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Research paper

A major trade-off between structural and photosynthetic investments operative across plant and needle ages in three Mediterranean pines

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Pine (*Pinus*) species exhibit extensive variation in needle shape and size between juvenile (primary) and adult (secondary) needles (heteroblasty), but few studies have quantified the changes in needle morphological, anatomical and chemical traits upon juvenile-to-adult transition. Mediterranean pines keep juvenile needles longer than most other pines, implying that juvenile needles play a particularly significant role in seedling and sapling establishment in this environment. We studied needle anatomical, morphological and chemical characteristics in juvenile and different-aged adult needles in Mediterranean pines *Pinus halepensis* Mill., *Pinus pinea* L. and *Pinus nigra* J. F. Arnold subsp. *salzmannii* (Dunal) Franco hypothesizing that needle anatomical modifications upon juvenile-to-adult transition lead to a trade-off between investments in support and photosynthetic tissues, and that analogous changes occur with needle aging albeit to a lower degree. Compared with adult needles, juvenile needles of all species were narrower with 1.6- to 2.4-fold lower leaf dry mass per unit area, and had ~1.4-fold thinner cell walls, but needle nitrogen content per dry mass was similar among plant ages. Juvenile needles also had ~1.5-fold greater mesophyll volume fraction, ~3-fold greater chloroplast volume fraction and ~1.7-fold greater chloroplast exposed to mesophyll exposed surface area ratio, suggesting overall greater photosynthetic activity. Changes in needle traits were similar in aging adult needles, but the magnitude was generally less than the changes upon juvenile to adult transition. In adult needles, the fraction in support tissues scaled positively with known ranking of species tolerance of drought (*P. halepensis* > *P. pinea* > *P. nigra*). Across all species, and needle and plant ages, a negative correlation between volume fractions of mesophyll and structural tissues was observed, manifesting a trade-off between biomass investments in different needle functions. These results demonstrate that within the broad trade-off, juvenile and adult needle morphophysiotypes are separated by varying investments in support and photosynthetic functions. We suggest that the ecological advantage of the juvenile morphophysiotype is maximization of carbon gain of establishing saplings, while adult needle physiognomy enhances environmental stress tolerance of established plants.

Keywords: cell wall thickness, heteroblasty, needle age, needle morphology, nitrogen content, *Pinus halepensis*, *Pinus nigra*, *Pinus pinea*, primary needles, secondary needles.

Introduction

Increases in tree age and height are associated with profound changes in foliage anatomy, morphology and chemistry with major impacts on foliage photosynthetic capacity (Niinemets and Kull 1995, Day et al. 2001, Niinemets 2002, Thomas and Winner 2002, Woodruff et al. 2009, Thomas 2010, Steppe et al. 2011, Mediavilla et al. 2014) that alter whole-tree carbon gain and competitive ability. Particularly in conifers, increases in tree age and size are associated with reductions in foliage photosynthetic capacity per dry mass (Steppe et al. 2011). There is a continuous age- and size-dependent change in leaf traits through plant life called ontogenetic drift, which has been

studied in the majority of investigations looking at tree age and size effects on foliage structure and function. However, changes in leaf traits are characteristically the largest during development from juvenile to adult leaves and many species exhibit an almost abrupt developmental change called heteroblasty (Zotz et al. 2011). Heteroblasty is particularly pronounced in some plant genera, but the variation in the extent of heteroblastic changes in different species is poorly known, and although heteroblasty is widely considered as adaptive, there are few studies examining its role in altering plant survivorship and competitive relations (Day and Greenwood 2011, Climent et al. 2011*b*).

Pine (*Pinus*) species exhibit remarkably large differences in needle morphology and function between juvenile and adult phases (Climent et al. 2011*b*), with juveniles characterized by less sclerophytic needles that have smaller cross-sectional area (Boddi et al. 2002) and lower leaf dry mass per unit area (Pardos et al. 2009, Mediavilla et al. 2014), but greater photosynthetic capacity (Niinemets 2002), ultimately leading to a shorter needle payback time (Day and Greenwood 2011). The factors responsible for greater assimilation potentials of juvenile needles are poorly known. They could involve any of the three key limitations of photosynthesis: stomatal, biochemical (total amount of photosynthetic enzymes) and mesophyll diffusion (Flexas et al. 2012). Past studies have demonstrated that both stomatal and biochemical limitations increase with increasing plant age and size (Day et al. 2001, Niinemets 2002, Woodruff et al. 2009, Steppe et al. 2011, Räim et al. 2012).

In fact, anatomical differences among juvenile and adult needles can directly impact photosynthesis by altering the fraction of photosynthetically competent mesophyll tissue. With increasing needle structural robustness upon juvenile-to-adult transition, the fractional investment in support tissues, including strongly lignified epidermal and hypodermal tissues and central cylinder, is expected to increase, inevitably resulting in lower fractions of mesophyll tissues. Such a possible trade-off could importantly further contribute to reductions of the carbon gain of adult needles. So far, it has been demonstrated that there is a significant variation in the mesophyll volume fraction among pine species, and this variation explains species differences in photosynthetic activity (Niinemets et al. 2007). However, for adult needles, tree size and age-dependent anatomical and structural modifications only moderately or not at all altered the share among mesophyll and support tissues (Richardson et al. 2000, Apple et al. 2002, Greenwood et al. 2008), but so far, there is no information on juvenile-to-adult transition effects on fractional distribution of photosynthetic and support tissues.

Compared with European boreal and temperate pine species, the juvenile period lasts longer in Mediterranean pines (Climent et al. 2011*b*), implying that this period of plant life plays a particularly significant role in seedling and sapling establishment in highly stressful Mediterranean environments. Here we studied anatomical and photosynthetic differences between juvenile and mature needles of three Mediterranean pine species—*Pinus halepensis* Mill., *Pinus pinea* L. and *Pinus nigra* J. F. Arnold subsp. *salzmannii* (Dunal) Franco. All three pine species bear single juvenile (primary) and paired adult (secondary) needles in fascicles. The seedlings and young juvenile saplings (as described in Hanley et al. 2004) can carry juvenile needles for several years until they are gradually replaced by adult needles (Boddi et al. 2002). Juvenile needles are bluish, short and elliptical or rhomboidal in shape, while adult secondary needles are dark green and significantly longer and thicker (Climent et al. 2006*a*). We hypothesized that there is a trade-off between support and photosynthetic investments upon juvenile to adult transition.

As needle age also influences foliage structure and chemistry (Teskey et al. 1984, Niinemets 2002, Weiskittel et al. 2008, Niinemets et al. 2012, Mediavilla et al. 2014), but the anatomical and structural modifications in ageing needles are poorly understood, we studied non-senescent adult needles of three different age classes (current-year, 1-year-old and 2-year-old needles). We hypothesized that ageing also leads to accumulation of structural biomass similar to juvenile-to-adult transition, thereby resulting in reduction in foliage nitrogen content. However, because ageing cannot change the basic leaf morphological framework, e.g., position, number and size of cells in the leaf cross-section, we expected that the needle age-dependent changes in older needles are quantitatively less significant than modifications occurring upon juvenile-to-adult transition.

The results of this study highlight a major trade-off between investments in support and physiologically active tissues, and also show that plant age-dependent modifications are more profound than needle age-dependent alterations.

Materials and methods

Study species

Pinus halepensis is found growing in marls, limestone, dolomites, acid sands and sandstone, mostly in subhumid and semiarid climates from lowlands (up to 700 m) to submontane (from 700 to 1300 m) areas, and is the most drought tolerant of the three investigated species (Barbéro et al. 1998, Atzmon et al. 2004). *Pinus nigra* subsp. *salzmannii* prefers limestone, dolomites, acid sands and volcanic (ultrabasic) rocks and mostly humid areas in mid-montane to mountainous (from 900–1000 m up to 1600–1800 m) areas (Kienast et al. 1987, Barbéro et al. 1998). *Pinus pinea* grows on dolomites and sandstone in subhumid climates, mostly at lowland to submontane areas, and its environmental requirements are in between *P. halepensis* and *P. nigra* (Cabanettes and Rapp 1978, Barbéro et al. 1998). Needle life span in mature plants is 2.7 years for *P. halepensis* (Niinemets 2010), 3.8 years for *P. nigra* (Withington et al. 2006) and 4.1 years for *P. pinea* (Mediavilla et al. 2014). The life span of juvenile needles in saplings is less, ~1–1.5 years (Mediavilla et al. 2014, and personal observations).

Measurement sites and plants

The mature cone-bearing plants were studied at Alto Tajo Natural Park, Los Cerrillos sampling site, in the vicinity of Villar de Cobeta, central Spain (Guadalajara, Castilla-La Mancha, 40.8480°N, 2.1812°W). The climate in the study area is continental Mediterranean with hot and dry summers and cold and dry winters. According to Molina de Aragón climatic station (40.8444°N, 1.8853°W, elevation 1063 m), the mean annual rainfall is 490 mm and mean annual temperature is 10.2 °C. The site has a mean elevation of 950 m, with steep slopes and cliffs covered with Mediterranean-type shrublands, and 5–20 m high open forests dominated by *Quercus ilex*, L. subsp. *ballota* (Desf.) Samp. on south-facing slopes and *P. nigra* subsp. *salzmannii* on north-facing slopes. The upper parts of the plateaus are dominated by *Juniperus thurifera* L., *Pinus sylvestris* L. and *Buxus sempervirens* L. Soils are basic, rocky and poorly developed over the limestone bedrock. Five trees were randomly chosen for each species for sampling of needles of different age classes. Mean tree diameter at breast height, mean tree height and estimated tree age were 40 cm, 15 m and 95 years for *P. halepensis*, 44 cm, 17 m and 90 years for *P. pinea* and 60 cm, 21 m and 100 years for *P. nigra*. Representative terminal branchlets carrying only needles (without cones) were sampled from mid-height and from the southern aspect of upper canopy and were exposed to a daily integrated quantum flux density of ~40 mol m^{-2} day^{-1}.

To study juvenile foliage, 2-year-old juvenile plants were bought from a local nursery at El Seranillo Forestry Center, Guadalajara and transplanted to 5 L pots filled with a mixture (4:4:1:1, v/v) of compost, vermiculite, perlite and sand, with slow release N:P:K (14:13:13) fertilizer with micronutrients (Osmocote, Scotts Miracle-Gro Company, Marysville, OH, USA, Baquedano and Castillo 2007, Baquedano et al. 2008 for details). The juvenile saplings were grown at the greenhouses of Museo Nacional de Ciencias Naturales (CSIC) in Madrid, Spain (40.440°N, 3.690°W, elevation 682 m). The pots had big holes in the bottom and were buried into soil. There were nine pots per m^2 and the soil temperature was ambient. The plants were grown under full sunlight with the mean daily integrated photosynthetic quantum flux density during spring of 41 mol m^{-2} day^{-1}. Current-year juvenile needles that had formed under the new growth conditions were used for the measurements.

An apparent limitation of this experiment is that juvenile and adult foliage could not be studied at the same environmental setting. However, this limitation was inevitable as the juvenile plants were very rare in the field and were only found in shaded habitats and we intended to avoid shade effects interfering with our analysis (Poorter et al. 2009, Niinemets et al. 2015, Keenan and Niinemets 2016). Shading effects on leaf structure are typically much greater than the effects of nutrients or water availability (Poorter et al. 2009 for a review), although in particularly tall tree canopies, shading and water availability can collectively affect foliage structure (Koch et al. 2004, Woodruff et al. 2007). To avoid the confounding shading effects, we used potted saplings grown under full sunlight and similar environmental conditions as those prevailing in the field. In fact, often the studies on plant size and age versus leaf structure and function are carried out along the chronosequence of different stands to avoid the shading effects that would be inevitable when different-aged individuals are sampled within the same stand. Although such experimental designs introduce a certain level of confounding variability due to possible differences in site conditions, we argue that avoiding the shading effects inevitable in studies with different-sized plants in the same stands (Poorter et al. 2009 for major sources of shading effects) is superior to controlling for other sources of variability (e.g., Chandler and Dale 1993, Helmisaari 1990, Niinemets et al. 2001 for illustration of moderate effects of site fertility on foliage structural traits in conifers). In addition, we argue that the general trade-offs among needle fractional investments in different tissues through needle ages, plant ages and species are not dependent on site effects.

Foliage sampling

At the field site, branches of randomly chosen mature plants growing at full sunlight (one branch from each individual tree) were collected from the mid-height of the canopy to reduce the influence of light on needle characteristics (Gebauer et al. 2011, Niinemets et al. 2015) in October 2005 and in August 2006. To maintain the needle hydration state, the branches were cut under water, the cut ends were maintained in water and the branches were transported to the laboratory for foliage structural measurements.

In adult trees bearing secondary needles, needles of age classes with at least 50% needles present were studied. Three different age classes (current-year, 1-year-old and 2-year-old needles) were investigated in *P. nigra* and *P. pinea*. In *P. halepensis*, which has a shorter leaf life span than the other two species, current-year and 1-year-old needles were studied. In the case of juvenile plants where the canopy mainly (>90%) consisted of juvenile (primary) current-year needles, only this needle age class was studied. In 2005 we measured five juvenile plants from each studied species and five branches from adult trees per species. In 2006, two branches from *P. halepensis*, three branches from *P. pinea* and four branches from *P. nigra* mature trees were measured. In total, 24 branches (from seven trees of *P. halepensis*, eight trees of *P. pinea* and nine trees of *P. nigra*) and 15 juvenile plants (five of each species) were used, and altogether 5–8 samples for each tree age × leaf age × species combination, on average 6.7, were available.

Measurements of needle morphological and anatomical characteristics

At least 10 needles from each age class were randomly sampled from each branch. Needle length (L), width (W) and thickness

(T) from the central needle part were measured with Starrett Digital calipers (model 727, L. S. Starrett Company, Athol, MA, USA), and average needle projected area, S_P, was calculated as WL. Altogether 190 (juvenile current year, and mature different age classes) needles were measured for *P. halepensis*, 290 for *P. pinea* and 310 for *P. nigra*.

Needles for preparation of free-hand cross-sections for anatomy were randomly selected from the set of harvested needles (a subsample of three needles) for each species, branch and age class. The sections taken from the central parts of the needles were stained with phloroglucinol and HCl to specifically visualize the lignified tissues in red (Weisner test) as in Niinemets et al. (2005*a*, 2007). The images were viewed at 20–100× magnification and digital pictures were taken with a Nikon Eclipse E600 microscope and Nikon digital DS-Fi1 camera (Nikon Corporation, Tokyo, Japan). Needle cross-section width, thickness, circumference (U), needle cross-sectional area (S_S) and the area of the lignified tissues were measured from the images using UTHSCSA Image Tool for Windows Version 3.00 software (UTHSCSA, TX, San Antonio, USA, 1995–2002). From these measurements, the ratio of needle total (S_T) to projected surface area, S_T/S_P, was calculated as U/W and needle volume to total area ratio, V/S_T, as S_S/U, needle volume to projected area ratio as S_S/W and needle total area as UL (Niinemets and Kull 1995, Niinemets et al. 2007). Comparison of thickness and width measurements from free-hand sections by microscopy and from intact needles by calipers indicated a strong correspondence between two types of measurements (within 5%).

In addition to the free-hand sections, needle samples were taken for electron microscopy next to the site of taking the free-hand sections from the middle part of the needle and fixed by infiltrating with 2% glutaric aldehyde in 0.2 M phosphate buffer (pH 7.2) under negative pressure in a syringe. After fixation, the samples were dehydrated in ethanol series and embedded in Epoxy Embedding Medium (Sigma-Aldrich Chemie, Steinheim, Germany) as described by Bozzola and Russell (1992). An OM U2 Ultra Microtome (American Optical Corporation, Reichert Products, Buffalo, NY, USA) with a 45° diamond knife (Diatome, Hatfield, PA, USA) was used for cutting semithin sections for light microscopy (1.5–2 μm) and ultrathin sections (70–90 nm) for transmission electron microscopy (TEM). The semithin sections were stained with Toluidine Blue as described in Burns (1978) and viewed and photographed with a Zeiss Axioplan 2 (Carl Zeiss Microscopy, Jena, Germany) microscope using a 40× magnifying objective with 0.63× lens and a digital camera AxioCam HRc (Carl Zeiss Microscopy). From these images, epidermis, endodermis, hypodermis, mesophyll, mesophyll air spaces, central cylinder and resin ducts areas were measured using the UTHSCSA Image Tool as described in Niinemets et al. (2007). For each sample, 5–10 sections were measured and averages were calculated.

Uranyl acetate and lead citrate were used to contrast the ultrathin (90–100 nm) sections as in Copolovici et al. (2011) and the samples were viewed under a Philips Tecnai 10 TEM (FEI, Eindhoven, The Netherlands) that was operated with an accelerating voltage of 80 kV. Digital pictures were taken with an Olympus Veleta 2 K × 2 K sidemounted TEM CCD camera (Olympus Soft Imaging Solutions GmbH, Germany, Münster, Germany). Magnifications of 7000–10,000× were used to measure cell wall thickness, chloroplast length, thickness and circumference with the UTHSCSA Image Tool (6–12 chloroplasts were measured from each section and 5–15 sections, on average 7.5 sections per sample were measured). Mesophyll (S_m/S_P) and chloroplast (S_c/S_P) surface area exposed to intercellular air space per unit of projected leaf area were also measured from the TEM micrographs at 1250–3700× magnification and calculated according to Evans et al. (1994) using a curvature correction factor of 1.27 (Thain 1983). The volume of mesophyll per needle projected area (V_m/S_P) and the volume of chloroplasts per projected area (V_c/S_P) were also calculated, and ultimately the fraction of chloroplasts in needle cross-section, f_{chl}, was calculated as $V_c/V_m f_{ma}$, where f_{ma} is the fraction of mesophyll in needle cross-section without intercellular air space.

Estimation of needle dry mass per unit area, density, dry to fresh mass ratio, carbon and nitrogen contents

Fresh mass of sample needles was immediately estimated after measurements of needle dimensions. Then needles were oven-dried for 48 h at 70 °C and nitrogen (N_m) and carbon (C_m) contents per dry mass were measured with a Perkin Elmer series II CHNS/O Analyzer 2400 (Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA, USA).

From these measurements, needle dry to fresh mass ratio, needle dry mass ($M_{A,P}$) and needle nitrogen content ($N_{A,P}$) per projected area were calculated. Considering that the trait values per total area are given as the values per projected area divided by S_T/S_P, needle dry mass ($M_{A,T}$) and nitrogen content ($N_{A,T}$) per total needle area were also calculated. Leaf dry mass per unit area can be expressed as a product of leaf density (D) and thickness (Witkowski and Lamont 1991, Niinemets 1999, Poorter et al. 2009), and for needle-leaved species with complex cross-sectional geometry as the product of needle density and volume to area ratio (Niinemets and Kull 1995). Thus needle density was calculated as $D = \frac{M_{A,T}}{V/S_T}$.

Statistical analyses

Average values for all needles taken from the given branch and needle age class were calculated prior to the statistical analyses. One-way ANOVAs followed by Tukey's post-hoc test were used

to analyze the differences in trait means among study years, needle age classes and among trees of different age using Statistica 6 (StatSoft Inc., Tulsa, OK, USA) and R 3.1.1 (R Development Core Team 2012). Because the study year was not significant in any of the analyses, the data for both years were pooled in all analyses. For simplicity, two-way ANOVAs were carried out to test for the global effects of species and effects of heteroblasty on the traits of current-year needles in saplings and mature trees (species and plant age as main factors), and effects of species and needle age on traits of adult needles in mature trees (species and needle age as main factors with needle ages as juv—current year juvenile needles, m0—current year needles in mature trees, m1—1-year old needles in mature trees and m2—2-year-old needles in mature trees). One-way ANOVAs were also conducted to separate the differences among the trait means for different combinations of tree age (juvenile vs adult), needle age and species. The relationships among traits were studied by linear and non-linear (log or power) regression analyses. All r^2 values reported in the text refer to linear regressions unless noted. All statistical tests were considered significant at $P < 0.05$.

Results

Age and species effects on needle shape and morphology

The cross-section of adult (secondary) needles was hemi-elliptical to hemi-circular in all three *Pinus* species, but the cross-section of juvenile (primary) needles was rhomboidal in *P. halepensis*, sectorial in *P. pinea* and intermediate between sectorial and rhomboidal shapes in *P. nigra* (Figure 1). Thickness of juvenile and adult current-year needles was similar, but juvenile needles were narrower with smaller circumference and cross-sectional area than adult needles (Table 1). Due to differences in needle shape, needle total to projected area ratio (S_T/S_P) was smaller in juvenile than in adult needles, except for *P. nigra* where this trait did not differ among juvenile and current-year adult needles (Table 1).

Needle dry mass per unit total ($M_{A,T}$, Figure 2A) and projected ($M_{A,P}$, Table 2) area was lower in juvenile than in adult needles in all species. Analysis of the variation in the components of $M_{A,T}$, needle volume to total area ratio (V/S_T) and needle density (D) indicated that differences in $M_{A,T}$ among juvenile and adult current-year needles were driven by lower V/S_T in all species

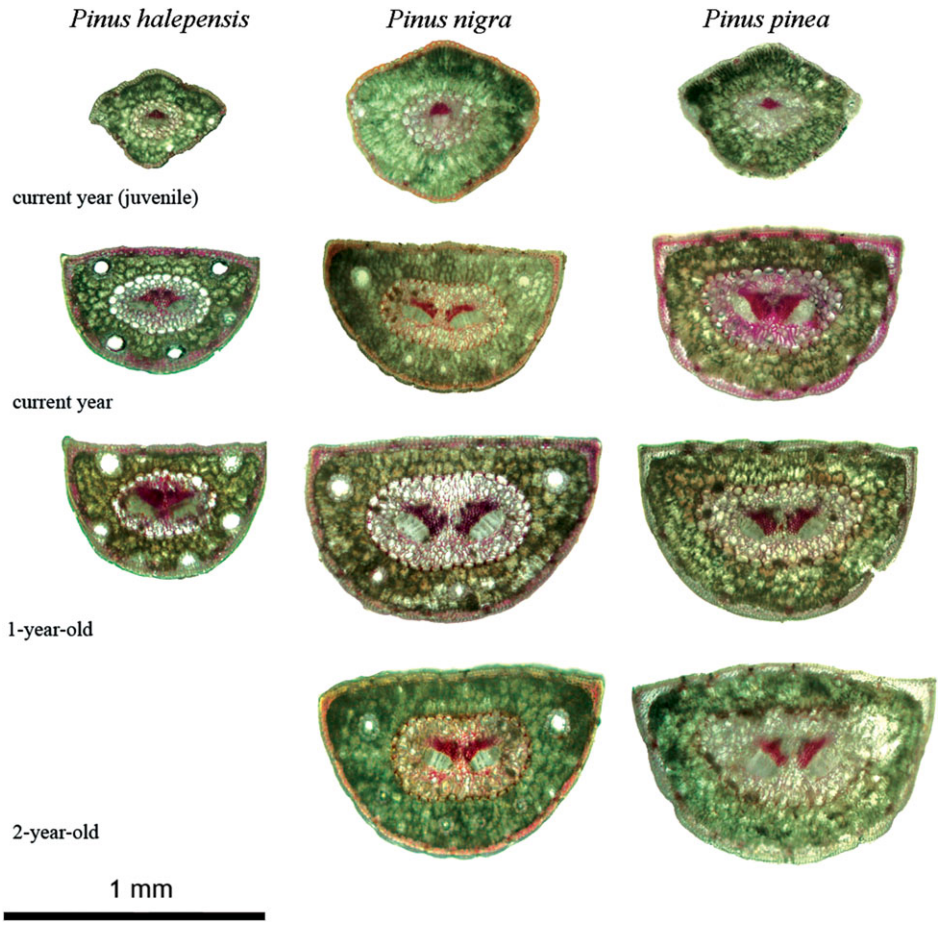

Figure 1. Representative free-hand needle cross-sections of juvenile (primary) current-year and different-aged adult (secondary) needles in three Mediterranean *Pinus* species. Juvenile needles were sampled from 2-year-old 0.2–0.3 m tall young plants, while the adult needles were sampled from 15 to 21 m tall cone-bearing mature trees. Needle cross-sections were stained with phloroglucinol that specifically stains lignified tissues.

Table 1. Effects of tree and needle age on average (±SE) needle linear dimensions (thickness (T), width (W), circumference (U)), needle total area (S_T) and total to projected area ratio (S_T/S_P) in the three Mediterranean *Pinus* species.

Species	Tree age class	Needle age	T (mm)	W (mm)	U (mm)	S_T (mm^2)	S_T/S_P
P. halepensis	Seedling	(juv)	0.431 ± 0.014^{a}	0.541 ± 0.015^{a}	1.412 ± 0.042^{a}	19.1 ± 0.8^{a}	2.608 ± 0.015^{a}
P. halepensis	Mature	(m0)	0.478 ± 0.016^{a}	0.738 ± 0.017^{a}	2.065 ± 0.043^{ab}	26.1 ± 1.0^{a}	2.798 ± 0.017^{bc}
P. halepensis	Mature	(m1)	0.479 ± 0.014^{a}	0.773 ± 0.018^{a}	2.21 ± 0.06^{bc}	28.7 ± 0.5^{ab}	2.862 ± 0.043^{c}
P. nigra	Seedling	(juv)	0.615 ± 0.012^{b}	0.727 ± 0.036^{a}	1.95 ± 0.10^{ab}	24.4 ± 1.7^{a}	2.69 ± 0.07^{ab}
P. nigra	Mature	(m0)	0.579 ± 0.010^{b}	1.008 ± 0.020^{b}	2.85 ± 0.08^{cd}	37.7 ± 1.2^{bc}	2.826 ± 0.026^{bc}
P. nigra	Mature	(m1)	0.637 ± 0.024^{b}	1.126 ± 0.036^{ab}	3.14 ± 0.09^{de}	44.3 ± 1.4^{c}	2.789 ± 0.034^{bc}
P. nigra	Mature	(m2)	0.660 ± 0.028^{b}	1.179 ± 0.042^{ab}	3.29 ± 0.10^{de}	43.0 ± 1.6^{c}	2.792 ± 0.032^{bc}
P. pinea	Seedling	(juv)	0.644 ± 0.019^{b}	0.74 ± 0.09^{a}	2.02 ± 0.26^{ab}	28.3 ± 1.5^{ab}	2.722 ± 0.036^{abc}
P. pinea	Mature	(m0)	0.612 ± 0.027^{b}	1.134 ± 0.032^{ab}	3.15 ± 0.08^{de}	42.4 ± 1.7^{c}	2.778 ± 0.021^{abc}
P. pinea	Mature	(m1)	0.674 ± 0.025^{b}	1.33 ± 0.12^{b}	3.68 ± 0.36^{e}	46.8 ± 5.6^{c}	2.760 ± 0.023^{abc}
P. pinea	Mature	(m2)	0.651 ± 0.019^{b}	1.250 ± 0.017^{b}	3.41 ± 0.06^{de}	44.1 ± 1.5^{c}	2.726 ± 0.017^{abc}
F-values for two-way ANOVA (juvenile vs adult current-year needles)							
Tree age ($F_{1,32}$)			0.22 ns	134.9***	155.0***	83.2***	18.7***
Species ($F_{2,32}$)			38.3***	59.0***	57.0***	39.8***	1.02 ns
Tree age × Species ($F_{2,32}$)			2.64 ns	4.91*	3.55*	3.04 ns	1.79 ns
F-values for two-way ANOVA (adult needles of different age)							
Needle age ($F_{2,51}$)			3.72*	4.85*	4.09*	2.77 ns	0.63 ns
Species ($F_{2,51}$)			29.7***	42.7***	36.6***	28.7***	3.17 ns
Needle age × Species ($F_{3,51}$)			1.16 ns	1.13 ns	0.96 ns	0.36	1.11 ns

n = 5–8 (on average 6.7) for all average values. Seedlings were 2 years old and 0.2–0.3 m tall at the time of sampling, while the mean height of mature trees was 15 m for *P. halepensis*, 17 m for *P. pinea* and 21 m for *P. nigra* at the time of sampling. Needle ages as: juv, juvenile current year; m0, mature current year; m1, mature 1-year-old; m2, mature 2-year-old needles. Different letters denote statistical significance of average values for different species and needle age class combinations at $P < 0.05$ (one-way ANOVA followed by a Tukey post-hoc test based on $F_{10,63}$). In the case of two-way ANOVA, significance levels as: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant.

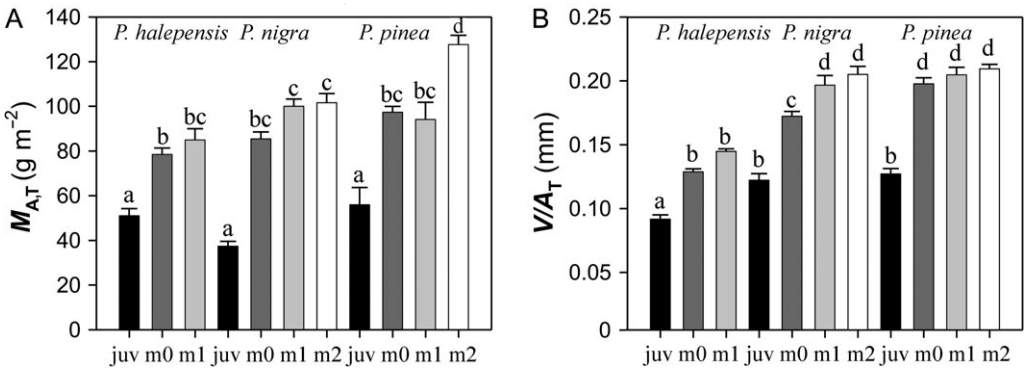

Figure 2. Differences in leaf dry mass per unit total needle area ($M_{A,T}$) and needle volume to total area ratio (V/A_T) in dependence of needle and plant age in three Mediterranean *Pinus* species. $M_{A,T}$ is the product of needle density (Table 2) and V/A_T. Needle ages as: juv, juvenile current year; m0, mature current year; m1, mature 1-year-old; m2, mature 2-year-old needles (Figure 1 for representative needle cross-sections). Data correspond to average ±SE values of 5–8 (on average 6.7) replicates, and different letters show statistical significance among needle age classes and species at $P < 0.05$ (ANOVA followed by a Tukey post-hoc test).

(Figure 2B), and by lower density in juvenile needles of *P. nigra* (Table 2). Needle dry to fresh mass ratio was lower for juvenile than for adult current-year needles in *P. halepensis* (Table 2).

Among adult needles in mature trees, needle linear dimensions, i.e., needle thickness, width and circumference, increased somewhat with needle age (Table 1). $M_{A,T}$ and $M_{A,P}$ were greater in 2-year-old needles than in current-year and 1-year-old needles of *P. pinea* (Figure 2A, Table 2), while V/S_T was greater in 1- and 2-year-old needles than in current year needles in *P. nigra* (Figure 2B). Needle dry to fresh mass ratio was greater in 1- and 2-year-old needles than in current-year needles in all species (Table 2).

Although the heteroblastic and needle age-dependent changes were broadly similar in different species, most needle dimensions ($M_{A,T}$, $M_{A,P}$ and V/S_T) were smaller for needles of given age in *P. halepensis* than in *P. nigra* and *P. pinea* (Figures 1 and 2, Tables 1 and 2). In addition, $M_{A,T}$ (Figure 2A) and $M_{A,P}$ (Table 2) of 2-year-old needles of *P. pinea* were greater than those in *P. nigra*, reflecting greater needle density of *P. pinea* 2-year-old-needles (Table 2).

Table 2. Variations in needle dry mass per unit area ($M_{A,P}$), dry to fresh mass ratio ($R_{D/F}$), density (D) and carbon content (C_m) in relation to tree and needle age in three Mediterranean *Pinus* species.

Species	Tree age class	Needle age	$M_{A,P}$ (g m^{-2})	$R_{D/F}$ (g g^{-1})	D (g cm^{-3})	C_m (%)
P. halepensis	Seedling	(juv)	133 ± 9^{a}	0.393 ± 0.007^{a}	0.555 ± 0.026^{bc}	48.12 ± 0.15^{a}
P. halepensis	Mature	(m0)	219 ± 8^{b}	0.4687 ± 0.0035^{cd}	0.611 ± 0.027^{c}	50.62 ± 0.21^{cde}
P. halepensis	Mature	(m1)	244 ± 16^{bc}	0.514 ± 0.010^{e}	0.586 ± 0.034^{c}	51.3 ± 0.6^{de}
P. nigra	Seedling	(juv)	99.9 ± 4.8^{a}	0.357 ± 0.007^{a}	0.308 ± 0.021^{a}	49.71 ± 0.31^{abc}
P. nigra	Mature	(m0)	241 ± 7^{bc}	0.3798 ± 0.0021^{a}	0.498 ± 0.023^{bc}	49.50 ± 0.11^{abc}
P. nigra	Mature	(m1)	279 ± 8^{bc}	0.472 ± 0.019^{cde}	0.518 ± 0.038^{bc}	51.54 ± 0.20^{de}
P. nigra	Mature	(m2)	284 ± 12^{c}	0.511 ± 0.010^{de}	0.501 ± 0.032^{bc}	52.24 ± 0.33^{e}
P. pinea	Seedling	(juv)	153 ± 23^{a}	0.4009 ± 0.005^{ab}	0.44 ± 0.06^{ab}	50.15 ± 0.11^{bcd}
P. pinea	Mature	(m0)	270 ± 8^{bc}	0.382 ± 0.007^{a}	0.495 ± 0.019^{bc}	48.78 ± 0.26^{ab}
P. pinea	Mature	(m1)	260 ± 21^{bc}	0.4442 ± 0.0006^{bc}	0.464 ± 0.041^{bc}	49.28 ± 0.08^{abc}
P. pinea	Mature	(m2)	347 ± 9^{d}	0.484 ± 0.008^{cde}	0.611 ± 0.023^{c}	49.09 ± 0.22^{abc}
F-values for two-way ANOVA (juvenile vs adult current-year needles)						
Tree age ($F_{1,32}$)			164.7***	26.4***	22.2***	0.013 ns
Species ($F_{2,32}$)			7.35**	64.6***	14.9***	0.40 ns
Tree age × Species ($F_{2,32}$)			3.67*	29.9***	3.37*	29.8***
F-values for two-way ANOVA (adult needles of different age)						
Needle age ($F_{2,51}$)			12.5***	78.6***	2.45 ns	13.1***
Species ($F_{2,51}$)			6.65**	37.8***	7.20*	32.5***
Needle age × Species ($F_{3,51}$)			3.38*	2.25 ns	2.14 ns	4.9**

Sample number, data presentation and statistical analyses as in Table 1, except for C_m ($n = 3$–6 for each average estimate, and $n = 19$ for the residuals of the *F*-values for juvenile vs adult comparison and $n = 26$ for comparisons of adult needle ages).

Age and species differences in distribution of needle volume among photosynthetic and structural tissues

Visual assessment of needle cross-sections indicates that epidermis, hypodermis, endodermis, xylem and partly the cells of the transfusion tissue that surrounds the vascular bundles in the central cylinder had lignified cell walls that stained red upon phloroglucinol treatment (Figure 1). Although a non-quantitative measure, the staining intensity of different mechanical tissue fractions was visibly greater in adult than in juvenile needles (Figure 1).

The volume fraction of epidermal tissues (f_e, sum of epidermis and hypodermis, Figure 1) was lower in juvenile than in adult current-year needles of *P. halepensis* and current-year needles of *P. nigra*, and the fraction of central cylinder (f_c) was lower in juvenile than in adult needles in all species (Table 3). Thus, the total fraction of mechanical tissues (f_{tm}), the sum of epidermal tissues and the central cylinder, was in all cases lower in juvenile than in adult current-year needles (Table 3). The volume fraction of resin ducts was smaller, and the fractions of total mesophyll, mesophyll without air spaces, mesophyll air spaces and chloroplasts were greater in juvenile than in adult current-year needles (Table 3).

Among the adult needles, leaf tissue distribution was weakly affected by needle age (Table 3). However, the species effect was significant in all cases, reflecting mainly greater fractions of mechanical tissues and a lower fraction of mesophyll in *P. halepensis* and *P. pinea* than in *P. nigra* (Table 3). Across all data, mechanical tissue fractions were positively correlated with each other ($r^2 = 0.39$ for f_e vs f_{tm} and $r^2 = 0.77$ for f_c vs f_{tm}, $P < 0.001$ for both), except for the correlation among f_e and f_c ($r^2 = 0.04$, $P > 0.1$). The mechanical and mesophyll tissue fractions were negatively correlated (e.g., for f_{tm}, $r^2 = 0.98$ for total mesophyll and $r^2 = 0.77$ for the mesophyll fraction without intercellular air space, $P < 0.001$ for both). The fraction of chloroplasts and the fraction of mesophyll with ($r^2 = 0.20$) and without air space ($r^2 = 0.21$) were positively correlated ($P < 0.001$ for both).

The f_e scaled positively with needle density ($r^2 = 0.18$, $P < 0.01$) and with needle dry to fresh mass ratio ($r^2 = 0.10$, $P < 0.05$), while f_c and f_{tm} scaled positively with density ($r^2 = 0.26$ for f_c and $r^2 = 0.34$ for f_{tm}, $P < 0.001$ for both), with dry to fresh mass ratio ($r^2 = 0.17$ for f_c and $r^2 = 0.23$ for f_{tm}, $P < 0.01$), with $M_{A,T}$ ($r^2 = 0.66$ and $r^2 = 0.52$ for f_{tm}, $P < 0.001$) and with V/S_T ($r^2 = 0.55$ for f_c and $r^2 = 0.33$ for f_{tm}, $P < 0.001$). The relationships of the fractions of total mesophyll and mesophyll without air spaces with these four structural traits were analogous to f_{tm}, but negative (see Figure 4A for the correlation with $M_{A,T}$).

Differences in needle ultrastructure among plant and needle ages and species

Juvenile needles had thinner cell walls than current-year mature needles in *P. nigra* and in *P. pinea*, but not in *P. halepensis* (Table 4). Chloroplasts in juvenile needles had greater width in *P. halepensis* and *P. nigra* and greater thickness and circumference in *P. halepensis* than the chloroplasts in mature needles (Table 4). Mesophyll surface (S_m/S_P) and chloroplast surface area (S_c/S_P) exposed to intercellular air space per projected leaf area did not differ among juvenile and mature needles, but the

Table 3. Average (±SE) volume fractions of different needle tissues in three Mediterranean *Pinus* species in dependence on needle and tree ages.

Species	Tree age class	Needle age	Tissue volume fraction[1]							
			Total mechanical	Epidermal tissues	Central cylinder	Resin ducts	Total mesophyll	Mesophyll air spaces	Mesophyll without air spaces	Chloroplasts
P. halepensis	Seedling	(juv)	0.317 ± 0.015^{a}	0.122 ± 0.006^{ab}	0.195 ± 0.012^{a}	0.0262 ± 0.0023^{a}	0.657 ± 0.015^{d}	0.162 ± 0.014^{cd}	0.495 ± 0.015^{de}	0.088 ± 0.021^{b}
P. halepensis	Mature	(m0)	0.4908 ± 0.0041^{d}	0.2160 ± 0.0022^{c}	0.2745 ± 0.005^{bc}	0.081 ± 0.011^{d}	0.428 ± 0.006^{a}	0.106 ± 0.0056^{abc}	0.322 ± 0.007^{a}	0.0223 ± 0.0030^{a}
P. halepensis	Mature	(m1)	0.474 ± 0.009^{d}	0.202 ± 0.009^{c}	0.272 ± 0.010^{b}	0.0729 ± 0.0033^{cd}	0.453 ± 0.007^{ab}	0.102 ± 0.010^{abc}	0.351 ± 0.007^{ab}	0.053 ± 0.006^{ab}
P. nigra	Seedling	(juv)	0.267 ± 0.008^{a}	0.1006 ± 0.0034^{a}	0.1664 ± 0.0051^{a}	0.0193 ± 0.0010^{a}	0.714 ± 0.007^{e}	0.1963 ± 0.0034^{d}	0.517 ± 0.005^{e}	0.058 ± 0.010^{ab}
P. nigra	Mature	(m0)	0.410 ± 0.006^{bc}	0.1494 ± 0.0047^{b}	0.260 ± 0.007^{b}	0.0341 ± 0.0039^{a}	0.556 ± 0.006^{c}	0.117 ± 0.015^{bc}	0.439 ± 0.019^{cd}	0.0418 ± 0.0030^{ab}
P. nigra	Mature	(m1)	0.3959 ± 0.0049^{b}	0.1310 ± 0.0026^{ab}	0.265 ± 0.006^{b}	0.0360 ± 0.0018^{ab}	0.568 ± 0.006^{c}	0.133 ± 0.007^{bc}	0.435 ± 0.011^{cd}	0.0301 ± 0.0043^{a}
P. nigra	Mature	(m2)	0.394 ± 0.008^{b}	0.113 ± 0.011^{ab}	0.281 ± 0.007^{bc}	0.0406 ± 0.0019^{ab}	0.565 ± 0.009^{c}	0.1251 ± 0.0029^{bc}	0.440 ± 0.009^{cd}	0.028 ± 0.007^{a}
P. pinea	Seedling	(juv)	0.3061 ± 0.0029^{a}	0.113 ± 0.014^{ab}	0.193 ± 0.011^{a}	0.0246 ± 0.0020^{a}	0.6693 ± 0.0032^{de}	0.191 ± 0.009^{d}	0.478 ± 0.009^{de}	0.083 ± 0.018^{b}
P. pinea	Mature	(m0)	0.468 ± 0.016^{d}	0.132 ± 0.008^{ab}	0.335 ± 0.009^{d}	0.0340 ± 0.006^{ab}	0.493 ± 0.017^{b}	0.127 ± 0.016^{bc}	0.365 ± 0.012^{ab}	0.0211 ± 0.0023^{a}
P. pinea	Mature	(m1)	0.4711 ± 0.009^{d}	0.1315 ± 0.0047^{ab}	0.340 ± 0.012^{d}	0.0755 ± 0.0021^{cd}	0.453 ± 0.008^{ab}	0.049 ± 0.012^{a}	0.404 ± 0.010^{bc}	0.044 ± 0.016^{ab}
P. pinea	Mature	(m2)	0.4545 ± 0.0033^{cd}	0.128 ± 0.006^{ab}	0.3262 ± 0.0047^{cd}	0.0559 ± 0.0015^{bc}	0.4896 ± 0.0045^{b}	0.0779 ± 0.0032^{ab}	0.412 ± 0.006^{bc}	0.0285 ± 0.0030^{a}
F-values for two-way ANOVA (juvenile vs adult current-year needles)										
Tree age ($F_{1,19}$)			284.7***	35.3***	176.0***	27.6***	339.6***	33.2***	90.0***	25.1***
Species ($F_{2,19}$)			17.4***	11.6***	14.8***	10.5***	27.5**	1.54 ns	12.9***	0.11 ns
Tree age × Species ($F_{2,19}$)			0.84 ns	6.48**	5.29*	6.61**	3.80*	0.32 ns	4.34*	3.05 ns
F-values for two-way ANOVA (adult needles of different age)										
Needle age ($F_{2,26}$)			1.42 ns	4.09*	0.18 ns	3.67*	0.44 ns	2.56 ns	2.60 ns	2.70 ns
Species ($F_{2,26}$)			45.6***	51.9***	44.9***	33.3***	79.2***	7.22**	27.8***	0.50 ns
Needle age × Species ($F_{3,26}$)			0.43	1.53 ns	1.23 ns	6.81**	3.35*	5.60**	1.40 ns	4.11*

$n = 3–6$ for each average estimate. Data presentation and statistical analyses as in Table 1. [1]The fraction of total mechanical tissues is the sum of epidermal tissues and central cylinder, the fraction of epidermal tissues includes epidermis and hypodermis and the total mesophyll fraction corresponds to mesophyll with intercellular air spaces

Table 4. Average (±SE) needle ultrastructural characteristics in three Mediterranean *Pinus* species as dependent on needle and tree ages.

Species	Tree age class	Needle age	Cell wall thickness (μm)	Chloroplast width (μm)	Chloroplast thickness (μm)	Chloroplast circumference (μm)	Exposed surface area per projected area ($m^2\ m^{-2}$)		S_c/S_m
							Mesophyll (S_m/S_P)	Chloroplasts (S_c/S_P)	
P. halepensis	Seedling	(juv)	0.359 ± 0.029^{b}	2.40 ± 0.13^{d}	4.69 ± 0.18^{d}	11.43 ± 0.45^{d}	35 ± 5^{abcd}	17.7 ± 3.2^{ab}	0.501 ± 0.028^{cd}
P. halepensis	Mature	(m0)	0.375 ± 0.031^{ab}	1.46 ± 0.12^{ab}	2.62 ± 0.15^{ab}	6.49 ± 0.38^{ab}	24.1 ± 2.9^{abc}	7.6 ± 0.8^{a}	0.323 ± 0.026^{abcd}
P. halepensis	Mature	(m1)	0.301 ± 0.016^{a}	1.44 ± 0.08^{a}	2.59 ± 0.14^{a}	6.50 ± 0.28^{a}	48.8 ± 3.8^{cd}	21.1 ± 0.7^{b}	0.441 ± 0.023^{abcd}
P. nigra	Seedling	(juv)	0.309 ± 0.012^{ab}	1.90 ± 0.12^{bc}	3.58 ± 0.13^{c}	8.67 ± 0.31^{c}	22.2 ± 2.3^{ab}	11.6 ± 2.0^{ab}	0.51 ± 0.05^{d}
P. nigra	Mature	(m0)	0.542 ± 0.044^{c}	2.02 ± 0.09^{d}	3.17 ± 0.13^{abc}	8.00 ± 0.28^{abc}	37.7 ± 4.5^{abcd}	10.5 ± 2.0^{ab}	0.278 ± 0.039^{a}
P. nigra	Mature	(m1)	0.368 ± 0.012^{ab}	1.81 ± 0.08^{abc}	3.61 ± 0.13^{c}	8.56 ± 0.27^{c}	44.1 ± 1.7^{bcd}	13.7 ± 1.2^{ab}	0.314 ± 0.031^{abc}
P. nigra	Mature	(m2)	0.424 ± 0.023^{abc}	1.63 ± 0.08^{abc}	3.42 ± 0.17^{bc}	8.05 ± 0.34^{abc}	51 ± 7^{d}	18.0 ± 3.1^{ab}	0.354 ± 0.035^{abcd}
P. pinea	Seedling	(juv)	0.356 ± 0.021^{ab}	1.64 ± 0.06^{abc}	3.41 ± 0.17^{c}	7.88 ± 0.35^{abc}	18.1 ± 1.2^{a}	9.0 ± 1.3^{a}	0.49 ± 0.06^{bcd}
P. pinea	Mature	(m0)	0.53 ± 0.06^{c}	1.88 ± 0.09^{abc}	3.52 ± 0.17^{c}	8.63 ± 0.31^{c}	29.3 ± 3.8^{abcd}	9.6 ± 1.5^{a}	0.288 ± 0.032^{ab}
P. pinea	Mature	(m1)	0.354 ± 0.025^{ab}	1.93 ± 0.17^{d}	3.31 ± 0.25^{bc}	8.3 ± 0.6^{bc}	52.1 ± 2.4^{d}	18.0 ± 1.5^{ab}	0.348 ± 0.030^{abcd}
P. pinea	Mature	(m2)	0.373 ± 0.021^{ab}	1.61 ± 0.09^{abc}	3.08 ± 0.14^{abc}	7.56 ± 0.32^{abc}	43 ± 6^{bcd}	12.6 ± 1.7^{ab}	0.296 ± 0.010^{a}
F-values for two-way ANOVA (juvenile vs adult current-year needles)									
Tree age ($F_{1,29}$)			12.2**	1.62 ns	14.6***	11.0**	1.41 ns	1.98 ns	23.5***
Species ($F_{2,29}$)			1.10 ns	2.80 ns	3.21 ns	4.57*	1.56 ns	1.54 ns	0.012 ns
Tree age × Species ($F_{2,29}$)			21.75 ns	6.78*	10.3***	12.3***	3.74*	1.94 ns	0.12 ns
F-values for two-way ANOVA (adult needles of different age)									
Needle age ($F_{2,28}$)			5.47**	2.53 ns	0.37 ns	0.83 ns	8.49**	11.48***	1.96 ns
Species ($F_{2,28}$)			2.72 ns	3.76*	5.91**	6.05**	1.41 ns	0.71 ns	2.24 ns
Needle age × Species ($F_{3,28}$)			0.31 ns	0.30 ns	0.96 ns	0.41 ns	2.78 ns	5.26**	1.35 ns

$n = 3–8$ for each average estimate (on average 5.3). Data presentation and statistical analyses as in Table 1.

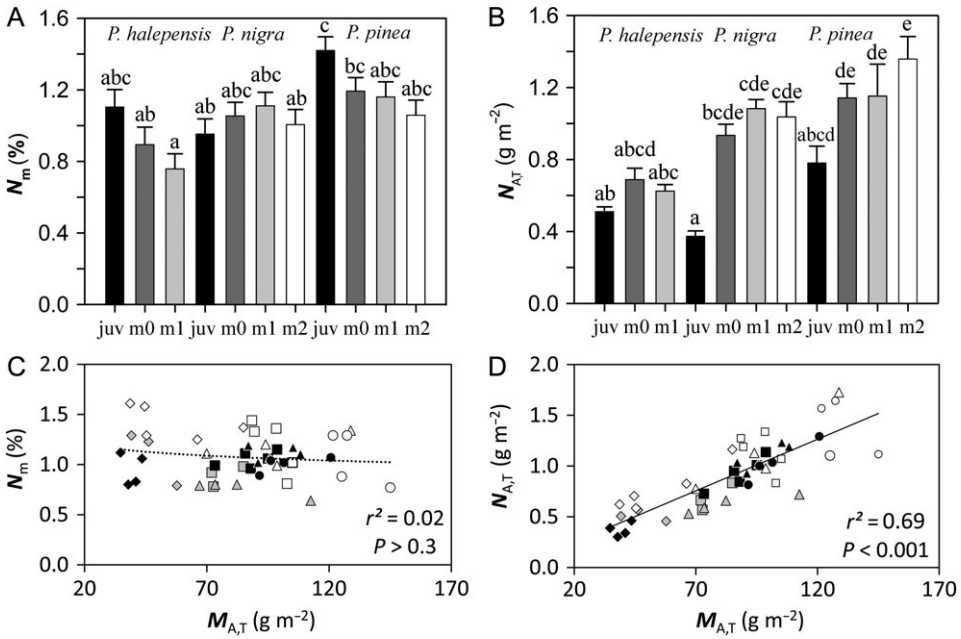

Figure 3. Effects of needle and plant age on needle nitrogen content per dry mass (N_m, A) and on nitrogen content per total needle area ($N_{A,T}$, B) and correlations of N_m (C) and $N_{A,T}$ (D) with needle dry mass per unit total area in three Mediterranean *Pinus* species. Data presentation, sample number and statistical analysis in (A) and (B) as in Figure 2. Data in (C) and (D) were fitted by linear regressions (n = 74) and different symbols correspond to: gray = *Pinus halepensis*, black = *Pinus nigra*, white = *Pinus pinea*; diamonds = juv, squares = m0, triangles = m1, circles = m2.

ratio S_c/S_m was greater in juvenile than in adult needles in *P. nigra* and *P. pinea* (Table 4).

Among different needle age classes in mature needles, current-year needles in *P. nigra* and *P. pinea* tended to have a greater cell wall thickness than older needles (Table 4). Needle age did not affect chloroplast dimensions, but older needles occasionally had greater S_m/S_P or S_c/S_P, and no consistent age-dependent patterns between needle age and these traits were observed (Table 4).

Across all data, the fraction of needle cross-section in chloroplasts (f_{chl}) was positively correlated with S_c/S_P (r^2 = 0.13, P < 0.05) and with S_c/S_m (r^2 = 0.32, P < 0.001), but not with S_m/S_P (r^2 = 0.01, P > 0.6). S_m/S_P was positively correlated with $M_{A,P}$ (r^2 = 0.30, P < 0.001), but S_c/S_P was not related to $M_{A,P}$ (r^2 = 0.01, P > 0.5), and thus, S_c/S_m was negatively correlated with $M_{A,P}$ (r^2 = 0.30, P < 0.001).

Needle nitrogen and carbon contents as affected by needle and plant age

Nitrogen content per dry mass (N_m) was not different among juvenile and adult current-year needles, and also did not differ among different ages of adult needles, but N_m varied among species (Figure 3A). In contrast, nitrogen content per unit total area ($N_{A,T}$), the product of N_m and $M_{A,T}$, was lower in juvenile than in adult current-year needles. $N_{A,T}$ also varied among species, but it did not differ among different ages of adult needles (Figure 3B). The differences in nitrogen content per projected area ($N_{A,P}$), the product of N_m and $M_{A,P}$ were analogous to those in $N_{A,T}$ (data not shown).

Across all data, N_m was not correlated with $M_{A,T}$ (Figure 3C) and $M_{A,P}$ (r^2 = 0.02, P > 0.2), but weak negative correlations were observed between N_m and needle density (r^2 = 0.10, P < 0.05), and the fraction of epidermal tissues (r^2 = 0.23, P < 0.005), and weak positive correlations were observed between N_m and the fractions of mesophyll without air spaces (r^2 = 0.09, P < 0.05) and with air spaces (Figure 4B). Positive correlations were observed among $N_{A,T}$ and $M_{A,T}$ (Figure 3D), and $N_{A,P}$ and $M_{A,P}$ (r^2 = 0.72, P < 0.001).

Needle carbon content per dry mass (C_m) was lower in juvenile than in adult current-year needles of *P. halepensis*, but not for other species × tree age combinations (Table 2). For adult needles, C_m increased with increasing needle age in *P. halepensis* and *P. nigra* (Table 2). Across all data, C_m was positively correlated with needle dry to fresh mass ratio (r^2 = 0.30, P < 0.001). This correlation was even stronger (r^2 = 0.42, P < 0.001) without the data for *P. pinea*, which had a lower C_m than the other species (Table 2). For the pooled data of *P. halepensis* and *P. nigra*, C_m also scaled positively with $M_{A,T}$ (r^2 = 0.30, P < 0.01), V/S_T (r^2 = 0.44, P < 0.001), volume fractions of central cylinder (r^2 = 0.36, P < 0.001) and total mechanical tissues (r^2 = 0.15, P < 0.05).

Discussion

Heteroblasty in Mediterranean pines: modifications in needle morphology

Heteroblastic change constitutes a major rearrangement of foliar characteristics. In our study with Mediterranean *Pinus* species,

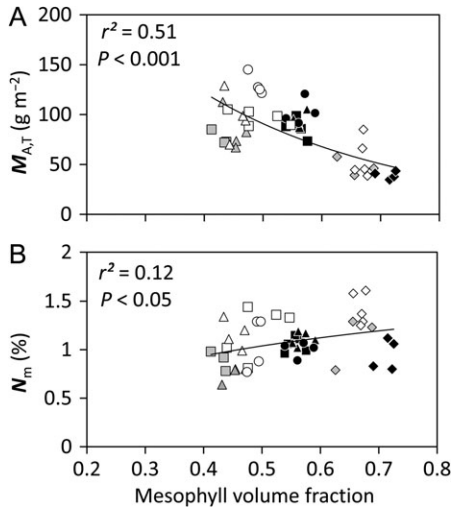

Figure 4. Correlations of leaf dry mass per unit total needle area (A) and needle nitrogen content per dry mass (B) with total mesophyll volume fraction across different needle ages in three Mediterranean *Pinus* species (Table 3 for data on volume fractions of mesophyll without intercellular air space and air space volume). Symbols as in Figure 3C and D. The data were fitted by non-linear equations in the form $y = ax^b$.

juvenile (primary) needles differed from adult (secondary) needles in key anatomical, morphological and photosynthetic traits in all three studied species. In particular, heteroblastic leaf development was associated with major changes in needle cross-sectional shape with juvenile needles having rhomboidal to sectorial shape, in strong contrast to the characteristic hemi-elliptical cross-section of adult needles (Figure 1). From the perspective of light harvesting, different cross-sectional shapes might have a similar efficiency of light interception as was the case for *P. pinea* where needle total to projected area ratio, S_T/S_P, was similar among juvenile and adult needles (Cescatti and Niinemets 2004 for a discussion). However, differences in needle shape among plant ages resulted in a greater exposed surface area at a given total needle area (lower S_T/S_P; Table 1) in *P. halepensis* and *P. nigra*, implying a greater efficiency of light interception in juvenile than in adult needles (Cescatti and Niinemets 2004 for a discussion). Of course, ultimate testing of differences in needle light harvesting efficiency would also require shoot-scale analyses, but so far, there is evidence that foliage spatial aggregation is less in young than in old conifer plants (Niinemets et al. 2005*b*).

Juvenile needles were also narrower, with smaller circumference and with lower volume to total area ratio (V/A_T; Table 1, Figure 2). They also had lower dry to fresh mass ratio, and density (D, Table 2). As a result, needle dry mass per unit area ($M_{A,T}$), the product of V/A_T and D, was smaller in juvenile than in adult needles (Figure 2). This evidence is in agreement with previous studies demonstrating increases in needle linear dimensions, dry mass per unit area and density with increasing tree age and size in several conifers (Steele et al. 1989, Niinemets and Kull 1995, Greenwood et al. 2008, Pardos et al. 2009, Räim et al. 2012).

Although foliage nitrogen content per dry mass was weakly affected by heteroblasty in our study, this low variability is in agreement with other studies demonstrating that nitrogen content per dry mass is not always associated with tree age and size (Day et al. 2001, Niinemets 2002) or it can be even lower in juvenile than in adult plants (Juárez-López et al. 2008, Mediavilla et al. 2014). In fact, with increasing leaf structural robustness, a greater fraction of leaf nitrogen can be associated with cell walls, and this is expected to lead to lower photosynthetic nitrogen-use efficiency (Takashima et al. 2004, Onoda et al. 2017).

Past studies on tree age effects on foliage structural characteristics in conifers have mainly focused on the ontogenetic drift that occurs continuously through plant lifetimes in adult foliage (Introduction, Zotz et al. 2011, see Greenwood et al. 2008 for specification of terminology). However, differently from the ontogenetic drift, heteroblastic changes in needle morphology observed in the current study are much greater, e.g., 1.6- to 2.4-fold for leaf dry mass per unit area (Table 2), than the changes typically observed for adult needles in aging trees (Introduction). We admit that differences in sampling locations (common garden for juveniles vs field environment for adult trees) might partly affect the observed differences. Due to strong shading effects that could interfere with plant age effects, we considered that sampling under comparable light conditions, which was only possible using common garden trials for juveniles, was most important to discern patterns arising from heteroblasty (see also Materials and methods). In fact, leaf shape—sectorial and rhomboidal in juveniles versus hemi-elliptical in adults—is not altered by growth conditions (field vs common garden). Furthermore, as shading reduces needle width and thickness and has a minor effect on needle density (Niinemets et al. 2001, 2007), we suppose that if needles of juveniles were sampled in the shade of adult trees, the differences among juveniles and adults would have been even bigger. So we conclude that the differences among adult and juvenile needles reported in our paper are robust and do not qualitatively depend on the study design.

Heteroblastic changes in needle anatomical and chemical traits

Our study demonstrates a major variation in biomass investments in structural and assimilative tissues across plant ages, ~1.5-fold lower investment in support, and concomitantly higher investment in mesophyll in juvenile than in adult needles (Figure 1, Table 3). The heteroblastic change in the share of different needle tissues (Table 3) observed in our study differs from the ontogenetic drift in conifers that only moderately alters the tissue volume fractions (Apple et al. 2002) or does not alter at all (Richardson et al. 2000, Greenwood et al. 2008), although a substantial decrease in air space volume fraction with increasing tree size has been reported (Apple et al. 2002, Greenwood et al. 2008).

Ultrastructural analysis of needle mesophyll characteristics demonstrated that increased mesophyll volume fraction was associated with a greater volume fraction of chloroplasts in juvenile needles (Table 4). The surface area of mesophyll cells (S_m/S_P) and chloroplasts (S_c/S_P) exposed to intercellular air space per projected leaf area scaled with V/A_T and did not differ among juvenile and adult needles (Table 4). However, juvenile needles had larger chloroplasts and filled a greater area of mesophyll cell walls that were exposed to intercellular air space (greater S_c/S_m ratio). This evidence suggests that the structure and ultrastructure of juvenile needles might result in greater photosynthetic activity per mass of juvenile needles. If so, this would imply a shorter payback time of juvenile foliage, and greater carbon availability for growth, and thus, be an important factor enhancing early growth and recruitment of the juveniles (Climent et al. 2011*a*, *b*, Mediavilla et al. 2014).

Lower assimilation rate in older conifer trees is a well-known phenomenon (Yoder et al. 1994, Niinemets 2002, Greenwood et al. 2008, Räim et al. 2012), but the underlying mechanisms of this decline are not entirely understood. Initially, it was suggested that the reduction in foliage photosynthesis rate in older trees mainly reflects decreases in stomatal conductance (Yoder et al. 1994, Greenwood et al. 2008). However, now there is increasing evidence that lower stomatal conductance is not the only factor responsible for the decline in photosynthesis rate with tree age (Niinemets 2002, Räim et al. 2012). In addition to stomata, lower mesophyll diffusion conductance from substomatal cavities to chloroplasts in more robust needles in older trees has been proposed as the factor constraining their photosynthesis (Niinemets 1997*b*, 2002), and the finding of lower cell wall thickness and greater S_c/S_m in juvenile needles is consistent with this hypothesis. On the other hand, differences in the volume fractions of mesophyll and chloroplasts suggest that differences in needle biomass distribution between support and photosynthetic tissues can constitute another key factor accounting for enhanced photosynthesis rates in juvenile needles. Detailed gas-exchange measurements are needed to test these hypotheses in the future.

Needle age-dependent changes in foliage structure and function

We observed that increases in the age of adult needles were associated with moderate increases in needle linear dimensions (width and thickness, Table 1) and volume to total area ratio, and greater needle dry mass per unit area (Table 2, Figure 2). However, the fractional tissue distribution (Table 3) and needle ultrastructural characteristics (Table 4) were weakly dependent on needle age. Increases in needle linear dimensions have been attributed to needle secondary growth (Gilmore et al. 1995, Niinemets 1997*a*). Secondary growth leads to enhanced needle volume to area ratio, explaining the increase of $M_{A,P}$ and $M_{A,T}$ with increasing needle age in our study. Increases in dry mass per unit area have also been associated with increases in accumulation of cell wall material and stronger lignification of cell walls (Niinemets 1997*a*, Niinemets and Lukjanova 2003). Increased dry to fresh mass ratio in older needles in our study is compatible with greater investment in cell walls (Table 2), but surprisingly, cell wall thickness was occasionally even greater in current-year needles than in older needles (Table 4). However, at a given thickness of cell walls, there might be major differences in the porosity of cell walls (Tosens et al. 2012), e.g., the porosity might decrease due to lignification and stronger cross-linkage of different cell wall matrix polymers in older needles. Among the chemicals having carbon concentration higher than the average (~65% for lignin, 53.5% for proteins and 88% for non-oxygenated terpenoids), lignin comprises a major fraction (e.g., cellulose contains 44% carbon). Thus, increases in leaf carbon content with increasing needle age (Table 2) might suggest a greater lignification of cell walls in older needles, although we cannot rule out increases in terpenoid accumulation. On the other hand, relaxation and expansion of cell walls with moderate addition of new cell wall material during secondary growth could also be involved in changed cell wall thickness, but so far, understanding of the process of needle secondary growth is very limited.

Despite the fact that important age-dependent changes were observed in needle functional traits, needle age-dependent changes were in most cases less pronounced than heteroblastic modifications, with the exception of leaf thickness (Table 1). Nevertheless, heteroblasty and needle aging brought about similar modifications in foliage structural and physiological traits, overall resulting in increasingly sclerophyllous needles with increasing plant and needle age, likely increasing the leaf payback time in mature plants (Day and Greenwood 2011).

Trade-offs between structural and functional investments across plant and needle ages and species

Greater structural robustness in adult needles and especially in older adult needles was associated with lower mesophyll volume fraction, suggesting that being structurally more robust is inevitably associated with a reduced amount of photosynthesizing biomass. This fundamental trade-off operating across species, plant ages and needle ages may be related to the changes in life history characteristics of juvenile versus adult plants during ontogeny. While greater investment in photosynthesizing tissue can enhance growth rates in seedlings and saplings, concomitantly lower foliage mechanical robustness can limit foliage longevity and compromise the tolerance of periods of environmental stress. Indeed, juvenile pine needles have a shorter life span (Mediavilla et al. 2014), and lower drought tolerance (Fernandez et al. 1999, Climent et al. 2006*a*, 2006*b*, Pardos et al. 2009) compared with the adults. There is also evidence

that juvenile needles of some conifer species are less frost resistant (Climent et al. 2009, Pardos et al. 2014), and this can be directly related to less robust foliage with greater elasticity of cell walls (Niinemets 2016). Furthermore, juvenile leaves with lower fiber fractions can be more sensitive to herbivore attacks (Karban and Thaler 1999, Loney et al. 2006). However, this might not always be the case as herbivory sensitivity also depends on differences in investments in secondary protective chemicals as well as leaf surface characteristics (Brennan et al. 2001, Lawrence et al. 2003).

Heteroblasty was the largest source of variation in tissue volume fractions, whereas the tissue distribution between photosynthetic and support components was remarkably similar among juvenile needles of the different species. This is in contrast with the important species differences observed among adult needles (Table 3) as has been observed in other studies (Richardson et al. 2000, Apple et al. 2002, Niinemets et al. 2007). The species ranked as *P. halepensis* > *P. pinea* > *P. nigra* according to the fraction of mechanical tissues in adult needles, while the reverse was the case for mesophyll volume fraction (Table 3). This ranking is in agreement with known distribution of species across climatic gradients, with *P. halepensis* inhabiting the harshest, severely water-limited environments, *P. pinea* intermediate sites and *P. nigra* more mesic habitats, again suggesting that the trade-off between structural and photosynthetic investments can affect species' ecological potentials.

Conclusions

Our study highlights a coordinated change in the combinations of foliage structural and photosynthetic traits upon heteroblastic change and demonstrates that differences among primary and secondary needles are quantitatively much larger than needle age-dependent modifications. Juvenile plants carrying primary needles have less robust foliage with lower volume fractions of mechanical and higher volume fractions of photosynthetic tissues than adult needles. These differences are compatible with lower leaf payback time, and all these traits can contribute to rapid increases in size, contributing to seedling and sapling establishment in the community. Furthermore, the juvenile needles of *P. halepensis* have greater efficiency of light interception due to greater exposed surface area at a given total needle surface area, likely also contributing to plant recruitment under the canopy of adult trees. Greater photosynthetic investments in juvenile needles allow for enhanced carbon gain and establishment within the community, but this might have a cost in terms of reduced leaf life span and in terms of reduced tolerance of environmental stresses such as drought and frost.

Conflict of interest

None declared.

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