SN+), and low N with well-watered (WN-), moderate (MN-) and strong drought stress (SN-). MN+ and MN- seedlings were exposed to 10 drought cycles, while SN+ and SN- plants experienced eight drought cycles. In most occasions, N+ plants usually reached drought target levels 1 day before N- plants and in one occasion the difference was 2 days. After the first drought cycle, predawn xylem water potential (Ψ_{pd}) of the W, M and S seedlings was: -0.30 ± 0.04 , -0.84 ± 0.08 and -1.35 ± 0.34 MPa ($n = 4-8$), respectively. Ψ_{pd} was measured with a home-built pressure chamber using twigs.

Treatments were arranged in a randomized complete block design with four replicate blocks. To avoid exposure to late spring frosts, plants were initially grown in a greenhouse until early June 1997 and thereafter grown outdoors except on rainy days where seedlings were covered with a transparent plastic sheet to avoid precipitation. At the end of the hardening period in mid-November 1997, all treatments were well watered and left to recover for 3 days before physiological and morphological analyses. Temperature and photoperiod conditions during most of the experiment were typical of summer and fall in the center of the Iberian Peninsula (Mollá et al. 2006).

Shoot water relations

Pressure–volume ($P-V$) curves were made at the beginning of the hardening period and again 5–10 days after the end of the hardening period at the end of the experiment, by following the free-transpiration method described in Koide et al. (1989). The shoot water potential was determined with a home-built pressure chamber. Eight randomly sampled plants per treatment were watered the afternoon before and maintained in the dark until the morning of shoot sampling. From each $P-V$ curve,

the osmotic potential at the turgor loss point ($\Psi_{\pi tp}$), the osmotic potential at saturation ($\Psi_{\pi sat}$), the modulus of elasticity (ϵ) and the symplasm volume fraction (V_s/V_t) were calculated as described by Koide et al. (1989). Shoot weight at full saturation was calculated by following the method described in Kubiske and Abrams (1990).

Electrolyte leakage, water potential and water vapor gas exchange

Ten days after the end of the drought stress period, 50 plants per treatment were randomly selected, thoroughly watered and left to dry out for 8 weeks in a greenhouse without any irrigation. Plants were kept in the same containers from cultivation during desiccation. The temperature inside the greenhouse varied between 5 and 25 °C and the photosynthetic photon flux density (PPF) was half of that outside, which was measured with a quantum sensor (Li 190 S-1, Li-Cor Inc., Lincoln, NE, USA). During the 8-week dry-out period, four to six seedlings per treatment were periodically sampled for their water content (WC), Ψ_{pd} , electrolyte leakage (E_L) and stomatal conductance to water vapor (g_s). At the first four sampling dates (Days 0, 11, 18 and 24), plants in each treatment were randomly selected. Afterwards, and due to the differential desiccation rates of fertilization treatments, subsequent sampling was directed to obtain an ample range of Ψ_{pd} , WC, E_L and g_s values in each treatment. WC and Ψ_{pd} were measured on the same twig. Ψ_{pd} was measured with a home-built pressure chamber on twigs, and WC was calculated as

$$\frac{(\text{Twig fresh weight}) - (\text{Twig mass})}{(\text{Twig mass})} \times 100\% \quad (1)$$

Twig mass was determined after oven drying at 80 °C for 48 h. g_s was measured with a porometer (LI-1600; Li-Cor Inc.) between 11:00 and 13:00 h (solar time), with the temperature, relative humidity and PPF varying during the measurements between 20 and 22 °C, 57 and 65% and 159 and 239 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively.

E_L was measured after g_s measurements in needles growing on the main stem using the methodology in Earnshaw (1993). Briefly, needles were cut into small pieces and washed twice in distilled water for 20 min. They were then placed in a vial with 20 ml distilled water on an illuminated bench at laboratory temperature and periodically shaken. E_L was determined as

$$\frac{EC_i}{EC_f} \times 100\% \quad (2)$$

where EC_i is the electroconductivity of the water bathing the needle pieces after 24 h and EC_f the electroconductivity of the same water after autoclaving the vials for 10 min at 120 °C.

Autoclaving produces tissue destruction and complete release of cell solutes.

Residual transpiration (R_T) was determined in 10 seedlings per treatment that were watered and enclosed in an opaque plastic bag to ensure saturation overnight. In the morning, shoots were excised and left to dry in a ventilated growth chamber in which the mean temperature and water vapor pressure deficit were 21.7 ± 0.22 °C and 0.56 ± 0.01 kPa, respectively, and PPF was $150 \mu\text{mol m}^{-2} \text{s}^{-1}$. Shoot fresh mass was measured to the nearest 1 mg at intervals of 0.5–1 h. By plotting shoot fresh weight vs. time, a curvilinear relationship is obtained in which the linear portion represents water loss from plant surfaces after stomatal closure. The residual transpiration rate of each shoot was calculated on a mass basis as the ratio of the slope of the linear portion and the shoot mass measured after drying at 80 °C for 48 h.

Plant morphology, nitrogen, non-structural carbohydrates and root growth capacity determinations

A set of 28 and 32 plants per treatment (seven to eight plants per block) was randomly selected immediately before the onset of the drought-hardening period and at the end of the experiment, respectively, and immediately frozen to -30 °C until processing for morphological and N concentration measurements. Once defrosted, shoots were cut at the cotyledon insertion point. Root plugs were washed from the growing media, rinsed in distilled water and, together with shoots, dried at 60 °C for 48 h and weighed. To assess N concentration, shoots and roots of plants sampled in a block were pooled separately and ground. Nitrogen concentration was determined by the standard Kjeldahl procedure. Starch and reducing soluble carbohydrates (RSCs) were determined by following the methodology of Coombs et al. (1987) and Vicente et al. (2000), respectively.

We initiated a root growth capacity (RGC) test 3 days after the end of the hardening period. Sixteen seedlings per treatment were transplanted into 3-l pots (one plant per pot) containing perlite. Plants were randomly arranged in a glasshouse where the temperature varied from 15 to 25 °C. PPF was not measured during the test but it was half of external ambient as measured with a quantum sensor (Li 190 S-1, Li-Cor Inc.). Seedlings were kept well watered by irrigating them every other day. After 21 days, plants were lifted, cleaned from the potting medium and any new roots longer than 1 cm protruding from the root plug were cut along with the shoots and oven dried at 50 °C for 48 h to measure their mass. The RGC of each plant was determined as the total mass of new roots.

Frost damage

Whole plants were subjected to a frost cycle and frost damage (F_D) was evaluated by measuring E_L . Five seedlings per treatment

were transplanted into a plastic box (40 × 100 × 40 cm; width, length, height) with moist river sand and were placed in a freezer. The sand was used to isolate the roots from the frost. The temperature was reduced at a rate of 4 °C h⁻¹ to -8 °C where it remained for 3 h and then it was raised to 10 °C at a rate of 4 °C h⁻¹. E_L was measured on needles utilizing the same protocol described above. The frost cycle was repeated with a second batch of five seedlings per treatment to complete 10 replicates per treatment.

Outplanting performance

The survival and growth of seedlings outplanted under xeric and mesic conditions was assessed in a ventilated greenhouse for 11 months. Six 0.6 m³ containers (1 m² surface and 0.6 m depth) were filled with washed river sand. In each container, the six treatments were randomly assigned to one of six rows of eight plants. Seedlings were transplanted in November 1997, 4 days after the end of the drought-hardening period. After transplanting, all containers were irrigated and three were assigned to a mesic environment and the remainder to a xeric environment. The experimental design was a split plot with three replicate blocks. Containers in the mesic environment were irrigated every week, whereas containers in the xeric environment were watered once the shoot apex of more than 50% of all the plants in a container showed wilting symptoms. Containers in the xeric environment were irrigated twice during the experimental period. The first irrigation took place in mid-June 1998 and the Ψ_{pd} of seedlings ($n = 12$) was measured with a home-built pressure chamber on lateral twigs to assess water stress differences between outplanting environments. The second irrigation occurred in mid-August 1998. The plant height and root collar diameter were measured 5 weeks after planting and again at the end of the experiment in October 1998, after one growing season. The stem volume was calculated as a measure of seedling shoot size as

$$0.33\pi(0.5 \times \text{diameter})^2 \times \text{height}(\text{cm}^3) \quad (3)$$

assuming that the stem of the seedling is a cone. Growth was defined as the stem volume increase (SVI). Survival was recorded at the end of the experiment.

Data analyses

The water relations, morphology, R_T and F_D data were analyzed by a two-way ANOVA. Ψ_{pd} and g_s data measured on different dates were analyzed by a three-way fixed ANOVA model. The experimental unit used in statistical analyses of morphological and N concentration data was the block. For the remaining variables, the experimental unit was the plant. The RGC was analyzed by analysis of covariance (ANCOVA), where shoot

mass was used as a covariable because we expected that variation in shoot size among treatments accounted for a significant part of the differences in RGC. Survival in the xeric environment was analyzed by a generalized linear model with a binomial distribution and a logit link function, the factors being block, fertilization and drought hardening. Growth in the outplanting trial was analyzed by ANOVA, the experimental design being a split plot with three blocks. The outplanting environment (xeric and mesic) was the main plot factor, and fertilization and drought hardening were applied to subplots. Differences in plasmalemma stability to tissue dehydration were determined by comparing E_L of treatments at 95% water content, which is the WC limit when seedlings began to die (approximately -3.8 to -4.0 MPa). We performed a generalized nonlinear model with a normal distribution and a logit link function, where $\log E_L$ was the dependent variable, fertilization and drought hardening were the independent variables and water content was a covariable. We used water content instead of Ψ_{pd} as covariable because goodness of fit was better for the former than for Ψ_{pd} .

Because larger plants desiccated faster than smaller seedlings (data not shown), differences in g_s among treatments across time were analyzed by ANCOVA with Ψ_{pd} as a covariable and adjusted means were computed. To compare the reduction of g_s with Ψ_{pd} in each treatment, the mean g_s obtained on each measuring date was divided by the mean maximum g_s value of each treatment, which was obtained on the first sampling date (Figure 2). Statistical analyses were performed using the general and generalized linear model modules of STATISTICA 7.0 (StatSoft, Tulsa, Oklahoma, USA).

Results

Water relations, residual transpiration and frost damage

Prior to the drought-hardening period, N+ and N- plants did not differ in $\Psi_{\pi sat}$ (-0.92 vs. -1.01 MPa), $\Psi_{\pi tlp}$ (-1.27 vs. -1.41 MPa) or ϵ (3.49 vs. 4.33 MPa). At the end of the drought-hardening period, $\Psi_{\pi sat}$, $\Psi_{\pi tlp}$ and ϵ were unaffected by either N fertilization or drought hardening (Table 1). W plants had significant seasonal reduction in $\Psi_{\pi sat}$, $\Psi_{\pi tlp}$ and ϵ from mid-August to mid-November (0.52 and 0.70 MPa for $\Psi_{\pi sat}$ and $\Psi_{\pi tlp}$, respectively, $P < 0.001$; 2.42 MPa for ϵ , $P = 0.028$).

N+ plants had higher E_L than N- plants ($P < 0.001$) and drought hardening reduced it. M seedlings had significantly lower E_L than W plants, while S seedlings had intermediate E_L values between M and W plants (Table 1). Before the drought-hardening period, N+ plants had lower R_T than N- seedlings (293 vs. $331 \mu\text{mol kg}^{-1} \text{s}^{-1}$, respectively; $P = 0.057$). At the end of the drought conditioning period, these differences persisted although the effect was marginal. Drought hardening reduced the R_T with W plants having higher R_T than M and S seedlings, which did not differ between them.

Nitrogen fertilization increased F_D , N+ plants having 24% higher F_D than N- ones (Table 1). In contrast, drought hardening reduced F_D , M and S seedlings having 21.3 and 18.9%, respectively, less F_D than W plants.

Morphology, RGC and tissue nitrogen and non-structural carbohydrate concentration

Before the drought-hardening period, N+ plants were significantly larger and had higher S/R than N- seedlings (plant mass: 2.66 vs. 2.41 g, $P = 0.02$ and S/R: 2.42 vs. 2.06 ,

Table 1. Plant morphology, water relations, frost damage and concentration of starch and reducing soluble sugars in *P. pinea* seedlings cultivated under two N fertilization regimes and subjected to three drought-hardening levels. Data are means \pm 1SE. Frost damage was measured after a frost cycle to -8 °C. $\Psi_{\pi sat}$, osmotic potential at saturation; $\Psi_{\pi tlp}$, osmotic potential at the turgor loss point; ϵ , modulus of elasticity near full turgor; V_s/V_t , symplasm volume fraction; E_L , electrolyte leakage at 95% water content; R_T , residual transpiration.

	Nitrogen fertilization		Drought hardening			ANOVA results $P > F$		
	Low N	High N	Well watered	Moderate drought	Strong drought	Fertilization (1)	Drought hardening (2)	1 \times 2
Plant mass (g)	3.48 \pm 0.09	4.05 \pm 0.10	4.99 \pm 0.13	3.42 \pm 0.09	3.39 \pm 0.09	<0.001	<0.001	0.085
Shoot/root mass ratio	1.77 \pm 0.02	2.13 \pm 0.03	1.87 \pm 0.05	2.02 \pm 0.06	1.97 \pm 0.06	<0.001	0.018	0.90
$\Psi_{\pi sat}$ (MPa)	-1.49 \pm 0.06	-1.55 \pm 0.05	-1.48 \pm 0.07	-1.61 \pm 0.06	-1.47 \pm 0.07	0.48	0.32	0.61
$\Psi_{\pi tlp}$ (MPa)	-2.06 \pm 0.07	-2.13 \pm 0.05	-2.04 \pm 0.08	-2.21 \pm 0.05	-2.04 \pm 0.07	0.46	0.21	0.65
ϵ (MPa)	6.7 \pm 0.6	6.7 \pm 0.5	6.3 \pm 0.6	7.2 \pm 0.6	6.6 \pm 0.7	0.94	0.57	0.33
V_s/V_t	0.51 \pm 0.04	0.51 \pm 0.04	0.48 \pm 0.06	0.52 \pm 0.05	0.53 \pm 0.04	0.95	0.69	0.043
E_L (%)	42.9 \pm 1.0	46.9 \pm 1.0	46.8 \pm 1.0	43.3 \pm 1.0	44.5 \pm 1.0	<0.001	0.035	0.26
R_T ($\mu\text{mol kg}^{-1} \text{s}^{-1}$)	290 \pm 15	323 \pm 15	359 \pm 18	274 \pm 16	286 \pm 16	0.06	<0.001	0.45
Frost damage (%)	21.9 \pm 0.7	27.2 \pm 1.8	28.4 \pm 1.9	22.3 \pm 1.5	23.0 \pm 1.7	0.009	0.004	0.29
Starch (mg g^{-1})	2.83 \pm 0.21	1.94 \pm 0.18	1.82 \pm 0.26	2.47 \pm 0.21	2.84 \pm 0.29	<0.001	0.004	0.61
Reducing soluble sugars (mg g^{-1})	37.6 \pm 2.6	30.7 \pm 3.3	28.2 \pm 5.5	37.9 \pm 3.1	35.9 \pm 1.8	0.058	0.09	0.33

$P < 0.001$ for N+ and N- plants). Differences between N fertilization treatments persisted at the end of the hardening period (Table 1). Drought affected total plant mass and S/R. W plants had higher mass than either M or S seedlings, which did not differ between them, whereas S/R was lower in W plants than in drought-hardened seedlings.

Drought hardening reduced RGC in N+ plants but had no effect on N- seedlings (interaction N fertilization \times drought hardening, $P = 0.04$) (Figure 1). The RGC was positively correlated with shoot mass ($r = 0.71$, $P < 0.001$). When the shoot mass was used as a covariable in the ANCOVA, shoot size and drought hardening had a significant effect on RGC ($P < 0.001$ and $P = 0.004$, respectively), while fertilization had no effect. Least square RGC means computed at covariates means were 57.6 ± 0.37 , 99.5 ± 0.19 and 106.2 ± 0.26 mg for W, M and S plants, respectively.

Prior to the drought-hardening period, N+ and N- seedlings differed significantly in plant N concentration (9.6 and 13.5 mg g⁻¹ for N- and N+, respectively, $n = 4$, $P = 0.017$). At the end of the water stress period, drought hardening had increased plant N concentration but this response only occurred

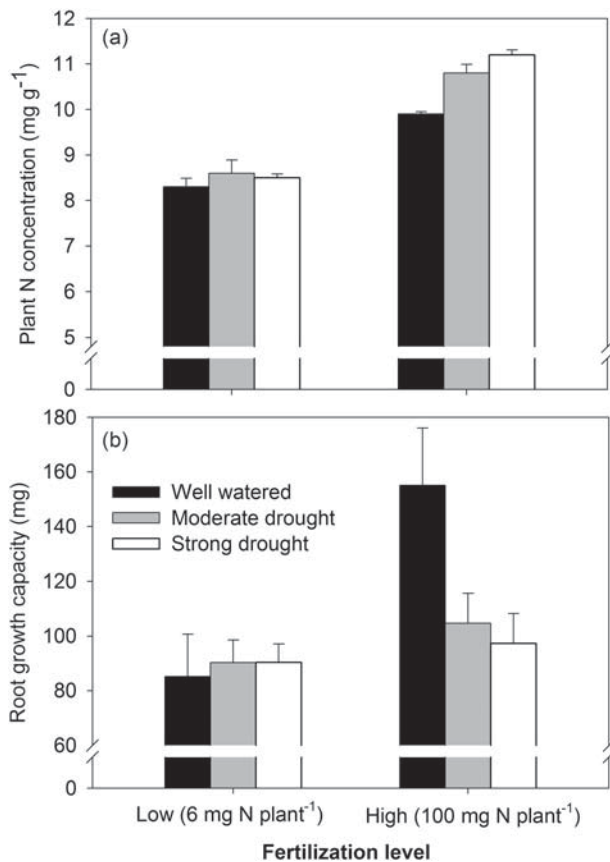


Figure 1. (a) Plant N concentration and (b) RGC of *P. pinea* seedlings after being cultivated under two N fertilization regimes (100 and 6 mg N seedling⁻¹) and subjected to three levels of drought hardening (well watered, moderate and strong). Data are means \pm 1 SE ($n = 4$ for N concentration and $n = 16$ for RGC).

in N+ and not in N- plants (interaction N fertilization \times drought hardening, $P = 0.017$) (Figure 1).

Plants accumulated less starch than soluble carbohydrates. Drought hardening increased starch and RSC concentration, while N fertilization reduced them (Table 1).

Stomatal conductance and water potential along a drought cycle after drought hardening

Seedling Ψ_{pd} diminished with increasing days of drought ($P < 0.001$), but the reduction was greater in N+ seedlings than in N- plants ($P < 0.001$, data not shown). g_s also declined with increasing days of drought. Drought hardening reduced g_s but this effect tended to be more pronounced in N- than in N+ seedlings (interaction drought hardening \times fertilization $P = 0.054$) (Figure 2). Drought hardening only reduced g_s in N+ on day 0 but had no effect thereafter. N+ seedlings had

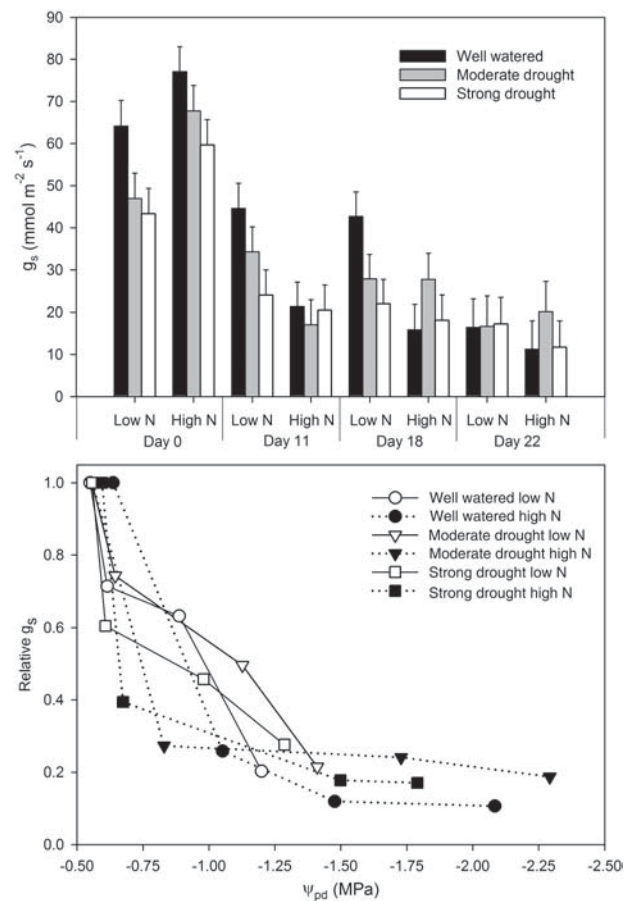


Figure 2. Stomatal conductance to water vapor (g_s) measured on four dates along a drought cycle (upper figure) and the variation of relative g_s with declining Ψ_{pd} (lower figure) in *P. pinea* seedlings after being cultivated under two N fertilization regimes (high: 100 mg N seedling⁻¹; and low: 6 mg N seedling⁻¹) and subjected to three levels of drought hardening (well watered, moderate and strong). g_s data are adjusted means \pm 1 SE after ANCOVA (see data analyses in the Material and methods). Relative g_s values were obtained by dividing the mean actual g_s by the mean maximum g_s of each treatment obtained on the first measurement date ($n = 4$ –6 plants per treatment).

higher g_s than N- on day 0, but this effect was reversed on Days 11 and 18 and no differences between fertilization treatments were detected after 22 days of drought (interaction fertilization \times date $P < 0.001$).

Stomatal conductance decreased with declining Ψ_{pd} . When g_s was calculated on a relative basis, the reduction of relative g_s with declining Ψ_{pd} was not of the same magnitude in all treatments. At $\Psi_{pd} > -1.2$ MPa, N+ seedlings had larger reduction in relative g_s than N- plants (Figure 2). Differences between drought-hardening treatments in the reduction of relative g_s were not apparent. At $\Psi_{pd} < -1.2$ MPa, all treatments had similar relative g_s .

Stomatal conductance was positively correlated with R_T and E_L . Frost damage was negatively correlated with the concentration of RSCs and positively correlated with E_L (Figure 3) and g_s ($r = 0.83$, $P = 0.034$, data not shown). E_L at 95% water content was negatively related with RSCs ($r = -0.81$, $P = 0.056$, data not shown).

Outplanting performance

Seedlings grown in the xeric environment had significantly more negative Ψ_{pd} than plants grown in the mesic environment (-2.14 vs. -0.73 MPa, $P < 0.001$). No mortality occurred in the mesic environment. In the xeric environment, S plants had lower survival than the other hardening treatments in N- plants but not in N+ seedlings (marginally significant drought hardening \times N fertilization interaction $P = 0.06$) (Figure 4). The SVI of the plants planted in the xeric environment was only 18% of the SVI in the mesic environment ($P < 0.001$). Fertilization increased SVI ($P = 0.004$), whereas drought hardening decreased SVI. However, the effect of drought hardening was apparent in the mesic environment but not in the xeric environment, where no significant differences existed among treatments (drought hardening \times outplanting environment interaction, $P = 0.031$) (Figure 4).

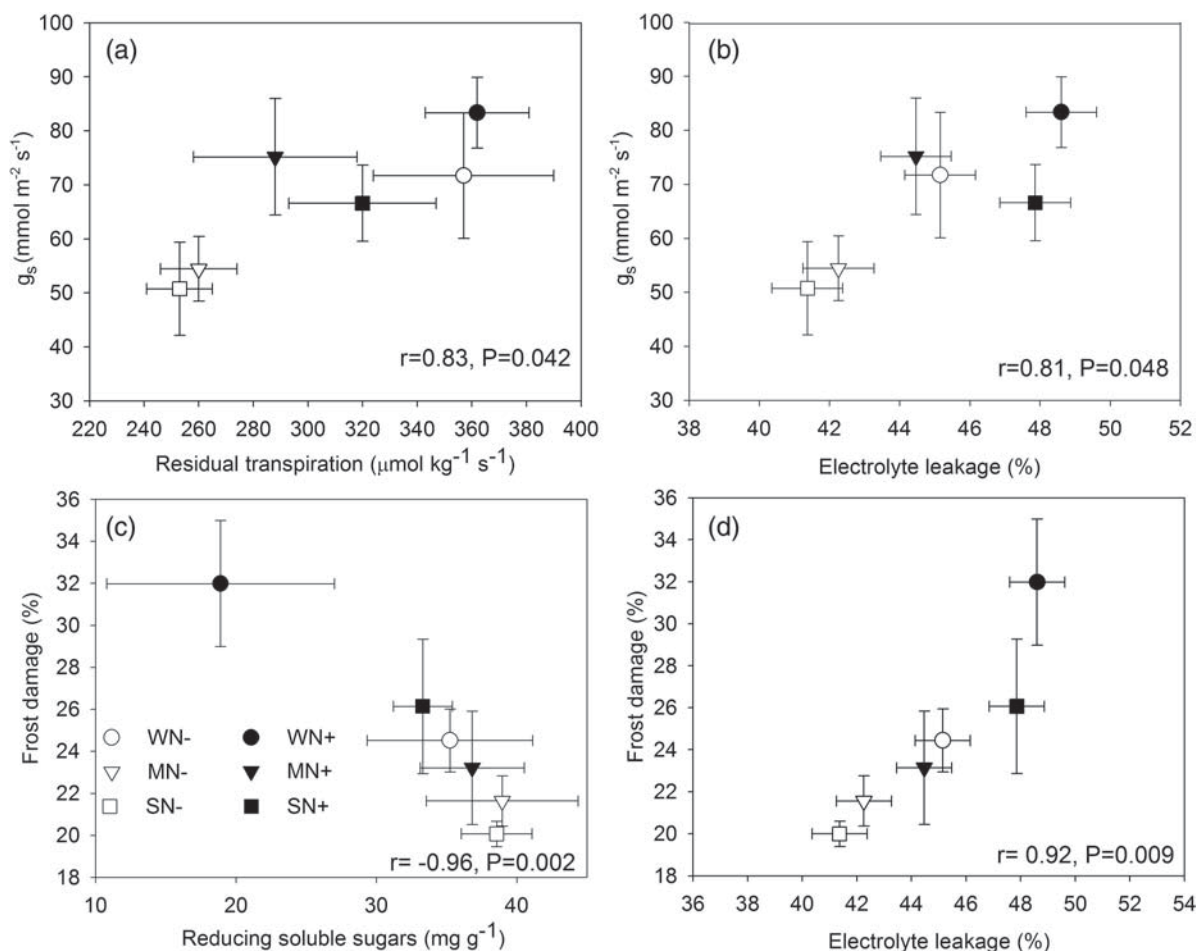


Figure 3. Relationships of stomatal conductance (g_s) with (a) residual transpiration and (b) electrolyte leakage at 95% water content in *P. pinea* seedlings cultivated under two N fertilization regimes (100 and 6 mg N seedling⁻¹) and subjected to three levels of drought hardening (well watered, moderate and strong). Subfigures (c) and (d) are the relationships of frost damage after a frost cycle to -8 °C with the concentration of plant reducing soluble sugars and electrolyte leakage at 95% water content, respectively. Each point represents the treatment mean \pm 1SE.

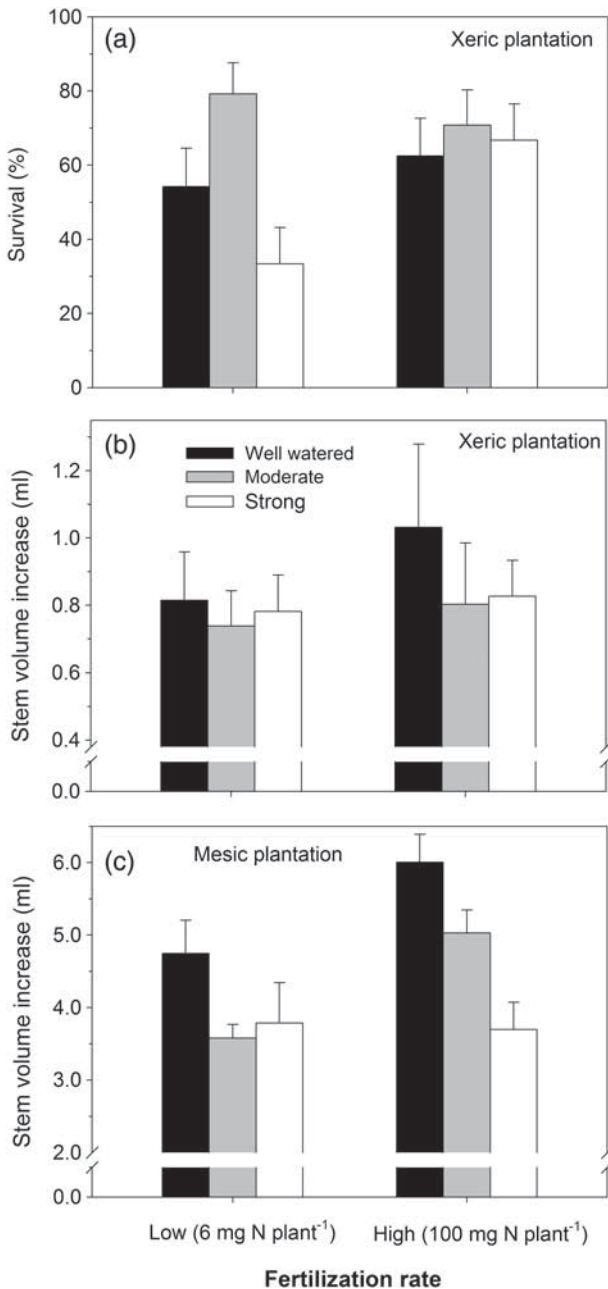


Figure 4. Survival (a) in the xeric outplanting environment and stem volume increase in both the xeric (b) and in the mesic (c) planting environment after the first growing season of *P. pinea* seedlings after being cultivated under two N fertilization regimes (100 and 6 mg N seedling⁻¹) and subjected to three levels of drought hardening (well watered, low and moderate). Each point represents the treatment mean ± 1 SE.

Discussion

This study illustrates that N nutrition and drought hardening had antagonistic effects on frost and drought tolerance of *P. pinea* seedlings. While N fertilization decreased the overall stress tolerance, drought hardening increased it, with no major differences between the moderate and strong drought stress levels. Stress acclimation of seedlings in response to

drought hardening applied under typical container nursery operational conditions was, in general, not affected by N nutrition because, in most cases, the magnitude of the change in drought and frost tolerance traits after drought hardening was similar among high- and low-N-fertilized plants.

Nitrogen nutrition affected the water economy of *P. pinea* seedlings. On the one hand, high-N-fertilized plants had a higher transpiration capacity due to increased plant size and g_s , and a reduced R_T relative to N- seedlings. The lower g_s in N- plants may be due to reduced hydraulic conductivity induced by low nutrient availability (Trubat et al. 2006, Samuelson et al. 2008). On the other hand, N nutrition affected the way seedlings respond to water stress, stimulating the drought stress avoidance capacity at the expense of reducing the drought tolerance capacity (sensu Levitt 1980). Two results support this assertion. First, N fertilization decreased the cell membrane stability to dehydration, as inferred from the higher E_L in response to strong dehydration in N+ than in N- plants. This is considered a drought tolerance mechanism (Levitt 1980, Premachandra et al. 1992). The response in *P. pinea* contrasts with the results in *Agrostis palustris* Huds., where high N increased the plasmalemma stability to dehydration (Saneoka et al. 2004). Second, N+ seedlings closed stomata at a higher water potential than N- plants, indicating a greater stomatal sensitivity to water stress (Figure 2). A similar response was reported in high-N-fertilized wheat plants (Morgan 1984a). The greater stomatal sensitivity to water stress in N+ plants is consistent with their lower dehydration tolerance and can be considered a dehydration avoidance mechanism that allows plants to reduce water consumption under mild drought but at the expense of reducing assimilation. The capacity of plants to maintain open stomata at lower water potential, such as that which occurred in N- seedlings, has been linked to decreased Ψ_{tsat} and ϵ (Koide et al. 1989, Serraj and Sinclair 2002, Brodribb and Holbrook 2003). Nitrogen nutrition has been reported to either increase, decrease, or have no effect on Ψ_{tsat} and ϵ (Morgan 1986, Correia et al. 1989, Tan and Hogan 1995). In this study, however, N nutrition had no effect on these parameters.

Irrespective of N fertilization, drought hardening decreased the seedling transpiration capacity after drought recovery due to a concomitant reduction in g_s and in R_T (Figure 3). This response might enhance the desiccation avoidance capacity of seedlings and help drought-hardened seedlings to expend less water and maintain better water status when outplanted. R_T and g_s also had a parallel reduction in *Quercus ilex* L. seedlings in response to drought hardening (Villar-Salvador et al. 2004a) and reductions in R_T or g_s after drought hardening have similarly been reported in other Mediterranean-climate pine species (Rook 1973, Villar-Salvador et al. 1999). In contrast, no R_T reduction after drought hardening was observed in three Mediterranean shrubs (Vilagrosa et al. 2003). Discrepancies

between studies suggest that changes in R_T in response to drought are species-specific or that the drought stress conditions differed across studies. The lower R_T after drought hardening presumably is due to enhanced cuticle thickness, deposition of hydrophobic compounds in the outer cell walls or both (Premachandra et al. 1992), while the reduction in g_s may be explained by lower hydraulic conductance, changes in stomatal behavior induced by ABA accumulation during the drought period or both (Rieger 1995, Chaves et al. 2003).

Drought hardening promoted drought tolerance through increased cell membrane stability to dehydration. A similar response has been reported in several trees and herbs (Zwiazek and Blake 1990, Premachandra et al. 1992, Villar-Salvador et al. 2004a). An increase in plasmalemma stability was independent of N nutrition as indicated by a lack of interaction between N fertilization and drought hardening. However, drought hardening affected neither osmotic adjustment nor changes in cell wall elasticity, which are important mechanisms involved in drought tolerance and species distribution (Morgan 1984b, Baltzer et al. 2008, Bartlett et al. 2012). Thus, our results point out that plasmalemma stability to dehydration is not linked to changes in Ψ_{rsat} in *P. pinea*, which is contrary to the results reported in other species (Premachandra et al. 1992, Gebre et al. 1994, Villar-Salvador et al. 2004a). Similar to our findings, no osmotic adjustment or changes in cell wall elasticity in response to drought cycles were observed in the Mediterranean pine *Pinus halepensis* Mill. (Villar-Salvador et al. 1999). In contrast, significant osmotic adjustment or changes in cell wall elasticity have been reported in other pine species (Meier et al. 1992, Fan et al. 1994, Fernández et al. 1999). Osmotic and cell wall elasticity adjustment might have been hindered because plants desiccated too fast (Abrams 1988, Collet and Ghuel 1997) as a consequence of cultivation in 300 ml containers. However, most physiological traits in our study were significantly modified after drought hardening, indicating that the speed of drought cycles did not limit drought acclimation. Although seedlings in our study did not show osmotic adjustment in response to drought, *P. pinea* is capable of seasonal osmotic adjustment as indicated by the decrease in Ψ_{rsat} of well-watered plants between mid-summer and mid-fall. Substantial seasonal shifts in drought tolerance attributes are typical for most plant species of cold and temperate biomes as they move from an active to an inactive growing state during cold acclimation in the fall (Sakai and Larcher 1987, Bigras et al. 2001).

Water stress usually increases the proportion of mass invested in roots at the expense of shoots, which might confer resistance towards drought (van den Driessche 1991a; Lloret et al. 1999). However, in this study we observed that well-watered plants had higher S/R than drought-hardened seedlings. This response can be explained by greater inhibition of root relative to shoot growth in drought-hardened seedlings (data not shown) compared with well-watered plants.

Frost tolerance is an important attribute for seedlings outplanted in the Mediterranean continental areas because plantations are established during the cold season. Previous studies have reported either an increase or decrease, or no effect of N nutrition on frost tolerance in plants (Pellett and Carter 1981, Fløistad and Kohmann 2004, Fernández et al. 2007, Islam et al. 2009). In this study, N fertilization decreased frost tolerance. Notably, drought hardening reduced the frost sensitivity of plants, thereby alleviating the negative effect of high N fertilization on cold tolerance. Drought hardening had a similar effect on cold hardiness in *Pseudotsuga menziesii* (Mirb.) Franco (Blake et al. 1979), but not in other conifers (Grossnickle et al. 1991). Therefore, an important consequence of the interaction of N nutrition and drought hardening in *P. pinea* was that frost and drought resistance covaried (Figure 3). Plants that had greater frost tolerance also had greater plasmalemma stability to dehydration and lower transpiration. Because E_L and F_D were negatively related with reducing soluble sugar concentration, we suggest that reducing soluble sugars might be the link between drought and frost tolerance. Soluble sugars and other organic compounds protect cell membranes from disruption during cell dehydration caused by frost and drought (Sakai and Larcher 1987, Chaves et al. 2003, Uemura and Steponkus 2003).

Plant N and non-structural carbohydrate concentrations were greater in drought-hardened plants, a response that has also been reported in *P. halepensis* (Villar-Salvador et al. 1999). However, an increase in plant N with drought hardening was evident in N+ seedlings but not in N- plants. Tissue N and non-structural carbohydrate concentration depends on the balance between C and N sink and source strengths (Luxmoore et al. 1995, Millard and Grelet 2010). It is possible that a concomitant decrease in the sink strength by water stress and an increase in N source strength under high N supply explains the higher plant N in N+ drought-hardened plants. In contrast, the absence of a drought-hardening effect on plant N in N- plants suggests that growth reduction from drought and N uptake maintained the same pace (Sanz Pérez et al. 2007).

Root growth capacity measures seedling functional integrity and vigor (Simpson and Ritchie 1997). Seedling survival in Mediterranean environments is linked to root growth achieved in summer (Padilla and Pugnaire 2007, Villar-Salvador et al. 2012). Drought stress can reduce new RGC after drought recovery (Tinus 1996, Villar-Salvador et al. 1999, 2004a). Consistent with previous studies, drought hardening reduced new RGC in *P. pinea* seedlings, but this effect only occurred in high-fertilized plants, suggesting that drought hardening overwhelmed the positive effect of high fertilization. Lower gas exchange capacity, hydraulic conductivity or root damage can explain the reduced RGC after drought stress recovery (Zwiazek and Blake 1989, Rieger 1995, Tinus 1996). We suggest that variation in shoot size among treatments accounted

for a significant part of the differences in RGC. Plant growth relies on photosynthesis and remobilization of N and carbon reserves (van den Driessche 1991b, Salifu and Timmer 2003, Millard and Grelet 2010), which increase concomitantly as plant size increases. This would explain the greater RGC of well-watered N+ plants relative to the other treatments. However, other physiological processes, which seem to be masked by the effect of shoot size, may also impact RGC in *P. pinea*. The fact that drought-hardened seedlings had greater RGC than well-watered plants when shoot size was accounted for supports this hypothesis.

No clear benefits of either drought hardening or N fertilization on outplanting survival or growth were observed under xeric conditions. This contrasts with the results reported by van den Driessche (1991a) and Bañon et al. (2006) that drought hardening increased seedling survival when planted under xeric conditions. Similarly, drought-hardened *Chamaecyparis nootkatensis* seedlings D. Don grew faster than well-watered seedlings (Arnott et al. 1993). Treatments in our study had similar survival except for the poorest N-fertilized and strongest drought-stressed plants (SN–), which had the lowest survival. We cannot find any relationship between morpho-physiological plant traits measured at the end of cultivation and outplanting survival to explain the low survival of SN– plants. These plants had the lowest transpiration and the highest plasmalemma stability to dehydration and frost resistance. But they also had low tissue N concentration and RGC. Low survival of SN– plants suggests that highly limiting growth conditions in the nursery may reduce the capacity of plants to thrive when transplanted under drought conditions, for instance due to low mycorrhizal colonization (Parladé et al. 2004). The lack of improved performance of drought-hardened plants may be associated with the loss of physiological differences among treatments several weeks after hardening (Grossnickle 2012). Reversibility of physiological traits related to drought tolerance might be accelerated when seedlings are not limited by water immediately after planting as probably occurred in the xeric environment in this experiment, where soil in containers was well hydrated at planting. In contrast, well-watered N+ plants had greater absolute growth under mesic planting conditions, probably because they had higher photosynthesis and N content to be remobilized for new growth (Salifu and Timmer 2003).

We conclude that drought hardening and N nutrition exert opposite effects on the stress tolerance of *P. pinea* seedlings. Nitrogen fertilization reduced the seedling frost and tissue dehydration tolerance and increased the transpiration, new RGC and responsiveness of stomatal closure to water stress. Drought hardening reversed part of these responses by alleviating the reduction in stress tolerance caused by high N fertilization and increasing the amount of N and non-structural carbohydrates stored in plant tissues. Drought acclimation in

response to drought hardening applied under typical nursery operational conditions was not affected by N nutrition. In spite of significant functional differences among treatments, neither N nutrition nor drought hardening had any clear benefits on seedling performance under xeric outplanting conditions. To improve stress tolerance and promote high tissue nutrient concentration, which are important attributes for performance in forest plantations established in the Mediterranean continental sites, we recommend cultivating *P. pinea* seedlings with 100 mg N and subjecting them to moderate drought stress for 2 months in the fall.

Acknowledgments

The authors are grateful to Professor José A. Pardos for permitting the use of the resources in his laboratory at the Polytechnic University of Madrid. Technical assistance of Javier Vallas and Jaime Puértolas in the evaluation of frost tolerance is appreciated.

Conflict of interest

None declared.

Funding

This research was supported by funds from the Ministerio de Agricultura, Alimentación y Medio Ambiente and by the project AGL2011-24296 (ECOLPIN) of the Spanish Ministry of Science and Innovation and the network REMEDINAL-2 (S2009/AMB/1783) of the Community of Madrid.

References

- Abrams MD (1988) Sources of variation in osmotic potentials with special reference to North American tree species. *For Sci* 34:1030–1046.
- Andivia E, Fernández M, Vázquez-Piqué J (2011) Autumn fertilization of *Quercus ilex* ssp. *ballota* (Desf.) Samp. nursery seedlings: effects on morpho-physiology and field performance. *Ann For Sci* 68:543–553.
- Arnott JT, Grossnickle SC, Puttonen P, Mitchel AK, Folk RS (1993) Influence of nursery culture on growth, cold hardiness, and drought resistance of yellow cypress. *Can J For Res* 23:2537–2547.
- Baltzer JL, Davies SJ, Bunyavejchewin S, Noor NSM (2008) The role of desiccation tolerance in determining tree species distributions along the Malay–Thai Peninsula. *Funct Ecol* 22:221–231.
- Bañon S, Ochoa J, Franco JA, Alarcón JJ, Sánchez-Blanco MJ (2006) Hardening of oleander seedlings by deficit irrigation and low air humidity. *Env Exp Bot* 56:36–43.
- Bartlett MK, Scoffoni C, Sack L (2012) The determinants of leaf turgor loss point and prediction of drought tolerance of species and biomes: a global meta-analysis. *Ecol Lett* 15:393–405.
- Beikircher B, Florineth F, Mayr S (2010) Restoration of rocky slopes based on planted gabions and use of drought-preconditioned woody species. *Ecol Eng* 36:421–426.

- Bigras FJ, Ryyppo A, Lindström A, Stattin E (2001) Cold acclimation and deacclimation of shoots and roots of conifer seedlings. In: Bigras FJ, Colombo SJ (eds) Conifer cold hardiness. Kluwer Academic Publishers, Dordrecht, pp 57–88.
- Blake J, Zaerr J, Hee S (1979) Controlled moisture stress to improve cold hardiness and morphology of Douglas-fir seedlings. *For Sci* 25:576–582.
- Brodribb TJ, Holbrook M (2003) Stomatal closure during leaf dehydration, correlation with other leaf physiological traits. *Plant Physiol* 132:2166–2173.
- Castro J, Zamora R, Hódar JA, Gómez JM (2004) Seedling establishment of a boreal tree species (*Pinus sylvestris*) at its southernmost distribution limit: consequences of being in a marginal Mediterranean habitat. *J Ecol* 92:266–277.
- Chaves MM, Maroco JP, Pereira JS (2003) Understanding plant responses to drought—from genes to the whole plant. *Funct Plant Biol* 30:239–264.
- Collet C, Ghuel JM (1997) Osmotic adjustment in sessile oak seedlings in response to drought. *Ann Sci For* 54:389–394.
- Coombs J, Hind G, Leegood RC, Tieszen LL, Vonshak A (1987) Analytical techniques. In: Coombs J, Hall DO, Long SP, Scurlock JMO (eds) Techniques in bioproductivity and photosynthesis. Pergamon Press, Oxford, pp 219–228.
- Correia MJ, Torres E, Pereira JS (1989) Water and nutrient supply regimes and the water relations of juvenile leaves of *Eucalyptus globulus*. *Tree Physiol* 5:459–471.
- DaMatta FM, Loos RA, Silva EA, Loureiro ME, Ducatti C (2002) Effects of soil water deficit and nitrogen nutrition on water relations and photosynthesis of pot-grown *Coffea canephora* Pierre. *Trees* 16:555–558.
- Domínguez-Lerena S, Herrero Sierra N, Carrasco Manzano I, Ocaña Bueno L, Peñuelas Rubira J, Mexal JG (2006) Container characteristics influence *Pinus pinea* seedling development in the nursery and field. *For Ecol Manage* 221:63–71.
- Earnshaw MJ (1993) Stress indicators: electrolyte leakage. In: Henry GAF, Grime JP (eds) Methods in comparative plant ecology: a laboratory manual. Chapman & Hall, London, pp 152–154.
- Fan S, Blake TJ, Blumwald E (1994) The relative contribution of elastic and osmotic adjustments to turgor maintenance of woody species. *Physiol Plant* 90:408–413.
- Fernández M, Gil L, Pardos JA (1999) Response of *Pinus pinaster* Ait. provenances at early age to water supply. I. Water relation parameters. *Ann For Sci* 56:179–187.
- Fernández M, Marcos C, Tapias R, Ruiz F, López G (2007) Nursery fertilization affects the frost-tolerance and plant quality of *Eucalyptus globulus* Labill. cuttings. *Ann For Sci* 64:865–873.
- Fløistad IS, Kohmann K (2004) Influence of nutrient supply on spring frost hardiness and time of bud break in Norway spruce (*Picea abies* (L.) Karst.) seedlings. *New For* 27:1–11.
- Gebre GM, Kuhns MR, Brande JR (1994) Organic solute accumulation and dehydration tolerance in three water-stressed *Populus deltoides* clones. *Tree Physiol* 14:575–587.
- Ghashghaie J, Saugier B (1989) Effects of nitrogen deficiency on leaf photosynthetic response of tall fescue to water deficit. *Plant Cell Environ* 12:261–271.
- Grossnickle SC (2012) Why seedlings survive: influence of plant attributes. *New For* 43:711–738.
- Grossnickle SC, Arnott JT, Major JE, Tschaplinski TJ (1991) Influence of dormancy induction treatments on western hemlock seedlings. I. Seedling development and stock quality assessment. *Can J For Res* 21:164–174.
- Harvey HP, van den Driessche R (1997) Nutrition, xylem cavitation and drought resistance in hybrid poplar. *Tree Physiol* 17:647–654.
- Hellergrén J (1981) Frost hardiness development in *Pinus sylvestris* seedlings in response to fertilization. *Physiol Plant* 52:297–301.
- Islam MA, Apostol KG, Jacobs DF, Dumroese RK (2009) Fall fertilization of *Pinus resinosa* seedlings: nutrient uptake, cold hardiness, and morphological development. *Ann For Sci* 66:704–712.
- Kleiner KW, Abrams MD, Schultz JC (1992) The impact of water and nutrient deficiencies on the growth, gas exchange and water relations of red oak and chestnut oak. *Tree Physiol* 11:271–287.
- Koide RT, Robichaux RH, Morse SR, Smith CM (1989) Plant water status, hydraulic resistance and capacitance. In: Pearcy RW, Ehleringer J, Mooney HA, Rundel PW (eds) Plant physiological ecology. Chapman & Hall, London, pp 161–179.
- Kubiske ME, Abrams MD (1990) Pressure–volume relationships in non-rehydrated tissue at various water deficits. *Plant Cell Environ* 13:995–1000.
- Larcher W (1981) Low temperature effects on Mediterranean sclerophylls: an unconventional viewpoint. In: Lieth H (ed) Components of productivity of Mediterranean-climate regions. Basic and applied aspects. Dr. W. Junk, The Hague, pp 259–266.
- Larchevêque M, Ballini C, Korboulewsky N, Montès N (2006) The use of compost in afforestation of Mediterranean areas: effects on soil properties and young tree seedlings. *Sci Total Environ* 369:220–230.
- Levitt J (1980) Responses of plants to environmental stresses. Vol. II. Water, radiation, salt, and other stresses. In: Kozlowski TT (ed) Physiological ecology. Academic Press, New York, 607 pp.
- Lloret F, Casanovas C, Peñuelas J (1999) Seedling survival of Mediterranean shrubland species in relation to root:shoot ratio, seed size and water and nitrogen use. *Funct Ecol* 13:210–216.
- Luxmoore RJ, Oren R, Sheriff DW, Thomas RB (1995) Source–sink–storage relationships of conifers. In: Smith WK, Hinckley TM (eds) Resource physiology of conifers. Acquisition, allocation and utilization. Academic Press, San Diego, pp 179–216.
- Meier CE, Newton RJ, Puryear JD, Sen S (1992) Physiological responses of loblolly pine (*Pinus taeda* L.) seedlings to drought stress: osmotic adjustment and tissue elasticity. *J Plant Physiol* 140:754–760.
- Millard P, Grelet GA (2010) Nitrogen storage and remobilization by trees: ecophysiological relevance in a changing world. *Tree Physiol* 30:1083–1095.
- Mollá S, Villar-Salvador P, García-Fayos P, Peñuelas JL (2006) Physiological and transplanting performance of *Quercus ilex* L. (holm oak) seedlings grown in nurseries with different winter conditions. *For Ecol Manage* 237:218–226.
- Morgan JA (1984a) Interaction of water supply and N in wheat. *Plant Physiol* 76:112–117.
- Morgan JA (1986) The effects of N nutrition on the water relations and gas exchange characteristics of wheat (*Triticum aestivum* L.). *Plant Physiol* 80:52–58.
- Morgan JM (1984b) Osmoregulation and water stress in higher plants. *Annu Rev Plant Physiol* 35:299–319.
- Oliet JA, Planelles R, Artero F, Valverde R, Jacobs DJ, Segura ML (2009) Field performance of *Pinus halepensis* planted in Mediterranean arid conditions: relative influence of seedling morphology and mineral nutrition. *New For* 37:313–331.
- Padilla FM, Pugnairé FI (2007) Rooting depth and soil moisture control Mediterranean woody seedling survival during drought. *Funct Ecol* 21:489–495.
- Parladé J, Luque J, Pera J, Rincón A (2004) Field performance of *Pinus pinea* and *P. halepensis* seedlings inoculated with *Rhizopogon* spp. and outplanted in formerly arable land. *Ann For Sci* 61:507–514.
- Pellett HM, Carter JV (1981) Effect of nutritional factors on cold hardiness of plants. *Hort Rev* 3:144–171.
- Premachandra GS, Saneoka H, Fujita K, Ogata S (1992) Leaf water relations, osmotic adjustment, cell membrane stability, epicuticular wax load and growth as affected by water deficits in *Sorghum*. *J Exp Bot* 43:1569–1576.

- Rieger M (1995) Offsetting effects of reduced root hydraulic conductivity and osmotic adjustment following drought. *Tree Physiol* 15:379–385.
- Rook DA (1973) Conditioning radiata pine seedlings to transplanting, by restricted watering. *NZ J For Sci* 3:54–69.
- Sakai A, Larcher W (1987) Frost survival of plants. Responses and adaptation to freezing stress. *Ecological studies* 62. Springer, Berlin.
- Salifu KF, Timmer VR (2003) Nitrogen retranslocation response of young *Picea mariana* to nitrogen-15 supply. *Soil Sci Soc Am J* 67:309–317.
- Salifu KF, Jacobs DF, Birge ZKD (2009) Nursery nitrogen loading improves field performance of bareroot oak seedlings planted on abandoned mine land. *Restor Ecol* 17:339–349.
- Samuelson LJ, Farris MG, Stokes TA, Coleman MD (2008) Fertilization but not irrigation influences hydraulic traits in plantation-grown loblolly pine. *For Ecol Manage* 255:3331–3339.
- Saneoka H, Moghaieb REA, Premachandra GS, Fujita K (2004) Nitrogen nutrition and water stress effects on cell membrane stability and leaf water relations in *Agrostis palustris* Huds. *Environ Exp Bot* 52:131–138.
- Sanz Pérez V, Castro-Díez P, Valladares F (2007) Growth versus storage: responses of Mediterranean oak seedlings to changes in nutrient and water availabilities. *Ann For Sci* 64:201–210.
- Serraj R, Sinclair TR (2002) Osmolyte accumulation: can it really help increase crop yield under drought conditions? *Plant Cell Environ* 25:333–341.
- Simpson DG, Ritchie GA (1997) Does RGP predict field performance? A debate. *New For* 13:253–277.
- Tan W, Hogan GD (1995) Effects of nitrogen limitation on water relations of jack pine (*Pinus banksiana* Lamb.) seedlings. *Plant Cell Environ* 18:757–764.
- Tinus R (1996) Root growth potential as an indicator of drought stress history. *Tree Physiol* 16:795–799.
- Trubat R, Cortina J, Vilagrosa A (2006) Plant morphology and root hydraulics are altered by nutrient deficiency in *Pistacia lentiscus* (L.). *Trees* 20:334–339.
- Trubat R, Cortina J, Vilagrosa A (2011) Nutrient deprivation improves field performance of woody seedlings in a degraded semi-arid shrubland. *Ecol Eng* 37:1164–1173.
- Uemura M, Steponkus PL (2003) Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from *Arabidopsis thaliana* leaves. *Plant Cell Environ* 26:1083–1096.
- van den Driessche R (1991a) Influence of container nursery regimes on drought resistance of seedlings following planting. I. Survival and growth. *Can J For Res* 21:555–565.
- van den Driessche R (1991b) New root growth of Douglas-fir seedlings at low carbon dioxide concentration. *Tree Physiol* 8:289–295.
- van den Driessche R (1992) Changes in drought resistance and root growth capacity of container seedlings in response to nursery drought, nitrogen, and potassium treatments. *Can J For Res* 22:740–749.
- Vicente C, Fontaniella B, Legaz ME (2000) Fructan-like polysaccharides produced by sugarbeet during deterioration. *Int Sugar J* 102:251–256.
- Vilagrosa A, Cortina J, Gil-Pelegrín E, Bellot J (2003) Suitability of drought-preconditioning techniques in Mediterranean climate. *Rest Ecol* 11:208–216.
- Villar-Salvador P, Ocaña L, Peñuelas J, Carrasco I (1999) Effect of water stress conditioning on the water relations, root growth capacity, and the nitrogen and non-structural carbohydrate concentration of *Pinus halepensis* Mill. (Aleppo pine) seedlings. *Ann For Sci* 56:459–465.
- Villar-Salvador P, Planelles R, Oliet J, Peñuelas-Rubira JL, Jacobs DF, González M (2004a) Drought tolerance and transplanting performance of holm oak (*Quercus ilex*) seedlings after drought hardening in the nursery. *Tree Physiol* 24:1147–1155.
- Villar-Salvador P, Planelles R, Enriquez E, Peñuelas Rubira J (2004b) Nursery cultivation regimes, plant functional attributes, and field performance relationships in the Mediterranean oak *Quercus ilex* L. *For Ecol Manage* 196:257–266.
- Villar Salvador P, Puértolas J, Cuesta B, Peñuelas Rubira J, Uscola M, Heredia-Guerrero N, Rey Benayas JM (2012) Increase in size and nitrogen concentration enhances seedling survival in Mediterranean plantations. Insights from an ecophysiological conceptual model of plant survival. *New For* 43:755–770.
- Walters MB, Reich PB (1989) Response of *Ulmus americana* seedlings to varying nitrogen and water status. 1. Photosynthesis and growth. *Tree Physiol* 5:159–172.
- Zhu XB, Cox RM, Meng FR, Arp PA (2001) Responses of xylem cavitation, freezing injury and shoot dieback to a simulated winter thaw in yellow birch seedlings growing in different nursery culture regimes. *For Ecol Manage* 145:243–253.
- Zwiazek JJ, Blake TJ (1989) Effects of preconditioning on subsequent water relations, stomatal sensitivity, and photosynthesis in osmotically stressed black spruce. *Can J Bot* 67:2240–2244.
- Zwiazek JJ, Blake TJ (1990) Effects of preconditioning on electrolyte leakage and lipid composition in black spruce (*Picea mariana*) stressed with polyethylene glycol. *Physiol Plant* 79:71–77.