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Biomass allocation and foliage heteroblasty in hard pine species respond differentially to reduction in rooting volume

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Abstract Rooting space is considered as a resource in plants, but comparative studies on the biomass allocation plasticity in response to rooting volume (RV) are rare. We compared responses in growth, biomass allocation and ontogenetic heteroblasty in nine hard pine species of contrasted ecology. Seedlings were cultivated in containers of 0.2, 0.5, 1.0, 2.8 and 7 L for two growing seasons (425 days). Reduction in RV caused a reduction in plant absolute and relative growth rate and biomass allocated to stems but it increased biomass allocated to roots. RV affected to a lesser extent and in a less consistent direction allocation to leaves. Species that grew faster (higher relative growth rate) had a steeper decrease in growth with the reduction in RV. Ontogenetic heteroblasty, evaluated as the proportion of secondary needles in the needle biomass,

showed highly different plasticity patterns in response to RV. Decrease in RV caused negligible or no change either in the most ontogenetically delayed Mediterranean pines or in the most ontogenetically advanced pines, the mesic *Pinus sylvestris* and *P. uncinata*. By contrast, ontogenetically intermediate species showed steep reaction norms in response to reduction in RV. While *P. pinaster* and *P. brutia* showed marked rejuvenation, *P. nigra* accelerated the development of adult foliage.

Keywords Container · Developmental plasticity · Growth · *Pinus* · Root restriction · Rooting volume

Abbreviations

| | |
|------|---------------------------------|
| RV | Rooting volume |
| RR | Root restriction |
| TM | Total plant biomass |
| AGR | Absolute height growth rate |
| RGR | Relative height growth rate |
| RMF | Root mass fraction |
| SMF | Stem mass fraction |
| LMF | Leaf mass fraction |
| SNMF | Secondary needles mass fraction |

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Introduction

Differences in allometry driven by environmental factors are considered key manifestations of developmental plasticity in plants (Chambel et al. 2007; Poorter and Nagel 2000; Sanchez-Gomez et al. 2006). In forest tree seedlings, allometry has long been investigated from different points of view. On the one hand, seedling allometry has been used as a plant quality selection criterion in forestation projects

(see, for example Ritchie 1984; Thompson 1985; Wilson and Jacobs 2006). On the other hand, biomass allocation patterns at the juvenile stage have been postulated as fundamental features of life-history diversification in forest trees (Cao and Ohkubo 1998; Grotkopp et al. 2002; Strauss and Ledig 1985).

Rooting volume can be considered as a resource on its own (McConaughay and Bazzaz 1991). Root restriction due to reduction in rooting volume can modify whole plant growth through chemical signals (Aiken and Smucker 1996) independently of other factors which covary with rooting volume such as growing media nutrient availability. Therefore, a rooting volume gradient can be considered as an environmental gradient. Mechanical restrictions imposed to root growth and structure by container volume is a central matter of concern in forest and crop plants (Aphalo and Rikala 2003; Dominguez-Lerena et al. 2006; Landis 1990; Beeson 1993; Ferree et al. 1992; NeSmith and Duval 1998). Root restriction reduces growth with no effect or an increase in shoot/root biomass ratio (Clemens et al. 1999; Hsu et al. 1996; NeSmith et al. 1992). The effect of root restriction in conifer species has been studied in several species separately (see Carlson and Endean 1976, Dominguez-Lerena et al. 2006; Endean and Carlson 1975; Lamhamedi et al. 1998; South et al. 2005). Growth response to reduced rooting volume might be species-specific, even at the infra-specific level (Climent et al. 2008; NeSmith and Duval 1998). However, as far as we know, no comparative study on the growth and allometry response to rooting volume has been accomplished in ecologically and functionally contrasted conifer species.

Pines are a paradigm of heteroblastic development during their pre-reproductive phase. A vegetative “adult” phase, characterized by the predominance of secondary needles in axillary dwarf shoots, follows a juvenile phase characterized by isolated primary leaves (euphylls). Phylogenetically closely related species can have marked ontogenetic differences in their heteroblastic phase change (Climent et al. submitted; Lester 1968), but little is known of how environmental factors, such as rooting volume, affect ontogenetic plasticity. Various works have assessed the effect of root restriction and rooting volume on the ontogeny and the proportion of vegetative and reproductive biomass in angiosperms but no clear general trend can be inferred, suggesting high specificity in response to root restriction (Clemens et al. 1999; McConaughay and Bazzaz 1991; NeSmith et al. 1992).

The objective of this study is to compare the growth, biomass allocation and ontogenetic heteroblasty in hard pine species of contrasting ecology, relative growth rate and ontogeny in response to decreasing rooting volume. To attain this objective, we cultivated pine species in a wide

gradient of container volumes and compared their growth, biomass allocation and shoot ontogeny.

Materials and methods

Plant material

We chose nine hard pine species that included five pines native to the Mediterranean basin (subsection *Pinaster*): *Pinus pinaster* Ait., *P. brutia* Ten., *P. halepensis* Mill., *P. pinea* L. and *P. canariensis* C. Sm., three Eurasian pines (subsection *Pinus*): *P. nigra* subsp. *salzmannii* (Dunal) Franco, *P. sylvestris* L. and *P. uncinata* Ram. ex D.C., and finally *P. radiata* D. Don (subsection *Trifoliae*). This latter species is widespread in mild and humid climate areas worldwide. Seeds used to grow seedlings were an equilibrated mixed lot, obtained from 25 to 30 open-pollinated mother trees, from a provenance representative of the average ecological range of each species (Table 1). Eurasian pines are highly tolerant to cold but sensitive to drought, while *P. radiata* is highly sensitive to frosts and moderately drought-tolerant. Three of the Mediterranean pines are highly tolerant to drought but sensitive to frosts (*P. canariensis*, *P. pinea* and *P. halepensis*), and the remainder are less drought-tolerant but moderately resistant to frost (*P. pinaster* and *P. brutia*). Seeds were stratified for a month to homogenize germination and then sown in a nursery located in Maceda (Ourense, Galicia, Spain). The experiment was entirely conducted in a heated glasshouse to control minimum temperature conditions and light. Sowing was done in August 11, 2006, and the experiment finished 425 days later in October 12, 2007. Mean air temperature and relative humidity ranged from 8–25°C and 39–62%, respectively. Luminosity at midday ranged from 0.1 to 22.9 klux.

Experiment layout

Five commercial plastic containers (Cetap®, Espinho, Portugal) of 0.2, 0.5, 1, 2.7 and 7 dm³ were selected for this study and were as similar as possible to minimize other differences apart from rooting volume. Containers were black, each with a truncated conical shape, with a height/diameter ratio of 2.5. We discarded keeping rooting depth constant, since it would have obliged us to use very thin tubes for the lower volumes; nevertheless, at a similar rooting volume, container depth seems to be less important than cultivation density or other container attributes in hard pines (Dominguez-Lerena et al. 2006). We chose the highest volume (7 dm³) because it did not induce any growth restriction, based in previous experience and nursery practice. All containers were grid-open at the bottom,

Table 1 Ecological data of the provenances representative of each species

| Species | Provenance | Altitude (m) | MAP (mm) | MAT (°C) | TCM (°C) | DP (month) | Heteroblastic change |
|-----------------------|------------------------|--------------|----------|----------|----------|------------|----------------------|
| <i>P. brutia</i> | Taurus (Bucak) | 980 | 600 | 13.5 | 3.9 | 3.0 | I |
| <i>P. canariensis</i> | Tenerife | 1350 | 691 | 14.8 | 10.3 | 5.1 | D |
| <i>P. halepensis</i> | Inland Eastern Spain | 830 | 503 | 13.7 | 5.5 | 3.2 | D |
| <i>P. pinea</i> | La Mancha | 690 | 594 | 14.3 | 6.3 | 4.9 | D |
| <i>P. pinaster</i> | Guadarrama Mnts. | 1230 | 620 | 12.7 | 3.2 | 2.6 | I |
| <i>P. nigra</i> | Southern Iberian Range | 1280 | 840 | 10.3 | 2.2 | 1.7 | P |
| <i>P. sylvestris</i> | Guadarrama Mnts. | 1520 | 920 | 8.5 | 0.9 | 0.8 | P |
| <i>P. uncinata</i> | Eastern Pyrenees | 1760 | 1175 | 5.5 | 0.0 | 0.0 | P |
| <i>P. radiata</i> | Basque Coast | 220 | 1390 | 13.0 | 7.5 | 0.0 | P* |

MAP mean annual precipitation, MAT mean annual temperature, TCM mean temperature of coldest month, DP drought period length (number of months in which precipitation less than two times the mean temperature). Heteroblastic change (formation of secondary needles) D delayed, I intermediate, P precocious, P* precocious but combined with delayed bud set

but we used a geo-textile mesh to prevent root growth out of the container and thus any fine root loss during harvest. The geo-textile mesh did not reduce water leaching or growing media aeration. The growing media consisted of a 1:2 (v/v) mixture of peat and perlite that was fertilized with 3 g l⁻¹ of a slow-releasing fertilizer (Osmocote® 15 + 9+9, Scotts International BV, The Netherlands) to keep the same nutrient concentration among container types.

To ensure the same water availability among container types, growing media water content was maintained between 35 and 40% throughout the experiment, following the best nursery practices for these species. Growing media water content and temperature were weekly measured in the 10 cm upper layer with a Time Domain Reflectometer connected to a PDA and a Temperature Sensor (Gemini, UK). No significant deviations in water content or air temperature were observed among container types.

We used a split-plot design with four replicates, main plots corresponding to container type (five plots), and subplots corresponding to six plants of each species (hence 24 plants per species and container type). In addition, we used periodical re-randomization within each main plot to avoid bias due to border effects. Plants were cultivated at a low and equal density, 10 plants m⁻², independently of container size using specific support grids, which also allowed upper part of all containers to be placed at the same height to keep similar light conditions.

Growth and biomass allocation measurements

Twelve plants per species and container type were harvested 425 days after seeding. Then, the root collar diameter and seedling height were measured, and plants were separated into roots, stems and leaves. Roots were cleaned from the growing media, and foliage was separated into secondary (adult) and primary (juvenile) needles. All parts

were washed with tap water, and their mass was measured after drying at 80°C for 72 h. Total leaf mass was obtained as the sum of secondary and primary needle dry mass. Total plant mass (TM) was calculated as the sum of all plant components, and the leaf, stem and root biomass fraction (LMF, SMF and RMF, respectively) were calculated by dividing their mass by TM (Poorter and Nagel 2000).

A quantitative index of seedling ontogeny within each species was calculated as the ratio of secondary needle mass to the total leaf mass (SNMF), which is a proxy of the proportion of adult metamers in the shoot. Absolute height growth rate (AGR) and relative height growth rate (RGR) were calculated between days 65 and 425 after seeding, as described in Hunt et al. (2002). Day 65 was chosen because all species underwent visible shoot elongation at that time.

Data analysis

When treatments in an experiment produce plants of different size, it is possible that some observed effects are due to size differences, rather than to the studied treatments. This bias can be important for organ mass proportions, which varies along plant ontogeny, thus being affected by plant size (McConnaughay and Coleman 1999). We checked this source of bias (so-called *ontogenetic drift*, *op.cit.*) by analysing regressions of RMF, SMF, LMF and SNMF against TM per species and container volume. We found no significant effects of plant size, and so we discarded that plant size was a meaningful source of bias for the traits considered in this experiment.

First, we analysed the fixed effects of rooting volume, species and its interaction on growth and mass allocation variables by ANOVA. Since the interaction term was always significant, we used the interaction mean square as the denominator for testing the effect of main factors. One-way ANOVA and a Tukey's multiple range tests were used

for inter-specific comparisons of root mass fraction at the highest rooting volume.

Models of phenotypic responses for each genetic entry against an environmental gradient are generally called reaction norms (Schlichting and Pigliucci 1998). In a second step, we compared reaction norms of each species by comparing the intercepts and slopes of linearized models of the growth and biomass allocation variables against our environmental gradient; this is, rooting volume (RV). RV was square root transformed to linearize the relationship and homogenize residuals. We used a parallelism analysis (Milliken and Johnson 1992) to find groups of homogeneous slopes among species. Whenever the intercepts were not significantly different among species, we re-analysed data assuming equal slopes for regression models.

Finally, to check the hypothesis that species with higher inherent RGR should be more sensitive to root restriction, we compared the regressions between RGR at the largest rooting volume (7 dm³) and the slopes of TM and AGR reaction norms among species.

Results

Total biomass and shoot height growth rates

TM, AGR and RGR were significantly affected by reduction in RV in all species, with RV explaining 87, 84 and 71% of the variation for TM, AGR and RGR, respectively. Significant differences among species occurred for TM, AGR and RGR (Table 2). However, species showed different slopes of the reaction norms—this is a significant RV × Species interaction for TM, AGR and RGR with RV decrease (Table 2). *Pinus radiata* had the steepest reduction in TM with RV (Fig. 1a; Table 3), followed by the Mediterranean pine species (*P. pinaster*, *P. pinea*, *P. canariensis*, *P. halepensis* and *P. brutia*), where *P. pinea* and *P. pinaster* had similar slopes. The Eurasian *P. sylvestris* and *P. nigra* showed similar reaction norms that were lower than Mediterranean species. Finally, *P. uncinata* showed the lowest reduction in TM with decreasing RV.

AGR showed a similar variation pattern than TM (Fig. 1b; Table 3). Species ranking by the slopes of AGR reaction norms was very similar to that of TM, except for ranking changes between *P. pinea*, *P. canariensis* and *P. pinaster* within the Mediterranean species.

Differences among species in the slopes of reaction norms for RGR were smaller than for TM and AGR (Fig. 1c). As for TM and AGR, *P. uncinata* had the lowest RGR and the lowest responsiveness of RGR to RV. By contrast, *P. halepensis*, *P. pinaster* and *P. sylvestris* showed the highest reaction norm slopes (Table 3).

Across species, there was a significant positive relationship between RGR at the largest rooting volume (7 dm³) and the reaction norm slopes for AGR ($R^2 = 0.82$, $P = 0.007$) and TM ($R^2 = 0.73$, $P = 0.024$).

Root and shoot allometry

Species, RV and their interaction affected significantly RMF (Table 2). RMF increased with RV reduction, in eight of the nine species, the only exception being *P. uncinata*. Differences among species in the RMF slope of the reaction norm showed no clear phylogenetic trend: *Pinus brutia*, *P. sylvestris*, *P. halepensis* and *P. radiata* had a steeper reaction norm than *P. canariensis*, *P. pinea*, *P. pinaster* and *P. nigra* (Table 3; Fig. 2a).

Both species and RV had a highly significant effect on SMF, while the interaction between both factors was less significant (Table 2); all species reduced SMF with decreasing RV at rather similar slopes, except *P. uncinata* which reduced its biomass allocated to stems at a lower rate (Table 3; Fig. 2b).

Finally, attending to ANOVA LMF was not significantly affected by RV but species had highly contrasting norms of reaction, even with slopes of different sign, hence the significant RV × Species interaction (Table 2). *Pinus brutia* and *P. sylvestris* showed a positive reaction norm, i.e. a LMF increase with increase in RV, while *P. canariensis*, *P. pinea*, *P. pinaster* and *P. uncinata* showed the opposite trend. Finally, *P. halepensis*, *P. nigra* and *P. radiata* showed no significant variation in LMF with the reduction in RV (Table 3; Fig. 2c).

In summary, reduction in RV produced smaller plants because they grew more slowly, together with a proportional lower investment in stems and a higher investment in roots, while biomass allocation to leaves was affected to a lesser extent and in a less consistent direction.

Attending to RMF at the maximum rooting volume, three species groups were defined according to the results of the multiple range tests (Fig. 3): a low RMF group comprised by *Pinus pinea*, *P. radiata*, *P. pinaster* and *P. canariensis*; an intermediate RMF group with *P. brutia*, *P. halepensis* and *P. sylvestris*, and finally, a high RMF group comprised by *P. nigra* and *P. uncinata*.

Ontogenetic heteroblasty

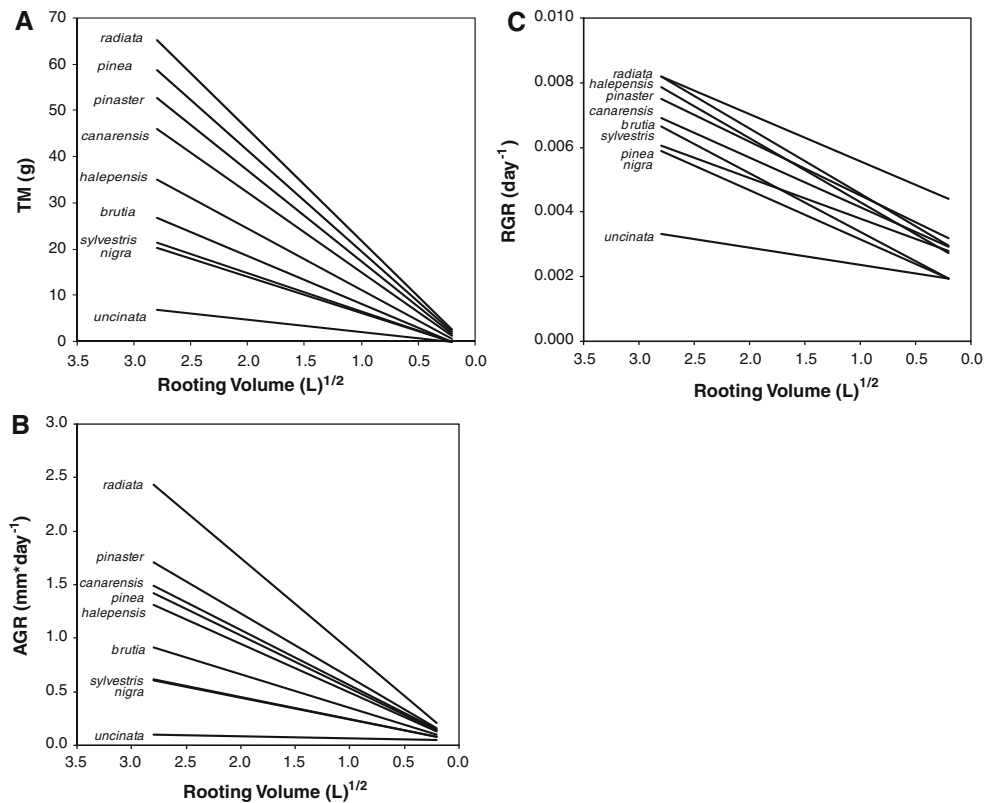
Species greatly differed in the response of the proportion of secondary needles, SNMF, to RV (interaction RV × Species in Table 2). Reaction norms differed not only in the magnitude but also in the sign of the slope among species. Four of the nine species did not show any significant relationship between SNMF and RV (the Mediterranean *P. canariensis* and *P. pinea*, and the Eurasian *P. sylvestris*

Table 2 ANOVA results (F-statistic value and probability levels) of the effect of rooting volume, species and their interaction on the growth and biomass allocation variables

| Trait | Rooting volume (RV) | Species (S) | RV x S |
|--|---------------------|-------------|-----------|
| Total plant mass (TM) | 25.63*** | 7.19 *** | 20.16 *** |
| Absolute height growth rate (AGR) | 703.6*** | 344.4 *** | 25.56 *** |
| Relative growth rate (RGR) | 467.7*** | 127.6 *** | 4.45 *** |
| Root mass fraction (RMF) | 11.23*** | 26.62 *** | 2.99 *** |
| Stem mass fraction (SMF) | 61.89*** | 52.89 *** | 1.62 * |
| Leaf mass fraction (LMF) | 0.84 ns | 13.91 *** | 2.78 *** |
| Secondary needles mass fraction (SNMF) | 0.82 ns | 33.58 *** | 10.72 *** |

F tests for rooting volume and Species used the mean square of the interaction as denominator. Probability levels are indicated as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns: $P > 0.05$

Fig. 1 Linearized regression models (norms of reaction) between growth variables and squared-root transformed rooting volume. **a** Total plant biomass (TM) at the end of the experiment, **b** Absolute height growth rate (AGR) and **c** Relative height growth rate (RGR) between days 65 and 425 after seeding



and *P. uncinata*). However, while *P. sylvestris* and *P. uncinata* displayed very high SNMF values across volumes, *P. canariensis* and *P. pinea* had very low SNMF values (Table 3; Fig. 2). Among the species with significant reaction norms, the two Mediterranean species, *P. pinaster* and *P. brutia* showed similar steep decreases in SNMF with RV reduction. This pattern was also followed by *P. radiata*, although less markedly and with higher absolute SNMF values. *Pinus halepensis* had very low SNMF values across rooting volumes as observed in *P. pinea* and *P. canariensis* but unlike these species it had a statistically significant linear relationship between SNMF and RV. Finally, contrary to the

reminder species, *P. nigra* showed a marked increase in SNMF (from 0.23 up to 0.60) with RV reduction (Figs. 2, 4).

Discussion

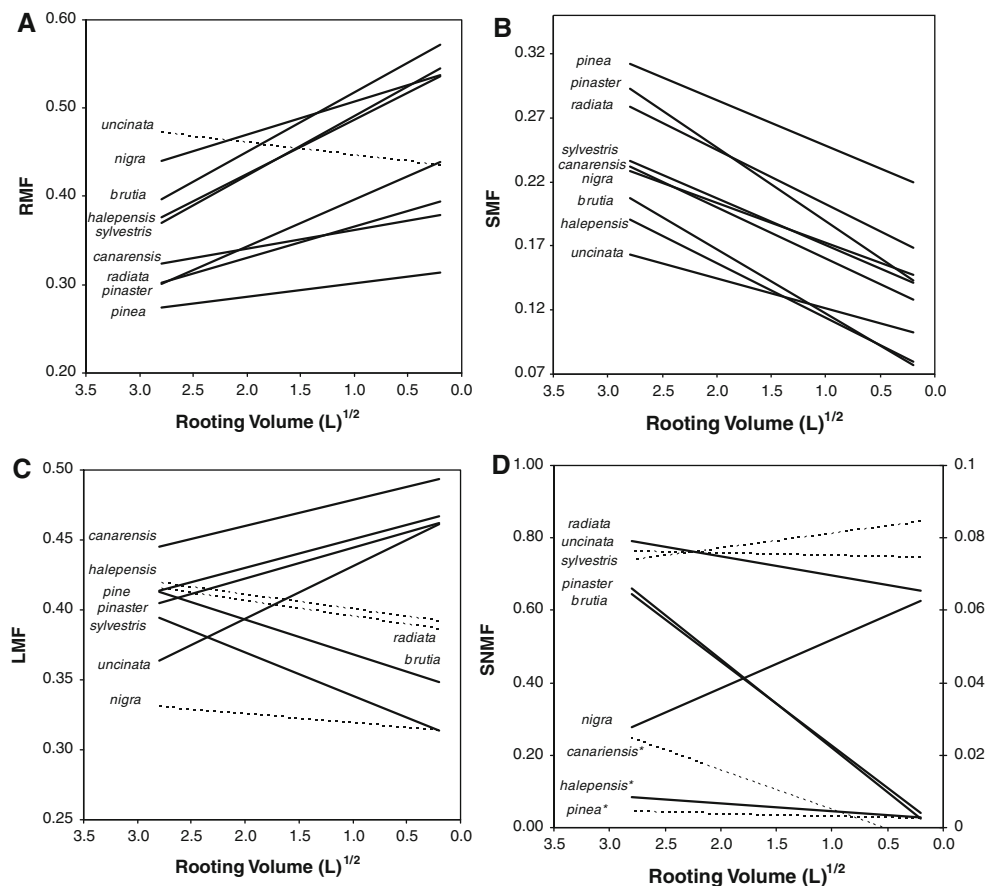
Our experiment showed contrasting developmental responses to a gradient of decreasing rooting volumes among pine species. In addition to expected differences in the intensity of the reaction under a common trend, we also detected cases of virtually no response and totally opposite responses between species, especially regarding shoot heteroblasty.

Table 3 Comparison of the slopes of linearized regressions for phenotypic variables against rooting volume (squared-root transformed) for the nine studied species

| Species | TM | AGR | RGR | RMF | SMF | LMF | SNMF |
|-----------------------|----------|----------|-----------|----------------|----------|--------------|------------|
| <i>P. brutia</i> | 0.33 f | 0.31 e | 0.0015 ab | −0.0021 a | 0.0013 a | 0.0008 a ** | 0.0073 a |
| <i>P. canariensis</i> | 0.54 d | 0.52 c | 0.0017 ab | −0.0007 c * | 0.0013 a | −0.0006 b * | 0.0003 ns |
| <i>P. halepensis</i> | 0.42 e | 0.45 d | 0.0020 a | −0.0019 a | 0.0016 a | 0.0004 ns | 0.0001 c |
| <i>P. pinaster</i> | 0.69 b | 0.60 b | 0.0020 a | −0.0011 bc | 0.0011 a | −0.0006 b ** | 0.0077 a |
| <i>P. pinea</i> | 0.62 c | 0.49 c | 0.0013 b | −0.0005 c ** | 0.0018 a | −0.0007 b | 0.0000 ns |
| <i>P. nigra</i> | 0.27 g | 0.20 f | 0.0015 ab | −0.0012 abc ** | 0.0010 a | 0.0002 ns | −0.0043 d |
| <i>P. sylvestris</i> | 0.25 g | 0.20 f | 0.0018 a | −0.0021 a | 0.0012 a | 0.0010 a ** | −0.0013 ns |
| <i>P. uncinata</i> | 0.10 h | 0.02 g | 0.0005 c | 0.0005 ns | 0.0007 b | −0.0012 b * | 0.0002 ns |
| <i>P. radiata</i> | 0.76 a | 0.85 a | 0.0015 b | −0.0017 ab | 0.0013 a | 0.0003 ns | 0.0016 b |
| Model R ² | 0.87 *** | 0.85 *** | 0.70 *** | 0.59 *** | 0.66 *** | 0.38 *** | 0.84 *** |

Cells sharing the same letter within a column show non-significantly different slopes, i.e. parallel reaction norms. Intercepts were only equal between species for TM and AGR, but intercept comparisons for the other variables are omitted. All values significant at $P < 0.001$ except when indicated (* $P \leq 0.05$, ** $P \leq 0.01$, ns: $P > 0.05$)

Fig. 2 Linearized regression models (norms of reaction) between biomass allocation variables and squared-root transformed rooting volume. **a** Root mass fraction (RMF); **b** Stem mass fraction (SMF) **c** Needle mass fraction (LMF) and **d** Secondary needles mass fraction (SNMF) as an index of ontogenetic heteroblasty. Species with asterisks are referred to the amplified scale at the secondary Y-axis (right). Non-significant regression models are shown in dotted lines



Specific trends for growth and biomass allocation

All species reduced growth with the diminution in RV. Similar results have been documented in other plant species, which have been attributed to restricted supply of water and nutrients (see, for example Aphalo and Rikala 2003; Ferree et al. 1992; NeSmith and Duval 1998 and

references therein). However, in our case, plants were maintained well-watered and fertilized, so probably changes in hormonal signals and lower oxygen concentration in the growing media might explain growth reduction with decrease in RV (Landis 1990; Aiken and Smucker 1996). These effects might become more intense in those pine species which grew faster, as suggested by the greater

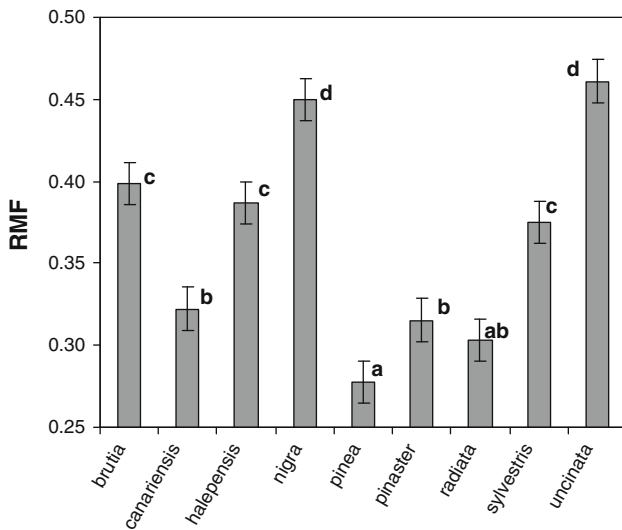


Fig. 3 Mean root mass fraction per species in 7-dm³ containers. Intervals are standard errors, and means sharing the same letter are not significantly different after a Tukey's multiple range test ($P < 0.05$)

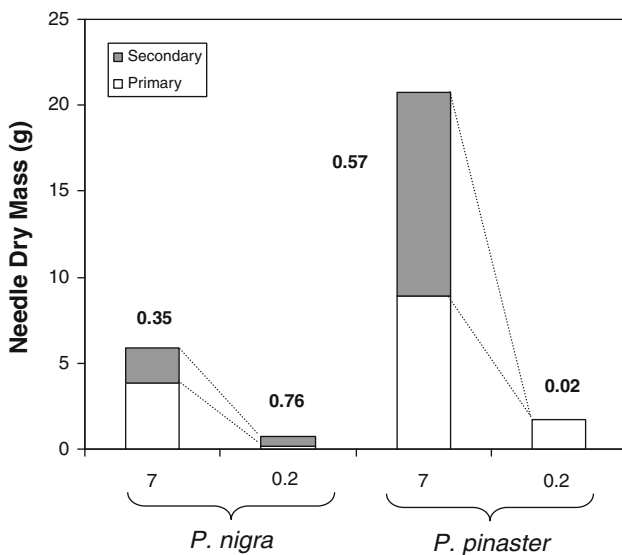


Fig. 4 Comparison of the contribution of primary (white) and secondary (dark) needles to the total needle dry mass for *Pinus nigra* and *P. pinaster* in 7- and 0.2-dm³ containers. Figures close to each bar correspond to SNMF (proportion of secondary needles in the total needle biomass). Error bars have been omitted for clarity

growth reduction responding to small RV in species with higher inherent RGR (this is, the relative growth rate with no restriction for root growth). This result is consistent with the hypothesis that species adapted to favourable environments tend to be more plastic in response to changes in resource availability (Alpert and Simms 2002; Lortie and Aarssen 1996; Valladares et al. 2007).

Species ranking based on the reaction norm slopes for total biomass and height AGR did not match exactly the

species ranking by RGR. Part of this difference is probably due to seed-size effects, since species with large seeds produce larger seedlings [see, for example Leishman et al. (2000) and references therein]. In our case, the main disparity is that of *P. pinea*, which has the largest seeds (0.8 g, compared to 0.012 g in *P. halepensis* and 0.010 g in *P. uncinata*), conducive to bigger seedlings compared to the other species at earlier developmental stages (data not shown).

Eight of nine species allocated proportionally more resources to roots when grown in a smaller rooting volume, as seen by RMF variation, but almost identically by root to shoot ratio (data not shown). This trend contrasts with previous studies where most species showed either no response or decreased allocation to roots accompanying rooting volume reduction (Clemens et al. 1999; Endean and Carlson 1975; Hsu et al. 1996; Lamhamedi et al. 1998; McConaughay and Bazzaz 1991; NeSmith and Duval 1998). In the present experiment, the greater plant mass in larger containers was mainly due to increase in shoot mass, and more precisely stem mass, with very different trends among species in the proportion of mass allocated to needles. This result is consistent with other experiments showing a neat decrease in root to shoot ratio accompanying an increase in plant size as a response to better cultivation conditions in containerized pine seedlings (Luis et al. 2009; Villar-Salvador et al. 2008).

Differences in plant size among treatments with contrasting resource availability frequently do not permit disentangling between apparent plasticity (caused by allometric differences linked to different plant size) from actual resource re-allocation (McConaughay and Coleman 1999). Similar to our findings, Ledig and Perry (1966) pointed out that root mass fraction increases with age or size in seedlings of woody species, but there is a marked lack of information on this issue. We checked the possible effect of plant size on allometric differences in each species by studying the regressions between each biomass fraction and total plant biomass, instead of incorporating total biomass as a covariate (Poorter and Nagel 2000). The lack of significance of these regressions confirmed that the allometric differences observed in our experiment were not merely due to plant size differences among treatments. However, there is not a perfect way to remove all size effects without eliminating the treatment effect itself.

Higher biomass allocation to roots is commonly associated to drought tolerance in woody plants (Lloret et al. 1999). However, the differences in biomass allocation to roots in the bigger containers (7-l) revealed no clear correspondence between RMF and the species' ecology (Fig. 3). Thus, drought-tolerant species such as *P. pinea* and *P. canariensis* had a significantly lower allocation to roots, compared to mesic Eurasian pines such as *P. nigra*,

P. uncinata and *P. sylvestris*. Efficient water-saving mechanisms through reduced transpiration and a high plasticity in shoot/root ratios rather than a high allocation to roots have probably been selected in response to the highly variable resource availability of dry Mediterranean environments (Pardos et al. 2009; Luis et al. 2009; Valladares et al. 2005, 2007).

Furthermore, the higher root allocation in the Eurasian mesic pines relative to the Mediterranean pines could also be due the different shoot and root growth phenology of the former species. Bud set in high latitude and high-mountain conifers, such as our Eurasian pines, is strongly influenced by day-length reduction, which causes shoot elongation cessation in late summer when temperature is not yet limiting for shoot growth (Smit-Spinks et al. 1985; Colombo et al. 2001). In contrast, root growth is not controlled by day-length but temperature. Thus, under mild temperature conditions such as those in the green house in fall roots may experience significant growth (Riedacker 1976; Livonen et al. 2001). On the contrary, shoot growth in Mediterranean pines is much more controlled by temperature and display a much more free shoot growth pattern, especially in seedlings, and a marked delay in first bud set (Cannell et al., 1976). This would explain a shift towards greater stem and foliage allocation in our Mediterranean pines, but it does not explain the relatively high RMF values of *P. halepensis*, which has a clear continuous shoot growth providing favourable environmental conditions (Serre 1976). The differential responsiveness of each species to particular environmental conditions suggests that inferences on actual values of biomass ratios obtained in a single common environment should be taken with caution in inter-specific experiments with Mediterranean pine seedlings.

Ontogenetic heteroblasty

The ontogenetic change from juvenile to adult foliage had high responsiveness to RV in some pine species but not in others. As ontogeny is tightly linked to the number of divisions of meristem cells (Poethig 2002), a delay in ontogenetic development can be expected under conditions that constrain carbon gain, as shown previously in *P. pinaster* (Lascoux et al. 1993), *Eucalyptus* (Williams et al. 2004) and *Acacia implexa* (Forster and Bonser 2009). Surprisingly, in our experiment, this occurred only in two species, *P. pinaster* and *P. brutia*, which showed a marked delay in the production of adult foliage (ontogenetic heteroblasty) with decreasing RV. This trend was independent of plant size; therefore, plants of these two pines were not only smaller, but also more juvenile when grown in smaller rooting volumes. *P. nigra* also had high ontogenetic heteroblasty responsiveness, but in contrast to *P. pinaster* and

P. brutia, it produced proportionally more adult structures in small rooting volumes. This counter-intuitive result might be linked to different inherent shoot ontogeny among the three mentioned species, since *P. nigra* tends to start the formation of adult needles much earlier during seedling development compared to *P. pinaster* or *P. brutia* (Climent et al. in press). The precocious formation of dwarf-shoot buds in *P. nigra* might have caused their presence even in the more stressful conditions (smaller RV), while the size of primary needles decreased as a consequence of root restriction. In other words, the dry mass of primary needles is more plastic than the dry mass of secondary needles in *P. nigra*, and this leads to a higher proportion of adult structures in small rooting volumes (Fig. 4). In future experiments, node counting of juvenile and adult needles in two different times would help significantly to understand the plasticity of heteroblastic processes.

Variation in RV did not stimulate any ontogenetic responses in the remainder species (this is, regression linear models were either non-significant or almost flat). The lack of ontogenetic plasticity in *P. canariensis* and *P. pinea* and to a lesser extent in *P. halepensis* might be a consequence of an insufficient ontogenetic age for changing the fate of axillary meristems from a juvenile to an adult condition, even in large containers (Table 1; Climent et al. in press). By contrast, *P. sylvestris* and *P. uncinata* showed a high proportion of adult shoots (hence accelerated ontogeny, Climent et al. in press) that allowed discarding a similar behaviour to that of the aforementioned Mediterranean pines. Moreover, if the lack of reaction of *P. uncinata* can be explained merely by a lack of environmental stimulus (low responsiveness to RV in growth and biomass allocation), this is definitely not the case with *P. sylvestris* (Fig. 1).

Functional and practical implications

The divergent reaction norms for ontogenetic heteroblasty among pines can have an adaptive significance that merits further investigation, especially considering that secondary needles are more frost and drought-tolerant than primary needles (Climent et al. 2009; Pardos et al. 2009). Our results are also relevant from a practical point of view because they provide a functional basis for choosing the optimum container size for the production of high-quality plants for Mediterranean Europe (Aphalo and Rikala 2003; Dominguez-Lerena et al. 2006). Little can be gained in terms of plant quality in the three Eurasian pines using larger containers in nursery cultivation. But, in several Mediterranean species, especially *P. pinaster*, *P. brutia* and *P. radiata*, larger containers provided a higher degree of maturity (ontogenetic age) for the same chronological age together with a bigger plant size. Recent findings support

that larger and ontogenetically older plants, such as those obtained by cultivating these species in bigger containers, perform better in woodland plantations than the plants with the opposite traits (Cuesta et al. 2010; Luis et al. 2009; Oliet et al. 2009; Villar-Salvador et al. 2004), even when enhanced cultivation conditions cause a significant decrease in root to shoot ratios. To validate these assertions, an economic study should quantify if increased production and plantation costs linked with larger containers could be compensated by higher field success. On the contrary, the observed tendency of *P. nigra* to be “more juvenile” when grown in large containers could exacerbate transplanting stress in harsh environments.

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