

Effect of nitrogen fertilization in the nursery on the drought and frost resistance of Mediterranean forest species

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Abstract

The objective of this study was to assess the effect of N fertilization in the nursery on frost and water stress resistance of seedlings in Mediterranean forest species. We reviewed the data of six independent fertilization experiments that were performed between 1997 and 2003 in six Mediterranean forest species: three oaks (*Quercus suber* L., *Q. ilex* L., and *Q. coccifera* L.), and three conifer (*Pinus pinea* L., *P. halepensis* Mill., and *Juniperus thurifera* L.). Plants were cultivated under two contrasting N fertilization regimes and at the end or during the cultivation period several parameters related to drought and frost resistance were measured. N fertilization affected more the morphological than the physiological characters. Changes in most morphological traits in response to N fertilization tended to have the same variation pattern, whereas physiological traits had variable responses. N Fertilization reduced frost hardiness in *Pinus* species and increased the osmotic potential both at full turgor and at turgor loss point in *J. thurifera*. High-fertilized (HN) seedlings in all species were larger and had greater shoot to root mass ratio than low-fertilized or unfertilized (LN) plants. HN pine seedlings also had higher stomatal conductance than LN plants. These characteristics might impair the water balance of HN plants if soil water content remains low immediately after transplanting. In contrast, HN plants showed higher new root growth capacity than LN seedlings and the proportion of new roots emerging from the plug with respect to shoot size did not differ between fertilization treatments or it was greater in HN plants than in LN seedlings. These responses could improve the efficiency of roots to explore the soil and, therefore, the drought avoiding capacity of HN plants.

Key words: Drought tolerance, electrolyte leakage, fluorescence, osmotic potential, root growth, transpiration.

Resumen

Efecto de la fertilización nitrogenada en el vivero sobre la resistencia a la sequía y a la helada en especies forestales mediterráneas

El objetivo de este estudio es abordar el efecto de la fertilización nitrogenada en vivero sobre la resistencia a la helada y a la sequía en diferentes especies forestales mediterráneas. Para ello se revisaron los datos de seis experimentos independientes realizados entre 1997 y 2003 con seis especies forestales mediterráneas (tres especies de *Quercus*: *Q. suber* L., *Q. ilex* L., *Q. coccifera* L.; y tres coníferas: *Pinus pinea* L., *Pinus halepensis* Mill., *Juniperus thurifera* L.). Las plantas fueron cultivadas bajo dos niveles contrastados de fertilización nitrogenada. Durante o al final del cultivo se midieron diferentes parámetros relacionados con la resistencia a la helada y la sequía. La fertilización con N afectó más a los caracteres morfológicos que a los fisiológicos. Los cambios en la mayoría de parámetros morfológicos en respuesta a la fertilización nitrogenada tendieron a seguir el mismo patrón de variación, mientras que las respuestas fisiológicas fueron más variables. El N disminuyó la resistencia a la helada en los pinos e incrementó el potencial osmótico, tanto a

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plena turgencia como en pérdida de turgencia, en *J. thurifera*. En todas las especies, las plantas altamente fertilizadas (HN) fueron mayores y con una relación tallo / raíz más alta que las plantas poco o nada fertilizadas (LN). En los pinos, las plantas HN también mostraron una conductancia estomática mayor que las plantas LN. Estas características pueden afectar negativamente al balance hídrico de la planta si el contenido hídrico del suelo es bajo en el momento de la plantación. En contraste, las plantas HN mostraron mayor capacidad de crecimiento radical que las LN y la proporción de nuevas raíces emitidas desde el cepellón en relación con el tamaño de la parte aérea o bien no mostró diferencias entre tratamientos o bien fue mayor en las plantas HN que en las LN. Estas respuestas pueden mejorar la eficiencia de las raíces para explorar el suelo y, por tanto, la capacidad de evitación del estrés hídrico de las plantas HN.

Palabras clave: Crecimiento radical, fluorescencia, liberación de electrolitos, potencial osmótico, tolerancia a la sequía, transpiración.

Introduction

Plants that grow in the Mediterranean continental climate are subjected to two main stresses: drought and cold (Mitrakos, 1980). The ability of seedlings to cope with these constraints determines in part the establishment capacity of plant species in this climate.

Water stress resistance in woody species can be achieved by avoiding drought or by tolerating it (*sensu* Levitt, 1980). Water stress avoidance can be achieved by either low water loss (*i.e.* by low stomatal conductance and shoot to root ratio and by high stomatal control and residual transpiration) or by a high water uptake capacity from the soil (*i.e.* by large and deep roots). Drought tolerant plants can maintain their physiological performance at low water potentials. However, Mediterranean forest species frequently have mixed features of both strategies (Valladares *et al.*, 2004). Growing conditions in the nursery can influence many of the physiological and morphological properties of seedlings related to stress resistance. Specifically, fertilization is one of the most important nursery tools for growing target seedlings. Most fertilization studies have focused on N, because it is the essential nutrient that plants need in higher amount and the changes in its availability induce large variations in seedling performance. N fertilization has been shown either to decrease frost hardiness in the fall or to accelerate cold dehardening in the spring (see citations in Pellet and Carter, 1981; Calmé and Margolis, 1993; Fløistad and Kohmann, 2004; Hellergren, 1981). Similarly, negative effects on water stress avoidance and tolerance have been also described. Tan and Hogan (1995) found that N-limited *Pinus taeda* seedlings maintained higher turgor when water potential declined than highly fertilized seedlings. van den Driessche

(1988) showed that high N fertilization in *Pseudotsuga menziesii* seedlings reduced transplanting survival and growth. In contrast to these studies, many others have reported either no effects (Birchler *et al.*, 2001; Fløistad, 2002) or positive effects (Bigras *et al.*, 1996; Rikala and Repo, 1997) of N fertilization on frost hardiness. N fertilization can improve the water stress avoidance of seedlings by increasing new root growth capacity (van den Driessche, 1992) and favouring an earlier stomatal closure in response to drought (Morgan, 1984).

Most studies on the effect of N fertilization on the stress resistance of forest species have been done with boreal and humid temperate species. In recent years, fertilization in the nursery of Mediterranean forest seedlings has received much attention. Nowadays, optimal fertilization is seen as a key factor for producing high quality seedlings. However, the influence of N on the stress resistance in Mediterranean woody species has received little attention. In this work we analyse the effect of N fertilization on the frost and water stress resistance in six Mediterranean forest species. To achieve this objective, we reviewed the physiological and morphological data of six independent fertilization experiments that were done by the authors from 1997 to 2003.

Material and Methods

Six experiments with six Mediterranean forest species were made independently from 1997 to 2003. Plants of experiments 1-4 were raised in Forest Pot 300® trays (Nuevos Sistemas de Cultivo S.L., Girona, Spain), which have 50 cavities of 300 ml. In experiments 5 and 6, seedlings were grown in Arnabat®

trays (Arnabat S.A., Barcelona, Spain) which have 54 cavities of 200 ml. In all experiments growing media was light *Sphagnum* peat moss and plants were kept well watered by irrigating them every 1-5 days depending on the weather conditions.

Experiment 1

Quercus ilex L. (holm oak) and *Q. suber* L. (cork oak) seedlings from La Mancha-Montiel and La Almoraima (Cádiz) provenances, respectively, were grown in a greenhouse from January to mid-May 1998. Seedlings were then moved outside and grown under full sun until the end of February 1999. Two fertilization levels were differentiated: no fertilization (LN) and high N fertilization (HN). HN plants were supplied with 154 mg N, 16 mg P and 34 mg K during all the cultivation period. This was achieved by growing seedlings in peat mixed with a slow release fertiliser (1kg per m³ of peat; Original Kasper B6), which released 34 mg N, 16 mg P and 34 mg K per seedling during the experiment. Each plant was supplemented with 120 mg of N by overhead sprinkling a NH₄NO₃ fertiliser once a week from the end of May to mid-September (7.05 mg N week⁻¹). LN seedlings were grown in unfertilised Original Kasper peat. Fertilization treatments were arranged in three blocks, each block being composed of two containers of 50 plants.

Experiment 2

Quercus coccifera L. (kermes oak) seedlings were grown in a greenhouse from February to mid June 2002 and then moved outside and grown under full sun until December 2002. Acorn provenance was Guadalajara. Two fertilization levels were differentiated: no fertilization (LN) and high N fertilization (HN). HN plants were supplied with 150 mg N, 40 mg P and 70 mg K during all the cultivation period. N was supplied as NH₄NO₃; P and K as K₂SO₄ and H₃PO₄, respectively, plus a mixture of micronutrients. Plants were individually fertilized once a week by applying the fertilizer with a syringe from mid June to the end of October 2002. Fertilization treatments were arranged in four blocks, each block being composed of two containers of 50 plants per fertilization treatment.

Experiment 3

Pinus pinea L. (stone pine) seedlings from La Mancha provenance were grown in a glass greenhouse from February to mid June 1997 and then moved outside and grown under full sun until the end of November 1997. Two N fertilization regimes were differentiated: high (HN) and low (LN). In the former, plants received a total of 100 mg N per seedling during all the cultivation period whereas in the latter, plants received 6 mg N per seedling. N was supplied using an NH₄NO₃ fertiliser. Plants from both fertilization treatments received 20 and 40 mg per plant of P and K, respectively, utilising a K₂P₂O₅ fertiliser and a mixture of micronutrients (Kanieltra, Hydro Agri, Oslo, Norway). Fertilization started in June 1 and was done once a week in two steps. First, all plants received the LN treatment, P, K, and micronutrient fertiliser by overhead sprinkling fertirrigation. Then, the HN plants received individually the remaining N that was applied manually with a syringe. Until August 15, each fertilization treatment received 70% of their total N. The remaining 30% was applied on four days from mid August to the end of November. Fertilization treatments were arranged in four blocks, each block being composed by two containers of 50 plants per fertilization treatment.

Experiment 4

Juniperus thurifera L. (thuriferous juniper or Spanish juniper) seedlings were cultivated from April 2002 to late May 2002 in a greenhouse and then moved outside and grown under full sun for two growing seasons until November 2003. Seed provenance was Pedraza (Segovia). Two N fertilization regimes were differentiated: high (HN) and low (LN). HN and LN plants received 150 mg N and 30 mg N per seedling, during each growing season, respectively. N was supplied using an NH₄NO₃ fertiliser. Plants from both fertilization treatments were supplied with a mixture of micronutrients and with 40 and 75 mg of P and K per plant, respectively, in each growing season. These nutrients were supplied as K₂SO₄ and H₃PO₄, respectively. Fertilization was done once a week from early May to the end of November in the first growing season and from mid April to mid October in the second growing season. Plants were fertilized

with a syringe. Each treatment was composed of two containers of 50 plants, which were randomly distributed in space.

Experiment 5

Pinus halepensis Mill. (Aleppo pine) seedlings from La Mancha provenance were grown outdoors in a commercial nursery (Genforsa, Casas de los Pinos, Cuenca, Spain) from March to September 1999. Seedlings were fertilized according to the nursery standard techniques, which led to a needle N concentration of 11 mg g⁻¹ at the end of September. In October, seedlings were separated into two groups (HN and LN) of three trays each one (162 plants) and were randomly placed into a phytotron for 14 weeks. Environmental conditions inside the phytotron were: temperature, 21.5/14.0 °C in the day/night cycle; relative humidity: 80%; photoperiod: 13 hours; photosynthetic photon flux density 75-100 µmol m⁻² s⁻¹. These conditions were maintained for three weeks. Afterwards, photoperiod and temperature were progressively reduced to 9.5 hours and 16.5/4.0 °C respectively. Seedlings were fertilized weekly with a soluble fertilizer (Peters Professional 4-25-35, Scott for HN; Fertraz Fruit 0-20-27, Cultifort for LN). HN seedlings received 8 mg N, 22 mg P and 59 mg K during the 14 weeks period, while LN received no nitrogen and the same amount of P and K as HN plants.

Experiment 6

Pinus halepensis seedlings were grown from March to September 2000 as described in experiment 5. In early October, they were transferred to a nursery at the E.T.S.I. Montes in Madrid, Spain and kept outdoors. In early March 2001, 80 seedlings were placed in four trays (blocks). Within a tray, seedlings were grouped in four rows of five seedlings and two rows were randomly assigned to each fertilization treatment (HN and LN). The trays were placed into a glass greenhouse for nine weeks. Seedlings were fertilized weekly. N was applied as NH₄NO₃ and P and K as KH₂PO₄. HN seedlings received 126 mg N, 19 mg P and 99 mg K, while LN received 18 mg N and the same amount of P and K.

Water relations

Pressure-volume (P-V) curves were made in experiments 1, 3, 4, and 6 following the free-transpiration method described in Koide *et al.* (1989). All measurements were done at the end of the experiments. Shoot xylem water potential was determined with a home-built pressure chamber. In experiments 1, 3 and 4, the sampled plants were watered the afternoon before and maintained in the dark until the excision of the shoot in the morning. In experiment 6, shoots were excised and rehydrated by immersing the cut end in distilled water for 1 hour. We used the upper half of six to eight shoots per treatment. From each curve, the osmotic potential at the turgor loss point ($\Psi_{\pi tlp}$), the osmotic potential at saturation ($\Psi_{\pi sat}$), and the modulus of elasticity (ϵ) were calculated as described by Koide *et al.* (1989). When plateaus in curves were detected the shoot weight at full saturation was calculated following the method described in Kubiske and Abrams (1990).

Stomatal conductance and residual transpiration

Stomatal conductance (g_s) was measured in experiments 2, 4, and 6 with an IRGA (LCA-4, ADC, Hoddesdon, UK), whereas in experiment 3, g_s was measured with a porometer (LI-1600; Li-Cor Inc., Lincoln, NE, USA). Stomatal conductance was measured around mid-morning or mid-day in five to twelve seedlings per treatment. In experiment 3, temperature, relative humidity, and photosynthetic photon flux density (PPF) during measurement varied between 20-22 °C, 57-65%, and 200-239 µmol m⁻² s⁻¹, respectively. In experiments 2 and 3, temperature and relative humidity, varied between 19-24 °C and 20-46%, respectively, and PPF was 1300 µmol m⁻² s⁻¹. In experiment 6, measurements were made at mid-morning inside a controlled-environment chamber where temperature, relative humidity and PPF was 22 °C; 65%, and 600 µmol m⁻² s⁻¹, respectively.

Residual transpiration (RT) was determined in experiments 1, 3, and 4 in 6-10 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. Shoot fresh mass

was measured to the nearest 1 mg at intervals of 0.5 - 1 h. By plotting shoot fresh weight *versus* time, a curvilinear relationship is obtained in which the linear portion represents water loss from plant surfaces after stomatal closure. 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 it at 80°C for 48 h.

Both g_s and RT measurements were done at the end of the experiments

Morphology and tissue N concentration

Morphology and N concentration were measured at the end of all experiments. Plants were randomly sampled ($n=12-30$) and immediately frozen to -30°C until processing. 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. The shoot to root mass ratio (S/R) was determined. To assess N concentration, shoots and roots of plants sampled in a block were pooled separately and ground. N concentration was determined by either the standard Kjeldahl procedure (experiments 3, 4 5 and 6) or by thermal conductivity using a LECO CHN-600 analyser (experiments 1 and 2).

Root growth capacity

The capacity of plants to produce new roots from the plug (RGC) was analysed at the end of the experiments 1 to 4 by transplanting seedlings into 3-liter pots (one plant per pot) containing perlite. We used this growing medium because roots are easily to clean from it. Eight to 16 seedlings per treatment were used in RGC tests. Seedlings were placed either in a glasshouse (experiment 1 and 3) where radiation was half that of the outside or left outdoors (experiment 2 and 4). Seedlings were kept well watered by irrigating them every 2-5 days and plants were not fertilized during the RGC test. Duration of RGC test varied from 21 days in experiment 3 to 102 days in experiment 4. At the end of RGC test plants were lifted, cleaned from the potting medium and all the new roots longer than 1 cm protruding out of the root plug were cut. RGC of each plant was

determined as either the number of new roots or the total mass of new roots after oven-drying at 50°C for 48 h. In all experiments shoot size was recorded and the ratio between the amount of new roots and shoot size (NR/S) was determined.

Frost damage

Freezing tests were performed in experiments 2 to 5. In experiments 2 to 4 whole plants ($n=8-10$) were subjected to a freezing cycle and frost damage measured by electrolyte leakage. Roots were protected against frost by transplanting seedlings to either a plastic box with moist sand or to a Styrofoam container and placed in a freezer. Temperature was reduced at a rate of $4-5^\circ\text{C h}^{-1}$ to either -8°C (*P. pinea*), -12°C (*Q. coccifera*) or -13°C (*J. thurifera*). Seedlings were kept in the target temperature for 3 hours and then it was raised to 10°C at a rate of $4-6^\circ\text{C h}^{-1}$. Electrolyte leakage was measured on leaves utilizing a protocol described in Villar-Salvador *et al.* (2004a). Frost damage (FD) was determined as

$$(EC_i \times 100)/EC_f (\%)$$

where EC_i is the electroconductivity of the water bathing the leaf pieces after 24 la and EC_f the electroconductivity of the same water after autoclaving the vials for 10 min at 110°C .

In experiment 5, freezing tests were performed on four dates during the experiment every two weeks. On each date, seedlings ($n = 9$) were subjected to a cooling rate of 1°C h^{-1} to -5°C where they were kept for three hours. Then temperature was increased at a rate of $4-6^\circ\text{C h}^{-1}$. Chlorophyll fluorescence was measured immediately before and 130 hours after the frost event with a portable fluorometer (Plant Stress Meter, Biomonitor AB, Umea, Sweden). Prior to each measurement, samples were dark adapted for 30 minutes. Basal (F_0) and maximum (F_m) fluorescence were measured. Variable fluorescence ($F_v = F_m - F_0$) and F_v/F_m , were calculated. The parameter used to measure the damage caused by frost was the difference in F_v/F_m between the measurements made before and after the test ($\Delta(F_v/F_m)$). This parameter, when measured 130 hours after the frost event it showed the strongest relationship with visible needle damage (data not shown).

Data analyses

Comparisons between fertilization treatments were made by ANOVA and by t-student tests or by the Mann-Whitney U test when variance homogeneity was not achieved. In the ANOVA fertilization and block were the main factors. In no case there was any interaction between block and fertilization. For simplicity, statistical results of the block are not presented. We considered results significant when p-values were ≤ 0.05 .

Results

LN seedlings in *J. thurifera* had more negative $\Psi_{\pi\text{sat}}$ and $\Psi_{\pi\text{tlp}}$ than HN plants. In the rest of species HN and LN plants did not have significant differences in $\Psi_{\pi\text{sat}}$ and $\Psi_{\pi\text{tlp}}$. Similarly, neither ε nor RT differed between HN and LN seedlings in any species (Table 1). HN plants in both pine species had higher g_s than LN seedlings, no differences existing between treatments in the rest of the species.

In all experiments HN seedlings had either higher shoot or leaf N concentration than LN seedlings, except in

Experiment 1 where no differences were found between fertilization treatments in *Q. ilex* and *Q. suber* (Table 1).

HN plants in all species had greater RGC than LN plants. However, only in *Q. coccifera* and in *Q. ilex* the new root growth capacity per unit of shoot size (NR/S) of HN plants was greater than LN seedlings, no differences existing between fertilization treatments in the rest of species (Figure 1).

Nitrogen fertilization increased plant size and tended to affect more shoot growth than root growth (Figure 2). As a consequence, in all species HN plants had higher S/R than LN plants. In *Q. ilex*, root mass in HN plants did not differ from that of LN seedlings. In experiment 5, HN seedlings in *P. halepensis* had smaller root mass than LN plants, while the reverse response was observed in shoot mass.

Fertilization did not affect FD of seedlings in *Q. coccifera* and *J. thurifera* but it was increased in *P. pinea* (Figure 3) and *P. halepensis*. In the latter species, differences were small and seedlings from both treatments hardened gradually, resulting in a complete resistance to a -5°C frost at the end of the experiment, in spite of the N needle concentration differences (Figure 4).

Table 1. Shoot or needle (only in *P. halepensis*) N concentration, components of water relations and water vapour gas exchange differences in high- and low-fertilized seedlings in several Mediterranean forest species. Data are means ± 1 standard error. Data in bold and with an asterisk indicate significant differences between fertilization treatments within a species

	N (mg g ⁻¹)	$\Psi_{\pi\text{sat}}$ (MPa)	$\Psi_{\pi\text{tlp}}$ (MPa)	ε (MPa)	RT ($\mu\text{mol kg}^{-1} \text{s}^{-1}$)	g_s (mmol m ⁻² s ⁻¹)
<i>Quercus ilex</i>						
High fertilization (154 mg N)	13.7 $\pm$ 0.6	-2.88 $\pm$ 0.11	-3.89 $\pm$ 0.14	14.0 $\pm$ 2.4	497 $\pm$ 67	
Low fertilization (0 mg N)	13.5 $\pm$ 0.4	-2.90 $\pm$ 0.08	-3.94 $\pm$ 0.15	14.7 $\pm$ 1.7	399 $\pm$ 41	
<i>Quercus suber</i>						
High fertilization (154 mg N)	12.9 $\pm$ 0.3	-2.80 $\pm$ 0.08	-3.38 $\pm$ 0.08	18.4 $\pm$ 1.1	675 $\pm$ 38	
Low fertilization (0 mg N)	11.7 $\pm$ 0.4	-2.90 $\pm$ 0.05	-3.56 $\pm$ 0.05	23.1 $\pm$ 2.6	630 $\pm$ 47	
<i>Quercus coccifera</i>						
High fertilization (150 mg N)	14.1 $\pm$ 0.2 *					114 $\pm$ 14
Low fertilization (0 mg N)	11.8 $\pm$ 0.2					119 $\pm$ 20
<i>Pinus pinea</i>						
High fertilization (100 mg N)	10.9 $\pm$ 0.1 *	-1.54 $\pm$ 0.10	2.09 $\pm$ 0.11	7.00 $\pm$ 0.94	362 $\pm$ 19	75 $\pm$ 5 *
Low fertilization (6 mg N)	8.8 $\pm$ 0.3	-1.42 $\pm$ 0.11	1.98 $\pm$ 0.13	5.45 $\pm$ 0.83	357 $\pm$ 33	59 $\pm$ 5
<i>Pinus halepensis</i>						
High fertilization (126 mg N) ¹	17.0 $\pm$ 0.8 *	-1.31 $\pm$ 0.06	-2.15 $\pm$ 0.04	2.82 $\pm$ 0.37		71 $\pm$ 5 *
Low fertilization (18 mg N)	8.8 $\pm$ 0.3	-1.37 $\pm$ 0.08	-2.19 $\pm$ 0.08	3.06 $\pm$ 0.37		44 $\pm$ 3
<i>Juniperus thurifera</i>						
High fertilization (150 mg N)	13.1 $\pm$ 1.8 *	-1.53 $\pm$ 0.10 *	-1.98 $\pm$ 0.10 *	6.71 $\pm$ 0.65	285 $\pm$ 25	124 $\pm$ 20
Low fertilization (30 mg N)	9.3 $\pm$ 0.6	-1.84 $\pm$ 0.09	-2.52 $\pm$ 0.16	7.10 $\pm$ 0.46	359 $\pm$ 45	112 $\pm$ 20

¹ Applied for 9 weeks at the beginning of the second growing season.

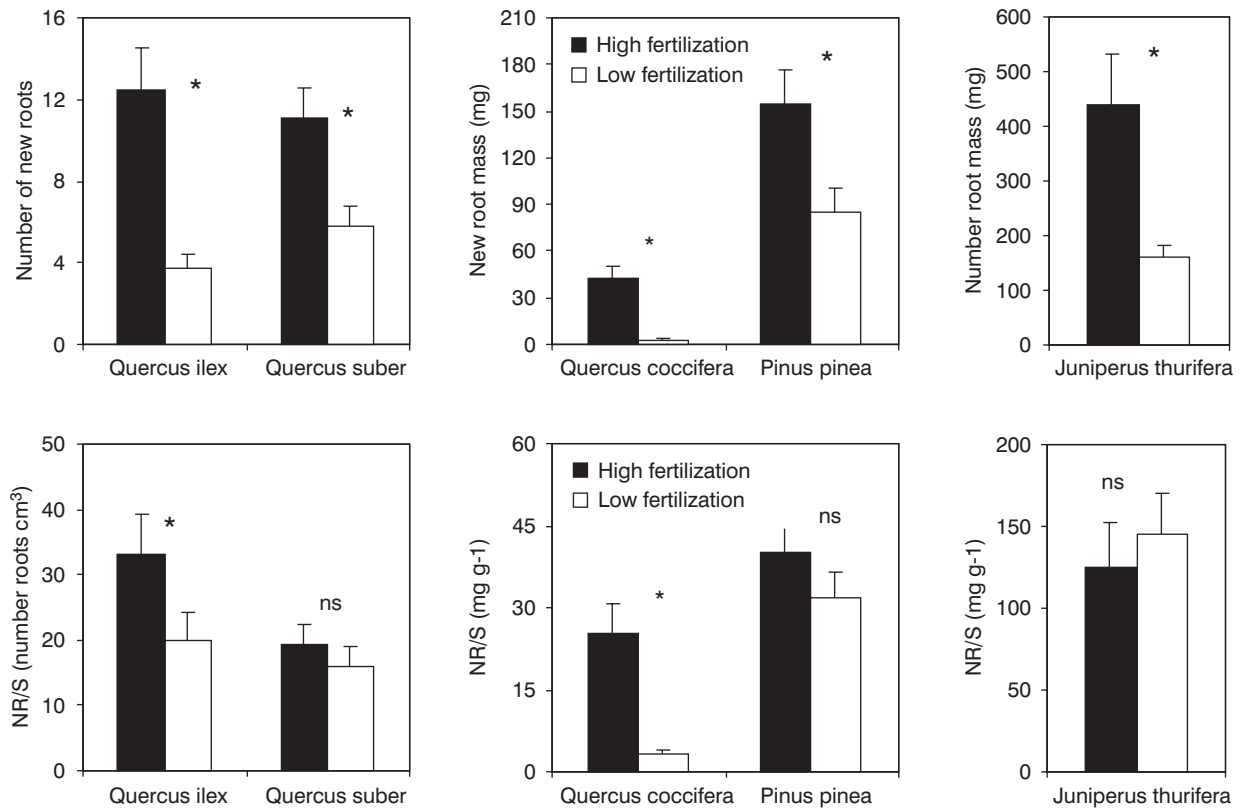


Figure 1. New root growth capacity (upper row) and the new root growth per unit of shoot size (lower row) in several Mediterranean forest species grown with high (HN) and low (LN) amounts of N fertilizer. New root growth capacity is represented as either the number (*Q. ilex* and *Q. suber*) or the mass (rest species) of new roots. Data are means ± 1 standard error. An asterisk represents statistical significant differences between fertilization treatments within a species.

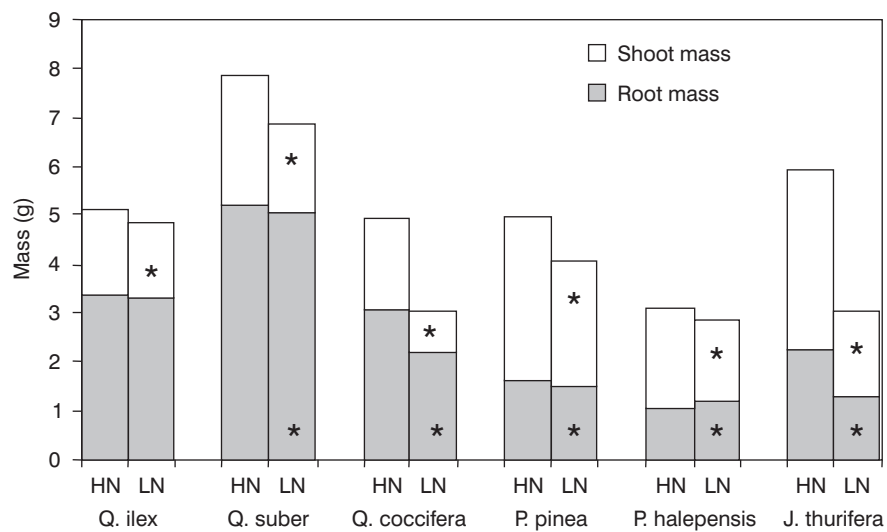


Figure 2. Shoot and root mass in several Mediterranean forest species grown with high (HN) and low (LN) amounts of N fertilizer. An asterisk represents statistical significant differences between fertilization treatments within a species and for each plant compartment.

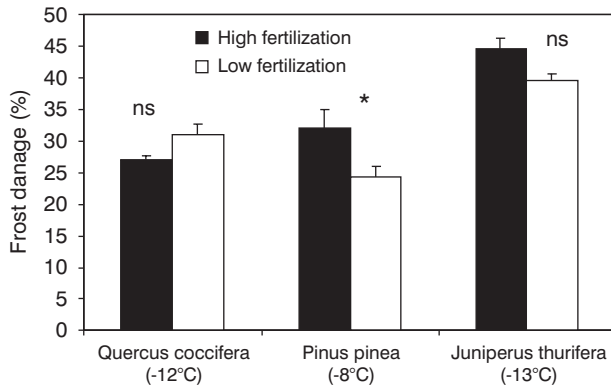


Figure 3. Frost damage in three Mediterranean forest species grown with high (HN) and low (LN) amounts of N fertilizer. Frost damage was determined as the relative release of electrolytes after subjecting each species to different frosts. Frost temperature in *Q. coccifera*, *P. pinea* and *J. thurifera* were -12°C , -8°C , and -13°C , respectively. Data are means $\pm$ 1 standard error. An asterisk represents statistical significant differences between fertilization treatments within a species.

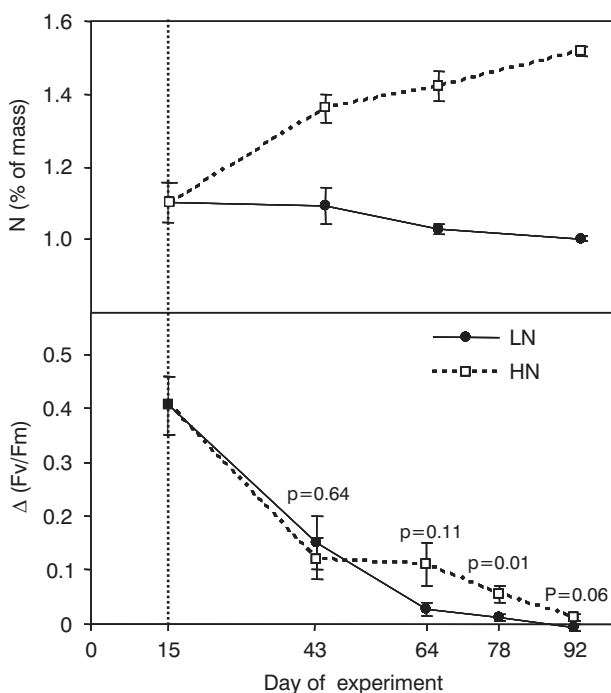


Figure 4. Evolution of needle N concentration and the difference between the F_v/F_m measurement before and after a frost cycle at -5°C ($\Delta(F_v/F_m)$) in *P. halepensis* seedlings grown with contrasted N fertilization (see experiment 5). The vertical dotted line indicates the beginning of the fertilization treatments. For $\Delta(F_v/F_m)$, p values of statistical comparisons in each measurement date are given.

Discussion

The experiments from which we obtained the data of this study were designed to address several questions related to fertilization of Mediterranean forest species. The heterogeneous experimental conditions might have influenced some of the results shown in this study, as it is discussed later. Therefore, any generalization of specific responses should be considered with caution. Overall results analysis of the six fertilization experiments revealed some general trends. Among the studied species, responses to contrasting N fertilization affected more the morphological characters than the physiological traits. Moreover, changes in most morphological traits tended to have the same variation pattern with increasing fertilization, whereas physiological traits had variable responses. Thus, HN plants were larger and had higher S/R than LN plants, which are responses that have been extensively documented in previous ecophysiological studies (see Canham *et al.*, 1996; Graff *et al.*, 1999). N fertilization increased g_s in both pine species but had no effect in *Q. coccifera* and *J. thurifera*. Both increase in and lack of response in g_s to N fertilization have been reported previously (see Green and Mitchell, 1992; Morgan, 1984). Within a species, greater plants with high S/R ratio and g_s transpire more than plants with the opposite traits (Leiva and Fernández-Alés, 1998) and this may increase their drought vulnerability to soil water shortage immediately after transplanting. S/R, as determined in this study, reflects the proportion of roots in the plug respect to the shoot size. Field transplanting is usually carried out when soil moisture is high and seedlings can produce new roots that rapidly embed into the surrounding soil if soil temperature is not limiting (Corchero de la Torre *et al.*, 2002). Most water uptake occurs through young roots and the physiological performance of seedlings after transplanting depends on the extent of these new roots (Bellot *et al.* 2002; Brissette and Chambers, 1992). Therefore, the proportion of new roots emerging from the plug with respect to shoot size (NR/S) in established seedlings might be a more meaningful measure of the balance between transpiration and water uptake than S/R. Contrary to S/R, in our study fertilization treatments in most species had similar NR/S and in *Q. ilex* and *Q. coccifera* NR/S was higher in HN seedlings than in LN plants. This suggests that HN plants do not necessarily become more vulnerable to drought than LN plants.

N fertilization has been shown to increase tissue N concentration (Miller and Timmer, 1994). In our study, HN plants had higher tissue N concentration than LN seedlings in all species except in *Q. ilex* and *Q. suber*. In these species, the lack of shoot N concentration differences between treatments occurred because the rate of shoot growth probably paralleled that of N uptake. As HN plants in most species are bigger and/or tend to concentrate more N than LN seedlings, their N content is higher than LN plants and this can benefit them more than LN plants when transplanted in oligotrophic soils or to sites with high herb competition (Timmer and Aidelbaum, 1996).

Some studies have reported negative effects of high N fertilization on the drought tolerance and frost resistance of plants. N fertilization reduced the capacity of *Pinus banksiana* and *Rosa rugosa* plants to maintain tissue turgor under water stress. In *P. banksiana* this was attributed to changes in ϵ whereas in *R. rugosa* this was related to a reduction in $\Psi_{\pi\text{sat}}$ (Augé et al., 1990; Tan and Hogan, 1995). In our study, only *J. thurifera* HN-plants had lower drought tolerance (less negative $\Psi_{\pi\text{tlp}}$) than LN seedlings and this was due to differences in $\Psi_{\pi\text{sat}}$ but not in ϵ (Table 1). Similarly, only pine species reduced their frost resistance in response to fertilization. In the rest of species fertilization had no effect in either drought tolerance or in frost resistance. Several studies have also reported no variation in drought tolerance traits in well-watered plants grown under contrasting fertilization regimes (Correia et al., 1989; DaMatta et al., 2002). Two reasons can be suggested to explain such results. First, developmental stage and time of year may influence plant responses to fertilization (Dalen and Johnsen, 2004; Tan and Hogan, 1995). Thus, in *P. halepensis* FD differences among fertilization treatments tended to disappear along the cold hardening period (Figure 4). In *Quercus* species, P-V curves and frost damage tests were made in late winter when growth had not yet resumed and plants were still cold hardened. In *J. thurifera*, frost tests were performed in mid-winter but P-V curves were made in October and plants had not probably hardened yet. Similarly FD in *Pinus pinea* was evaluated in the fall and frost hardiness probably had not fully developed. Second, species may have different responses to N fertilization. For instance, $\Psi_{\pi\text{sat}}$ and $\Psi_{\pi\text{tlp}}$ in *P. halepensis* and *P. pinea* have small seasonal variations or little changes in response to drought (Villar-Salvador et al., 1999;

Villar-Salvador et al., 2000). Kleiner et al. (1992) found no differences in $\Psi_{\pi\text{sat}}$ and $\Psi_{\pi\text{tlp}}$ in two oak species in well-watered plants grown under contrasted fertilization. This suggests that in several species these traits may have low responsiveness to changes in resource availability. Future research should be directed to understand the different cold acclimation strategies of Mediterranean species and how nursery practices, especially nitrogen fertilization, interact with this process.

Most studies on Mediterranean species have demonstrated that HN plants have greater growth and their survival after transplanting is frequently higher than LN plants, in spite of that some of their morphological and physiological features may increase their vulnerability to drought (Luis et al., 2004; Oliet et al., 1997; Puértolas et al., 2003; 2000). In *Q. ilex*, HN had lower mortality and higher RGC than LN plants (Villar-Salvador et al., 2004b). This suggests that the better out-planting performance of HN plants might be, in part, explained by their higher root growth capacity. HN plants in all studied species had higher RGC than LN seedlings, which is in accordance with previous studies (van den Driessche, 1992). Plant performance after planting depends on the formation of an extensive and deep root system (Kaushal and Aussenac, 1989). We do not know if high RGC in HN plants determined in the nursery implies a higher field root growth capacity (FRG) than in LN seedlings. If HN plants have also higher FRG than LN plants, it can be hypothesized that HN plants would have better drought avoidance capacity than LN plants. This may be more important for seedling establishment in Mediterranean environments than the potential reduction in drought tolerance caused by high fertilization, especially if high FRG permits plants to explore deep humid soil layers during the dry season. Studies are needed to know if HN and LN plants have different FRG and if these differences explain their out-planting performance differences.

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