Plasticity in *Pinus pinaster* populations of diverse origins: Comparative seedling responses to light and Nitrogen availability

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ABSTRACT

The effects of light and N availability, and population on seedling morphology and biomass allocation were assessed in eleven months seedlings from ten *Pinus pinaster* populations. We conducted a garden experiment using a light gradient (full sunlight, HL= 337.1 µmol m^2 s^{-1}; medium light, ML= 120.6 µmol m^2 s^{-1}; and low light, LL= 30 µmol m^2 s^{-1}) and two contrasting soil fertility regimes: low-N and high-N availability. Light availability was an important factor controlling phenotypic plasticity. However, differences between productivity environments were observed, especially in full sunlight and medium light conditions, which suggest that soil fertility may be very important at early successional stages at open and medium shaded microsites, but would exert low influence in deep shade conditions. Population-specific responses were observed for many traits, which suggest a genetic control of morphological plasticity. All populations produced more total dry weight with HL and high-N. When light and/or N richness varied, growth depended upon which resource was more limiting. Results indicated ontogenetic drift but when seedlings were compared at the same size, results fitted with optimal partitioning theory. Allocation to needles was higher in HL and ML/ high-N conditions, while allocation to root was higher in low-N seedlings in HL and ML conditions. With shade (ML and LL) all populations allocated more dry weight to stem, regardless N availability. A significant increment in stem height was observed with shade increase.

Keywords: Allometry/ Mediterranean/ Optimal partitioning/ Environment variation/ Root: shoot ratio.

1 INTRODUCTION

There is little information on intra-specific variation in morphology and biomass allocation as a mechanism involved in shade avoidance at the seedling stage, and the influence of soil fertility. Understanding how seedlings respond to resource variations is very important for predicting seedling establishment and growth of future stands structure (Jose et al., 2003), and evaluating the success of seedling and sapling establishment from open to low-light environments (Rodríguez-García et al., 2011a; Rodríguez-García et al., 2011b; Monnier et al., 2013). Phenotypic plasticity can be defined as the ability of a single genotype to express different morphological, anatomical, physiological and/or behavioural traits in response to environment variation (Chapin et al., 1987). New biomass is allocated to organs with the most limiting resources according to the optimal partitioning theory, OPT or balanced-growth hypothesis (Chapin et al., 1987). A different approach is the allometric biomass partitioning theory (APT) model, based on allometric theory that examines how organism attributes change with body size (Evans, 1972; Coleman et al., 1994; Gedroc et al., 1996; McConnaughay and Coleman, 1999; Müller et al., 2000; Moriuchi and Winn, 2005; McCarthy and Enquist, 2007; Poorter et al., 2012), and which with proportionally more biomass is allocated to leaves than to roots as plants grow (Shipley and Meziane, 2002). Nonetheless, If allocation changes with size, any factor that influences size should thereby change allocation (Müller et al., 2000). Then, If the variation in biomass distribution demonstrates OPT, plants should invest more in roots if water and nutrients become limiting; while biomass allocation should favour leaves and/ or stem if light becomes more limiting or there is surplus of Nitrogen.

Light has been the focus of most research on forests dynamics, and the dependence of different species on gaps for regeneration have been associated with a range of shade tolerant physiologies (Catovsky et al., 2002) and competitive abilities related to differences in juvenile responsiveness to light (Valladares and Niinemets, 2008; Monnier et al., 2013). Competitive ability can be separated into two components (Goldberg and Werner, 1983; Goldberg and Fleetwood, 1987; Goldberg and Landa, 1991; Keddy et al., 1998), the competitive effect (ability to grow and suppress neighbours) and competitive response (ability to tolerate suppressive effect from neighbours: survival). Pioneer, fast growing, tall plants will often have high competitive effect values (Gaudet and Keddy, 1988). These are commonly shade

avoiders which characterize early to intermediate succession stages (Grime, 1979), and that maximize vertical growth (to suppress neighbours) and light interception through plastic crown adjustments (Niinemets et al., 2002; Monnier et al., 2013). But the magnitude of seedling response to light quantity and quality also depends on nutrient requirements of species and on nutrient availability, specially of N availability (Valladares et al., 2000; Dehlin et al., 2004; Sardans et al., 2006). Then, structural and physiological plasticity may depend on the species shade tolerance (Portsmuth and Niinemets, 2007) and may vary along soil fertility and seasonal drought gradients (Coomes and Grubb, 2000). Pioneer species normally respond faster and stronger to fertilisations in term of growth and nutrient uptake than the species of more advanced succession stages (Sardans et al., 2006). On fertile sites stem growth is essential in allowing individuals to avoid overtopping by similarly rapidly growing neighbours (King, 2003) and intercept light. For example, height growth and total biomass production of *Pinus halepensis* Mill. (Monnier et al., 2011; Monnier et al., 2013) and *Pinus sylvestris* L. and *Betula pendula* seedlings (Dehlin et al., 2004) responded stronger to shade in high-fertility sites than in low-fertility sites. These plastic shade and nutrient-induced adjustments may procure an important adaptive advantage to early successional species seedlings by higher niche pre-emption and occupation during natural forest regeneration (Monnier et al., 2013).

Maritime pine (*Pinus pinaster* Ait.) is a forest species that grows naturally in the western part of the Mediterranean, but that has been planted in many countries around the world (CAB-International, 2002). *P. pinaster* grows in a great array of soils conditions varying from Mediterranean to Temperate-Oceanic climates (Gandullo and Sánchez-Palomares, 1994). The species is considered as a main colonizing species after disturbance with high light regime for growth (Gil et al., 1990) but that can take advantage of microenvironmental amelioration imposed by shelter's shade (tree overstorey, understorey or both) at seedling stage (Rodríguez-García et al., 2011b). Soil fertility and chemistry properties (e.g pH) have been shown to affect early establishment of *P. pinaster* (Rodríguez-García et al., 2011a). But overall, growth responses to shade and nutrients availability, and seed origin at seedling stage are still not well defined for this species. Additionally, *P. pinaster* shows elevated levels of genetic variability (González-Martínez et al., 2002) and many different populations can be found due to an important genotype by environment interaction that has enabled local adaptation to ecological conditions (Gil et al., 1990; Gandullo and Sánchez-Palomares, 1994;

Alía et al., 1996). This is important because soil fertility, geographical location and climate may influence on the response to light variation and therefore, populations located in different ranges may present different coping strategies to light and soil fertility variation.

In this study, we have investigated seedling phenotypic plasticity in, eleven-months old seedlings, ten *P. pinaster* populations to light availability and soil fertility variation, simulating shelter cover (three levels of light) and two contrasting nutrient regimes to obtain a productive (high fertility or high-N) and unproductive (low fertility or low-N) environment. Our objectives were to know: 1) whether the mechanisms by which light affects *P. pinaster* growth and biomass partitioning at seedling stage operate differently when soil fertility varies, and 2) whether they vary with population. We expected higher seedling growth and biomass with full sunlight and high-N availability, but some of our hypotheses were that 1) with increasing shade, shoot growth and biomass allocation to leaves and stem would be optimally adjusted and would be higher on fertile than on unfertile sites; 2) On the contrary, on unfertile sites there will be higher relative allocation to root than on fertile sites; 3) populations from milder and northern latitudes may present traits that enhance growth and high competitive effects (shoot growth and stem elongation) in the shade and rich-nutrient soils, as light might be the most limiting factor; while populations from drier and southern latitudes, which support higher levels of irradiance, and which may present traits that favours root system growth to cope with stress of water and nutrients limitations, may present lower competitive effects in shade.

2   MATERIAL AND METHODS

2.1   *Plant material, experimental design and culture conditions*

Ten populations of *Pinus pinaster* were selected along a latitudinal cline (Table 1), from France (*Mimi*), North-western Spain (*Scip*), Central Spain (*Bayu, Cuel, Arsp, Gred, Vald*), Eastern Spain (*Espa*), Southern Spain (*Oria*) and Morocco (*Tamb*). The experiment was conducted on a plot of land neighbouring a greenhouse located at the *Universidad de Valladolid* in Palencia, Spain (42º01'N-4º32'W, 739 masl). The average annual precipitation of the experimental site is 450 mm; average annual temperature is 12 ºC. The original seed sites and the experimental site share a Mediterranean-type climate with precipitation in autumn and spring, hot dry summers and cold winters. Commercial seeds from each population were received in January 2006 from the *Centro Nacional de Mejora Forestal*

(INIA. Madrid, Spain). Following germination and initial growth, seedlings were transferred (April 2006) to Arnabat® forty-eight cavity forest trays (308 cm³). Seedlings planted in the twenty-four central cavities of the trays were used for the experiment; seedlings planted in the border cavities were excluded from the measurements. Each cavity was 18 cm² with vertical anti-spiraling ribs along the inside walls. The trays were filled with a 3:1 (v:v) unfertile peat-vermiculite mixture, to which 3.5 kg/ m³ of NPK (14-8-15) slow release fertilizer (SRF) was added, following commercial indications (Plantacote®) of dose for a normal nutrition state of culture of conifers longer than 6 months in tray. The range suggested by the commercial brand varied between 3.0-7.Kg/m³. The formulation had and equivalent stated nutrient release period: 8-9 months at 21ºC.

A blocked, split-plot experiment with a factorial combination of light (three levels) population origin (ten levels) and nutrient availability (two levels) was designed to test for main effects and interactions on seedling morphology and biomass traits (Table 2). Three blocks were used. A light gradient consisting of three light environments (main plots) was randomly established per block: high light (HL) or full sun irradiance, medium light (ML) with a 64% reduction of sun irradiance, and low light (LL) with an 91% reduction of sun irradiance. ML and LL environments were obtained by constructing shade-houses or 2.60 m² x 1.90 m high tents, with one (ML) or two (LL) layers of black raffia mesh (PE monofilament and HDPE polyethylene raffia) and wire. Light plots were oriented north-south in three 15 m long parallel lines or replicate blocks. The distance between the blocks was 2 m. Twenty forest trays, consisting of two trays for each of the 10 populations were randomly placed within each light plot. A two-level nutrient treatment (low-N and high-N) was implemented in the split-plot design and randomly assigned to half the trays, one per population, in each light plot. Control or low-N treatment was established by the amount of SRF added to the substrate when the trays were filled. The average volume of compacted substrate per cavity was of 453.3 ml. So on average, each cavity received 222 mg N, 127 mg P and 238 mg K. The high-N (twofold N than low-N) treatment was obtained by adding N to the control substrate in a concentrated NH_4NO_3 solution (32 %; 1.3 g/ml) divided into 18 equal weekly doses (June to mid November 2006) and administered to the other half of the trays, one per population. Total N administered was 222 mg per control (low-N) seedling and 444 mg per high-N seedling. The amount of N administered in this study to obtain low-N treatment may not seem so low in comparison with other studies (Oliet, 2004; Villar-Salvador et al., 2004; Monnier et al.,

2011). In our study the amount for low-N was fixed following standard procedures of use of slow release fertilizer for culture of conifers longer than 6 months in trays, considering that *Pinus* species have medium nutrients needs and, that conifers with medium nutrient needs would need 5-6 kg/m^3 for an optimal growth without stress (Jiménez Gómez, 1992)

Photosynthetic photon flux density (PPFD, μmol m^2 s^{-1}), was measured in each light environment with cross-calibrated radiation sensors (LI-190SA PAR Quantum Sensors; LI-COR Biosciences, USA) connected to a data logger that measured conditions every 10 minutes and gathered the data every hour (Figure 1). PPFD measurements were taken during a whole 24h period on (1-2) sunny days in June, July, August and September, 2006. Seedlings in the medium light environment (ML) received an average of 120.6 μmol m^2 s^{-1}, a daily PPFD nearly three times lower than seedlings in full sun (HL, 337.1 μmol m^2 s^{-1}). The daily PPFD received by seedlings in low-light (LL) averaged 30.0 μmol m^2 s^{-1}, eleven times lower than seedlings in full sun. Relative air humidity (RH, %) and air temperature (T, ºC) were measured and recorded throughout the whole experiment (April 2006-March 2007) using field data loggers (Testo 175-H2; Testo S.A., Spain). Average air T and RH were 15.33 ºC and 68.85% in the HL environment, 15.04 ºC and 69.83% in ML, and 14.99 ºC and 69.28% in LL. Seedlings were kept well-watered by watering them every 1-3 days. Water was obtained from a domestic source and delivered by hand with a slosh nozzle at a constant flow rate of 2 L min^{-1} to field capacity. Seedlings cultured under full sunlight were watered mostly every day to ensure that the growing media in full sunlight would be always optimal (over 15 % in volume), (Puértolas et al., 2009). Water content in the growing media was higher in shaded trays before each watering (Puértolas et al., 2009). Irrigation water pH was 7.7; electrical conductivity at 25ºC (EC) was 2.7 μS/ cm, and chemical composition was NO$_3$-N 14 mg/l, P 0.0 mg/l; Mg 4.4 meq/l; Ca 51.4 mg/l; Na 5.1 mg/l; K 1.25 ppm.

Table 1. Main climate and physiographic characteristics of the *P. pinaster* populations studied.

Population	Acronym	AI	AIZ	Alti	P-spr	P-sum	P-aut	P-win	T-spr	T-sum	T-aut	T-win	Location
San Cipriano	Scip	61	PH	727	119.40	37.10	142.80	203.67	13.50	20.67	15.77	9.47	42°12'N-8°30'W
Arenas de San Pedro	Arsp	57	H	728	88.63	39.03	130.13	177.50	13.20	20.57	14.20	5.93	40°12'N-5°05'W
Mimizán	Mimi	40	H	20	73.67	52.67	81.67	94.33	11.42	19.25	13.75	6.58	44°12'N-1°13'W
Sierra de Gredos	Gred	48	H	1400	69.20	39.13	95.40	108.50	9.97	17.73	10.87	2.47	40°15'N-5°00'W
Tamrabta	Tamb	45	H	1760	110.00	19.00	40.17	151.00	8.75	19.67	12.58	4.42	33°20'N-5°01'W
Valdemaqueda	Vald	31	H	1220	53.67	35.17	70.40	72.83	12.40	19.80	13.10	4.97	40°30'N-4°15'W
Sierra de Espadán	Espa	25	SH	1000	46.30	34.57	75.37	47.57	11.73	21.43	14.70	7.13	39°55'N-0°25'W
Bayubas	Bayu	25	SH	900	53.07	32.23	44.17	46.37	9.60	19.83	12.07	4.07	41°30'N-3°50'W
Cuéllar	Cuel	22	SH	834	47.17	23.37	44.03	46.37	10.70	20.97	12.43	4.57	41°10'N-4°30'W
Sierra de Oria	Oria	19	SemA	1150	41.17	18.80	37.13	36.57	10.80	20.97	13.30	5.10	37°30'N-2°20'W

AI: Aridity Index. AIZ: Aridity Index Zone (De Martonne, 1926). PH: per-humid; H: humid; SH: sub-humid; SemA: semiarid; Alti: elevation, m.a.s.l.; P-spr, P-sum, P-aut and P-win refers to average rainfall (mm) in spring, summer, autumn and winter, respectively. T-spr, T-sum, T-aut and T-win refers to average temperature (ºC) in spring, summer, autumn and winter, respectively.

**Table 2**. Seedling morphological and biomass traits measured.

Acronym	Variable name	Units
H	Total stem height	cm
D	Basal diameter	mm
LS	Lateral stems	counts
Leaf-dw	Total leaves mass (primary + secondary needles dry weight)	g
Stem-dw	Total stem mass (main shoot + lateral stems dry weight)	g
Root-dw	Total root mass (root dry weight)	g
T-dw	Total dry weight (needles + stem + root mass)	g
LMR	Leaf mass ratio (leaf-dw/ T-dw)	$g^{-1} g^{-1}$
SMR	Stem mass ratio (stem-dw/ T-dw)	$g^{-1} g^{-1}$
RMR	Root mass ratio (root-dw/ T-dw)	$g^{-1} g^{-1}$
R:S	Root: shoot ratio (root mass/(needles +stem mass)	-
LAR	Leaf area ratio (primary + secondary needle area/ plant mass)	$cm^2 g^{-1}$
SLA	Specific leaf area (needle area/needle dry weight)	$cm^2 g^{-1}$
TSN	Total secondary needles	counts

2.2 Data collection and measured variables

At the end of the experiment (March 2007), three seedlings were randomly selected from each combination (light x soil fertility x population x block, n=540 seedlings). Stem height (length of the main stem measured to the nearest 5 mm with a hand tape) and basal diameter (root neck measured with a calliper to the nearest 0.01 mm) of each plant were measured. Harvested plants were divided into root, stem, lateral stems (secondary stems grown from axillary buds of the main stem) and needles. Primary needles (inserted in the primary and secondary stems) were separated from secondary needles (from brachiblasts), and a subset of ten primary needles and ten secondary needles from each seedling (or the number found per seedling) were scanned using a Windendro scanner with a resolution of 300 dots per inch. The average area of an individual needle (one-side needle area) was estimated using Winneedle software. All seedlings and the subsets of scanned primary needles were oven-dried for 48 h at 70 ºC and weighed. The weight of the scanned needle subsets after drying was used to calculate the average specific leaf area (SLA) of the needles. Different variables were obtained from these measurements (Table 2). Finally, an aridity index (AI) for characterizing general climate of the study populations was constructed for each population, and variables that describe the climate (Table 1) were obtained (June 2012) from weather data gathered from El Atlas Digital de la Península Ibérica (Ninyerola et al., 2005), used for Spanish populations, and from www.worldclim.org site (for French and Moroccan populations) from the closest meteorological stations. The AI was based on the index of De Martonne (1926),

computed using the annual average temperature (T, ºC) and precipitation (P, mm), as P/ (T+10).
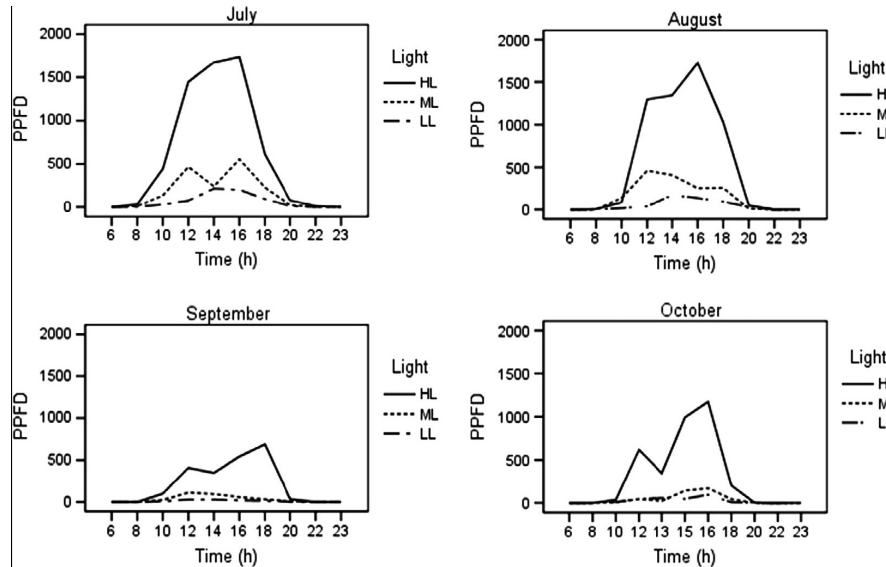
Figure 1. Daily time course of light availability in experimental light treatments on representative sunny days, measured on July, August, September and October, 2006. PPFD, Photosynthetic photon flux density (μmol m^2 s^{-1}); HL, high light or full sunlight; ML, medium light conditions; LL, low light conditions.

2.3 Statistical analysis

First, multifactor analyses of variance (GLMs) were used to investigate the effects of light, soil fertility and population origin on seedling traits. The main effects of light were tested against the block x light interaction term due to the split-plot design. Variables that did not meet normality and homoscedasticity assumptions were transformed to their natural logarithms before analysis. Post-hoc comparisons were carried out using Tukey multiple comparisons. Then, allometric relationships between plant compartments biomasses (needles, stem and root dry weight; hereafter Leaf-dw, Stem-dw and Root-dw) and total plant biomass (total plant dry weight, T-dw) were looked, that is, linear leaf mass ratio (LMR), stem mass ratio (SMF) and root mass ratio (RMR) regression models were constructed. The common allometrical relationship between the dry weight (dw) of Y (referring to either root, stem or needles) and the total dw of X (total plant dry weight, T-dw) is Y= a X^b. We used logarithmic-transformed values of X and Y to make relationships linear (Lenssen et al., 2003). As we wanted to test whether the continuous predictor variable (T-dw) had different effects

9

on the response variables (needles, stem and root dry weights) at different levels of categorical independent variables (population, light and soil fertility as categorical factors), homogeneity-of-slopes models (general linear models-GLMs), were employed. Populations were initially analysed together but when a significant population effect was observed, allometric equations of LMR, SMR and RMR were developed for each population separately. We obtained scatter plots and linear regression equations. All equations fits were checked by means of residuals, normality and model's adjustment parameters. Then, paired-slopes comparisons were carried out between treatments using least square estimates. Changes in linear regression slopes were investigated according to the methodology described by (Coleman et al., 1994; McConnaughay and Coleman, 1999). If b is equal to 1 a gives the ratio between Y and X; but if b significantly differs from 1, the ratio of Y and X changes with size, in this case with T-dw (McCarthy and Enquist, 2007). When there were signs of ontogenetic drift and significant effects of T-dw on light, N or population effects, then, the effects of the treatments were tested with ANCOVA analyses using values of needles, stem and root dry weights as dependent variable and values of T-dw as covariable (Coleman et al., 1994; Lenssen et al., 2003). This process is known as size correction after ontogenetic drift detection. Spearman rank correlation analysis was carried out to check out the relationships among morphological and biomass traits, and climate and geographical (habitat) factors of the studied populations. After that, a Factor Analysis (FA) with all climatic and geographical variables was conducted in order to reduce the number of variables and to search for combinations of climatic and/or geographical variables that explained the largest variation in the data set of the population's habitat factors (Härdle and Simar, 2007). The FA was based on the correlation matrix and the factors were rotated according to the *varimax normalized* method. The extraction of the principal factors was based on the *Principal Component* method. Finally, we carried out a Canonical Correspondence Analysis with the HL/ low-N data subset to graphically describe population's similitude in climatic and physiographic factors defining their habitats, and therefore creating a population-habitat's characteristics biplot to show it. We used populations as the response variable, morphological variables of the HL-low N data subset as the explanatory variables, the seedling total dry weight of that subset as covariable, and the climatic and physiographic as supplemental variables. The relationship between each explanatory variable with the population data was tested using a permutation test (Monte Carlo) with 499 unrestricted permutations under a reduced model.

Multivariate analyses of variance, regression models, Spearman correlation analysis and Factor Analysis (FA) were conducted with Statistica 6.0 software, while multivariate Canonical Correspondence Analysis (CCA) was carried out with Canoco 4.5 software.

3 RESULTS

3.1 *Light, soil fertility and population interaction effects*

Multifactor analyses of variance showed a great number of morphological traits (9 of 14) impacted by soil fertility and light interaction effects (Table 3). The highest average total plant dry weight (T-dw) observed was in full sunlight, high-N seedlings (Table 4). Higher N availability increased seedling T-dw significantly in full sunlight (11 %) and medium-light (18 %), but not in low-light seedlings (increment of 2 %). *Tamb* T-dw was notably increased when soil fertility was higher (Figure 2). Seedling stem height (H) increased with soil fertility in high-light (HL) and medium-light (ML) conditions (Table 4), and with increasing shade from HL to ML or LL, but without significant differences between ML and LL (Table 4). The highest RMR was observed in full sunlight and low-N seedlings. Higher N availability in full sunlight reduced R:S a 50% compared to low-N seedlings (Table 4), while the reduction in medium- and low-light seedlings was of 27 % and 12 %, respectively. Effects of soil fertility on the proportion of root biomass production (RMR) depended on both light and population effects (L x N x P, p<0.05). *Tamb* population was the one which showed the highest differences with the rest of populations in stem height, RMR and R:S (Figure 3). With increasing shade, relative biomass allocation to stem and needles (SMR and LMR, respectively) increased at expense of RMR (Table 4). Higher N availability increased LMR and SMR at expense of RMR in full sunlight and medium- light conditions. However it did not affect to LMR, SMR and RMR in low-light seedlings. Regarding leaf structural traits, the average leaf area ratio increased significantly with shade (light effect; p<0.0001; F=54.56) and with soil fertility (N effect; p<0.001; F= 5.38). Specific leaf area (SLA) was similar in high and medium-light. SLA was higher in low-light seedlings but without important differences with those from high and medium light (data not shown). Finally, both light and N effects separately (but higher with light) were population-specific for some secondary growth traits (Table 3) such as seedling basal diameter (D), not affected by soil fertility and light interaction effects (Table 4), number of lateral stems (LS) and number of secondary needles (TSN). These two last variables did not differ importantly among treatments.
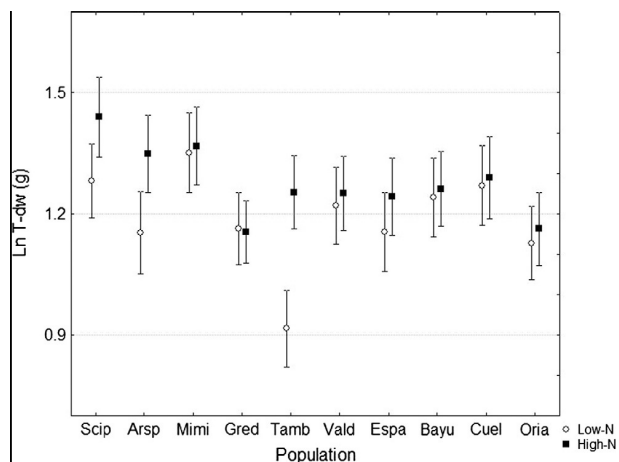
Figure 2. Mean total seedling dry weight (Ln T-dw) of ten populations of *Pinus pinaster* in different N availability environments. P x N effect: *F*=2.86, *p*<0.01. Vertical bars denote 0.95 confidence intervals.

Table 3. Significance for soil fertility, light, population and their interactions effects (error terms are not included). Light term refers to three irradiance levels (high, medium and low light availability). Soil fertility refers to two nitrogen levels (low and high N availability). ***p<0.001; **p <0.01; *p <0.05; *ns*: not significant (*p* >0.05).

Variable	Block	Soil fertility (N)	Light (L)	Population (P)	N x L	N x P	L x P	N x Lx P
H	ns	**	**	***	**	***	***	ns
D	ns	***	**	***	ns	***	***	ns
LS	ns	*	ns	***	ns	**	**	ns
Leaf-dw	ns	***	**	***	***	***	***	ns
Stem-dw	ns	***	*	***	***	ns	***	ns
Root-dw	ns	**	**	***	ns	*	***	ns
T-dw	ns	***	**	***	**	**	***	ns
LMR	ns	***	ns	***	***	ns	*	ns
SMR	ns	***	***	***	**	ns	*	ns
RMR	ns	***	***	***	*	ns	ns	*
R:S	ns	***	***	***	***	ns	ns	ns
LAR	ns	**	***	***	ns	ns	ns	ns
SLA	ns	*	***	*	ns	ns	**	ns
TSN	ns	**	*	***	ns	ns	***	ns

See Table 2 for variables names.

12
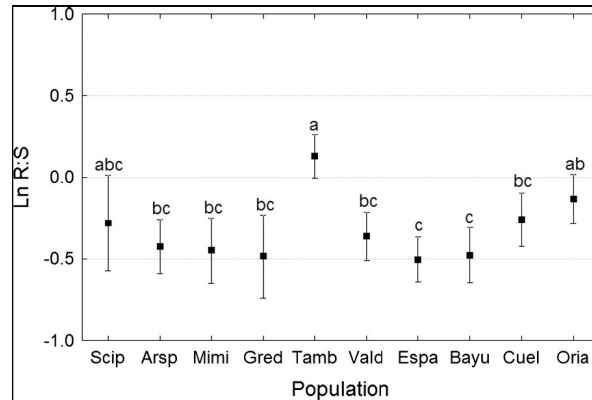
Figure 3. Adjusted means (Ln T-dw as covariable: 1.21) of the root: shoot (R:S) of ten *P. pinaster* populations. P effect: $F=2.1235$, $p<0.05$. Vertical bars denote 0.95 confidence intervals.


3.2   Allometric analyses of the dry weight partitioning patterns

Allometric relationships between plant compartments dry weights and total plant biomass were looked. A linear leaf mass ratio (LMR) model (Ln Leaf-dw *vs.* Ln T-dw; $R^2_{adj.}=0.97$; $p<0.001$; $F=150.20$) showed a non-significant population term ($p>0.05$), but a significant interaction between T-dw and light (L x Ln T-dw; $F=5.21$; $p=0.005$), and light N interaction effects (L x N x Ln T-dw; $F=3.53$; $p=0.03$). All populations were then pooled and regression curves were fitted for N and light treatments (Figure 4a). Slopes of the LMR equations of low-N and high-N seedlings differed significantly in high-light (HL) and medium-light (ML) conditions (Figure 4a). HL/ low-N equation had greater slope ($\beta>1$) than the rest of treatments ($\beta<1$), indicating that LMR was proportionally greater as seedlings grew (ontogenetic drift); greater than seedlings with high-N regardless the light environment. Allocation analyses showed a significant inter-population variation in the stem mass ratio model (SMR, Ln Stem-dw *vs.* Ln T-dw; $R^2_{adj.}=0.94$; $p<0.001$; $F= 59.02$), in response to light (L x P x Ln T-dw; $p=0.017$; $F=1.86$). Then, different linear SMR models were constructed for each population. Significant differences among treatments in dry weight partitioning were detected in four populations: *Vald, Espa, Cuel* and *Tamb*, but allometric relationships indicated that differences in the growth rates were due to ontogenetic drift ($\beta\neq1$), (see Figure 4b,c and Appendix). Finally, root mass partitioning analyses with the RMR model (Ln Root-dw *vs.* Ln T-dw; $R^2_{adj}=0.97$; $p<0.001$; $F=128.49$) indicated significant population-specific and light-dependent responses (L x P x Ln T-dw interaction; $p=0.014$; $F=1.91$). A significant response

13

to N availability, which varied depending on the light conditions (L x N x Ln Tdw; p=0.003; F=5.64), was also observed. Then, different linear models constructed for each population revealed significant adjustments in half of the populations: *Scip, Arsp, Tamb, Vald* and *Espa* (Figure 4d, e, f and Appendix), but analyses indicated ontogenetic drift (β≠1) as well. Overall, the slopes of the RMR equations under HL were lower in low-N than in high-N seedlings, although it differed significantly only in seedlings of *Arsp* (Figure 4f). These indicated that low-N seedlings allocated proportionally lower dry weight to roots as seedlings developed in size, and moreover, lower allocation to roots than high-N seedlings.

3.3   Optimal allocation patterns and stem height after size correction

After detection of ontogenetic drift, allocation relationships were checked out at a common size. ANCOVA analyses were employed using total plant dry weight (Ln T-dw) as covariable (Ln T-dw mean: 1.21). The covariable presented significant interaction effects with light, soil fertility and population (L x N x P x Ln-Tdw, p<0.001) in all analyses carried out. Significant adjustments in Leaf-dw occurred in all populations in response to higher N availability (N x L interaction; p<0.001; F=5.61) in full sunlight and medium-light conditions (Figure 5a). Significant adjustments were also observed in low-N seedlings in response to shade (medium light with respect to high light), but not to deep shade (low light). Stem dry weight (Stem-dw) increased (Figure 5b) when light availability decreased (p<0.001; F=12.25). Higher N availability did not increase stem dry weight, and significant differences among populations were not observed. Significant adjustments in root dry weight partitioning were observed depending on the light and N availability (L x N interaction; p<0.001; F=7.48), and the population origin (p<0.001; F=2.00). Seedlings with low-N significantly invested more dry weight on roots (Root-dw) than high-N seedlings with high and medium light conditions (Figure 5c), but significant differences were not observed in deep shade (low light). Finally, when stem height was compared at the same seedling size, it increased significantly with shade for most of the populations, and especially in deep shade conditions (Figure 6), although differences between N treatments within the light treatments were not observed. Regarding the rest of morphological traits, when seedling size was controlled, differences in number of lateral stems, basal diameter, LAR, and SLA, left to be significant.
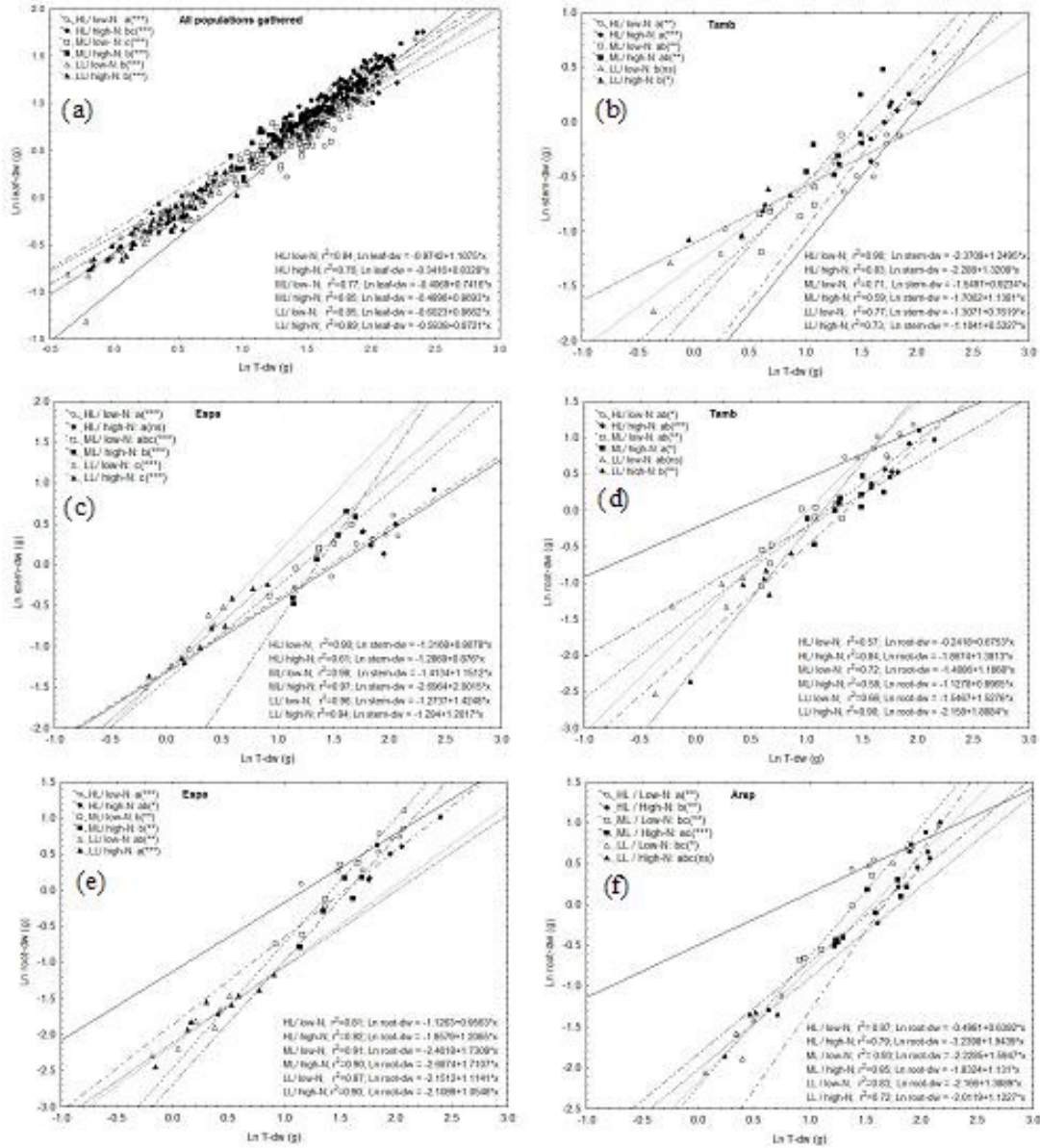
Figure 4. Allocation equations of: (**a**) leaf dry weight (LMR; with all *Pinus pinaster* populations gathered), and (**b**, **c**) stem dry weight (SMR) and (**d, e, f**) root dry weight (RMR) in eleven months seedlings of three of the *P. pinaster* populations that presented plastic adjustments in biomass allocation to soil fertility (Low and high-N) and light variability: HL, high light or full sun; ML, medium light conditions; LL, low light conditions.

15

Table 4. Average values and standard errors of morphological traits measured in eleven months *Pinus pinaster* seedlings, cultured in different conditions of soil fertility (low and high-N availability) and light variability: HL, high light or full sun; ML, medium light conditions; LL, low light conditions. Letters in bold indicate significant differences among treatments (p<0.05).

| Light	Soil fertility	H (cm) ± S.E.	D (mm) ± S.E.	T-dw (g) ± S.E.	LMR ± S.E.	SMR ± S.E.	RMR ± S.E.	R:S ± S.E.
HL	Low-N	26.14 ± 0.50 **a**	3.71 ± 0.04 **a**	6.06 ± 0.10 **a**	0.46 ± 0.005 **a**	0.20 ± 0.004 **a**	0.34 ± 0.005 **a**	0.54 ± 0.01 **a**
HL	High-N	28.33 ± 0.49 **b**	3.86 ± 0.04 **a**	6.75 ± 0.10 **b**	0.52 ± 0.005 **bd**	0.22 ± 0.004 **b**	0.26 ± 0.004 **b**	0.36 ± 0.01 **b**
ML	Low-N	32.18 ± 0.52 **cd**	3.31 ± 0.04 **a**	3.76 ± 0.10 **c**	0.48 ± 0.006 c	0.28 ± 0.004 **c**	0.24 ± 0.005 **c**	0.33 ± 0.01 **c**
ML	High-N	33.66 ± 0.51 **c**	3.60 ± 0.04 **a**	4.44 ± 0.10 **d**	0.51 ± 0.006 **b**	0.29 ± 0.004 **d**	0.20 ± 0.005 **d**	0.26 ± 0.01 **c**
LL	Low-N	32.90 ± 0.96 **cd**	2.63 ± 0.08 **a**	1.69 ± 0.19 **e**	0.52 ± 0.006 **bd**	0.33 ± 0.005 **e**	0.15 ± 0.005 **e**	0.19 ± 0.02 **d**
LL	High-N	31.97 ± 0.84 **d**	2.67 ± 0.07 **a**	1.66 ± 0.16 **e**	0.53 ± 0.006 **d**	0.33 ± 0.005 **e**	0.14 ± 0.005 **e**	0.17 ± 0.01 **d**

See Table 2 for variables names.
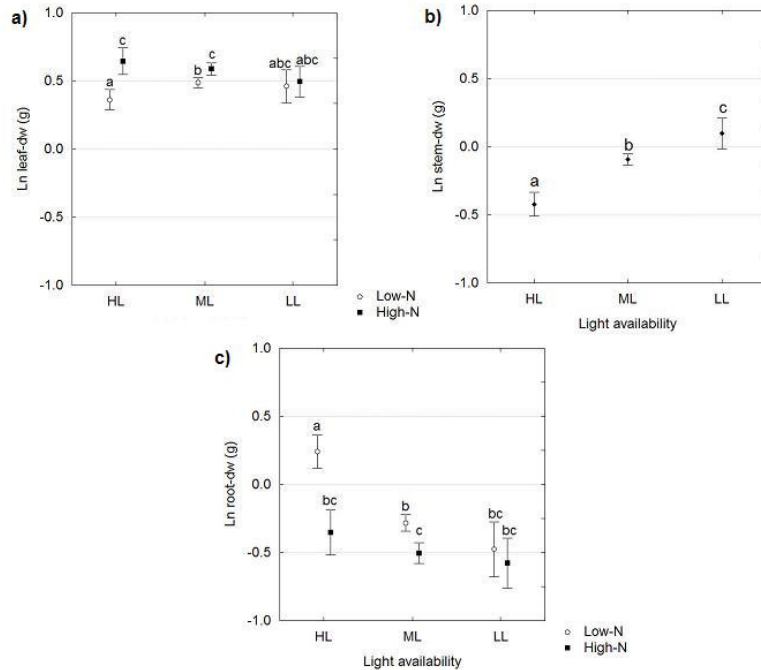
Figure 5. Adjusted means (Ln T-dw as covariable: 1.21) of: (**a**) total leaf dry weight production in seedlings of *P. pinaster* with different soil fertility (Low and high-N) and light availability environments. L x N effect: $F=5.61$, $p<0.01$; (**b**) total stem dry weight production. Light effect: $F=12.225$, $p<0.001$; (**c**) total root dry weight. L x N effect: $F=7.4790$, $p<0.001$. HL: full sunlight; ML: medium light; LL: low light. Vertical bars denote 0.95 confidence intervals.

3.4 Relationships among morphological and biomass traits, and climate and geographical (habitat) factors

Spearman's correlation analysis revealed a relatively strong and positive relationship ($p<0.001$) between stem height and northern latitude ($r_s=0.46$), while a negative relationship between stem height and elevation ($r_s=-0.47$). Stem height was positively correlated with precipitation in summer ($r_s=0.45$), precipitation in autumn ($r_s=0.35$), precipitation in spring ($r_s=0.30$) and with the average temperature in spring ($r_s=0.37$). Principal differences in climate and geographical (habitat) factors among study populations were observed with a Factor Analysis (Figure 7). The first three (only the first two are explained in the text) and main factors of the factor analysis (FA) explained up to 91.38 % of the total variance (Figure 7). The main factor (Factor 1, 55.54% of EV) described a gradient of precipitation in spring, autumn and winter that increased with the longitude to the west (Figure 7). The second factor (Factor 2), which described a gradient of precipitation in summer, was associated positively to higher northern latitudes but negatively with high elevation. *Tamb* and *Oria* presented the lowest precipitation in summer; *Tamb*, which was located at higher elevations and at the lowest

latitude, presented also the lowest average temperature in spring (8.75 ºC) (Table 1 and Figure 8). The first two CCA axes obtained with the Canonical Correspondence Analysis using populations as the response variable, morphological variables of the HL-low N data subset as the explanatory variables, and the seedling total dry weight of that subset as covariable, explained 63% of the variability in the population data; stem height was the main trait (p< 0.002; F= 5.17) in charge of almost 50% of the variation among populations (Figure 8 but morphological variables are not shown). Then, adding geographical and climatic variables (see Figure 7) we created this biplot (Figure 8), whose first two CCA associated axes explained 22% of the variation in the population data, and shows graphically population's similitude in habitat variables.
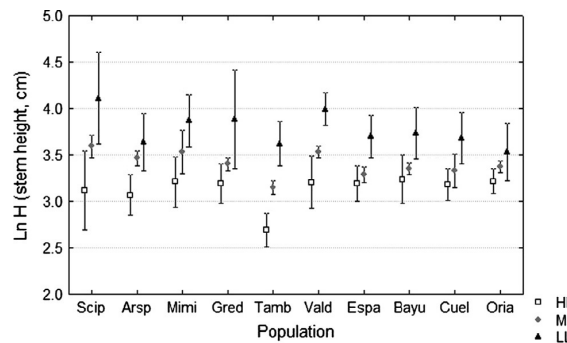
Figure 6. Adjusted means (Ln T-dw as covariable: 1.21) of the stem height (H) of ten *P. pinaster* populations in different light environments. L x P effect: *F*=2.693, *p*<0.001. HL: full sunlight; ML: medium light; LL: low light. Vertical bars denote 0.95 confidence intervals.
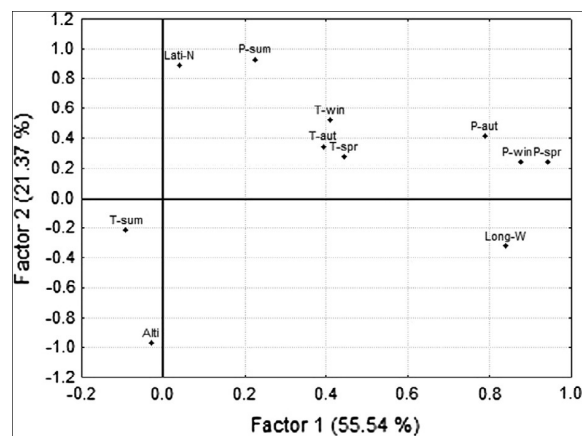
Figure 7. Biplot with factor loadings of the variables correlated with the two main factors that describe gradients which characterize ten *Pinus pinaster* populations habitat (see Table 1). In braquests, the percentage of explained variance by each principal factor. P-spr, P-sum, P-aut and P-win refers to average rainfall (mm) in spring, summer, autumn and winter, respectively. T-spr, T-sum, T-aut and T-win refers to average temperature (ºC) in spring, summer, autumn and winter. Alti: elevation, m.a.s.l.; Long-W: west longitude (º), Lati-N: north latitude (º).
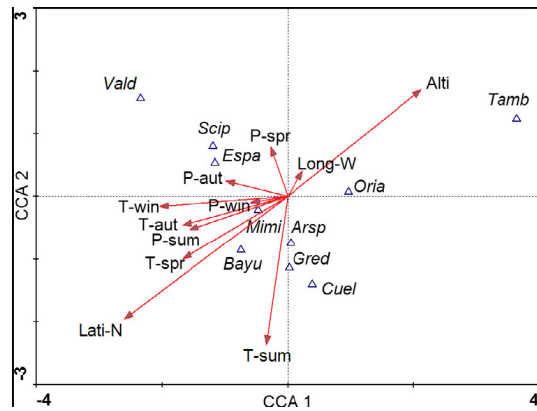
Figure 8. Population's climatic and habitat variables biplot of CCA axes 1 and 2 in ten populations of *P. pinaster*. P-spr, P-sum, P-aut and P-win refers to average rainfall (mm) in spring, summer, autumn and winter, respectively. T-spr, T-sum, T-aut and T-win refers to average temperature (ºC) in spring, summer, autumn and winter. Alti: elevation, m.a.s.l.; Long-W: west longitude (º), Lati-N: north latitude (º). The biplot was built with the HL/ low-N data subset.

4   DISCUSSION

In this study, we have investigated phenotypic plasticity in seedlings from ten *P. pinaster* populations in response to light and Nitrogen variation. Light availability was an important factor controlling seedling growth and morphological structure, which agrees with previous results (Ruano et al., 2009; Rodríguez-García et al., 2011a; Rodríguez-García et al., 2011b). However, differences between productivity environments were observed, especially in full sunlight and medium light conditions. This indicates that light is needed for the expression of N effects on *P. pinaster* seedling growth, and suggests that soil fertility may be very important at early successional stages at open and medium shaded microsites (Rodríguez-García et al., 2011a), but would exert low influence in deep shade conditions. Light levels used in this study are likely comparable with field conditions, especially for full-sun light and medium light conditions (Ruano et al., 2009; Rodríguez-García et al., 2011b). The 9% light availability treatment (low light or deep shade) may be also found in Mediterranean forests with dense overstorey and understorey (Gómez-Aparicio et al., 2006).

The higher biomass production was observed in full sunlight and high-N seedlings. At a common age, there were numerous significant differences in morphological traits which were dependent on the light and soil fertility interaction effects. Overall, growth and biomass allocation was detected to change ontogenetically (slopes ≠1), which coincides

with other findings (Gedroc et al., 1996; Müller et al., 2000; Shipley and Meziane, 2002; Poorter et al., 2012).

When light, soil fertility and population effects were assessed in seedlings of the same size, seedling growth and biomass allocation did not occur uniformly in root and shoot among the different experimental treatments as the relative allocation of resources to root versus shoot depended upon which resource (light or N) was more limiting (Cronin and Lodge, 2003), which agrees with optimal partitioning theory. With increasing shade in low fertility conditions, seedling responded with stem elongation and higher relative allocation of dry weight to leaves and stem, but at expense of a lower inversion to root system. This indicates that the *P. pinaster* seedlings were not severely stressed by the shade. Higher soil fertility affected to allocation to leaves (increasing it), and especially to allocation to root (decreasing it), in full sunlight and medium light; while it did not affect to dry weight allocation to stem neither to stem height elongation, or allocation to root in deep shade. Lack of or negative height growth response to fertilization is consistent with other fertilization studies in pines Kishkuch et al. (2002). However, there are other studies where seedlings of shade-intolerant species responded to increased N availability by increasing height and the shoot: root mass ratio (S:R), (Canham et al., 1996; Berger and Glatzel, 2001; Villar-Salvador et al., 2004).

Intra-specific responses to water and light variation at seedling stage have been observed previously in *P. pinaster* at seedling stage (Fernández et al., 1999; Awada et al., 2003; Chambel et al., 2007; Aranda et al., 2009; Sánchez-Gómez et al., 2010). In our study, a great population-specific variation in growth response to increased shade and N availability was observed at (same-age) seedling stage, which may reflects *P. pinaster* intra-specific differences in nutrient and light (gaps) requirements for growth and establishment. The main trait affected by light and N interaction effect, which was at the same time dependent on the population effect was the relative allocation to root system (RMR). Seedlings from *Tamb* presented the lowest shoot development potential (e.g. stem height, diameter, lateral stems) and the greater investment in the root growth, which coincides with results from Sánchez-Gómez et al. (2010), even in deep shade conditions. Species that have high root: shoot ratios grow well in infertile habitats (Chapin, 1980; Craine et al., 2002), and investment in the root system has traditionally been viewed as a drought and nutrients scarcity tolerance mechanisms for increasing the uptake of water and nutrients and thus improving the status of plants under stress

conditions (Ibrahim et al., 1997; Aranda et al., 2009; Kobe et al., 2010). The observed pattern of low stem height may be connected to this population's habitat, in the Atlas Massif, as this trait may be adaptive in low nutrients, cold and high elevation conditions (e.g. high mountains). This agrees with the negative correlation observed between stem height and elevation. With increasing elevation and cold conditions, plants need a low stature, among other different morphological and physiological adaptations, to cope with wind, snow, low temperatures and high light intensity (Körner, 2003; Kleyer et al., 2012). Genotypic adaptation can also play a role, as *Picea abies* from high elevation cold locations shows greater intrinsic root allocation (Oleksyn et al., 1998). On the other hand, for many vegetation ecosystems including forest and woodlands ecosystems, a decrease in precipitation is associated with an increase in RMR (Oleksyn et al., 1998; Mokany et al., 2006; Poorter et al., 2012). In our analysis, *Tamb* was the population with the lowest precipitation levels in summer, and located at higher elevation, lower northern latitude and lowest western longitude. Moreover, the fact that this population maintained high root system growth even in deep shade conditions may indicate that *Tamb* may present as well a strong competitive response in shade (shade avoidance mechanism and high ability to tolerate suppressive effect from neighbours). Populations from northern latitudes: *Scip, Gred* and *Bayu*, but also *Arsp, Vald, Espa*, and *Mimi*, presented the higher shoot development potential, and grew taller when shade increased; such a response may confer high competitive ability when light is limited and on high nutrient soils (Chapin, 1980; Aerts and Chapin, 2000). Populations of *Oria* and *Cuel* presented medium shoot development potential, and some similitude in root development potential with *Tamb* in medium and low light environments (data not shown). This fact may be related to the short growing season in *Cuel* due to a more continental climate (typically a short, hot growing season combined with low temperatures in winter), and elevation and the southern location in the case of *Oria*. Further research is needed to better understand the effects of environmental variation on early seedling stages. Nonetheless, our results may give examples of how population behaviours are related to different light and soil fertility environments.

5 CONCLUSIONS

Morphological traits impacted by N and light interaction effects were numerous. Results suggest that soil fertility may be very important at early successional stages at open and medium shaded microsites, but would exert low influence in deep shade. Ontogenetic

drift was observed in growth and biomass allocation but, after size correction growth depended upon which resource was more limiting, according to OPT. Two main growth strategies to achieve functional responses to resources variation were observed among populations: shoot vs root growth.The patterns in the amounts of within-species variation presented in this study may be related to the amounts and types of plasticity exhibited by the individuals that dominate in these different populations. Therefore, provenance selection for reproduction material could be determinant depending on the importance that a trait is given as adaptive or searched for a certain purpose.

6 ACKNOWLEDGMENTS

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