Trees
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ORIGINAL ARTICLE

Higher needle anatomic plasticity is related to better water-use efficiency and higher resistance to embolism in fast-growing *Pinus pinaster* families under water scarcity

D. Bert[1] · **G. Le Provost**[1] · **S. Delzon**[2] · **C. Plomion**[1] · **J.-M. Gion**[1,3]

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Abstract

Key Message **Needle anatomic plasticity enables fast-growing maritime pine trees to cope with water limitations by enhancing both water-use efficiency and embolism resistance.**

Abstract Maritime pine is a major forest tree grown for wood production in Southern Europe. A breeding program for the selection of fast-growing varieties was established in the 1960s, in France. In the context of climate change, the magnitude of phenotypic plasticity is a key issue for the prediction of putative decreases in productivity in the improved genetic material. We characterized the phenotypic plasticity of anatomical and physiological traits in two families with contrasting growth rates, under different water regimes, at the juvenile stage. An analysis of 38 traits showed that the fastest growing family had the greatest phenotypic plasticity for morphological, anatomic, chemical and physiological traits, enabling it to increase its water use efficiency and embolism resistance in response to water deficit. The observed modifications to the extravascular (proportion of spongy parenchyma in needles) and vascular (xylem in the needles and stem) compartments in response to water constraints were consistent with a higher water use efficiency and greater embolism resistance. The ability to optimize meresis and auxesis according to environmental conditions during needle development could be related to growth performance over time in different environmental conditions. These results suggest that selection for growth in maritime pine leads to the selection of individuals with greater phenotypic plasticity related to higher performances in non-limited conditions.

Keywords High yield *pinus pinaster* · Phenotypic plasticity · Needle anatomy · Water-use efficiency · Embolism resistance · Drought

Introduction

Phenotypic plasticity, defined as the ability of a genotype to modify its phenotype in response to environmental changes (reviewed by Nicotra et al. 2010), and the genetic variability associated with it are the most important factors characterizing the adaptive potential of populations and/or species (Valladares et al. 2014). Phenotypic plasticity has two components: an environmental component (E), and a second component consisting of the genetic by environment interaction (G × E). This second component is under genetic control and is, therefore, subject to selective pressure. Phenotypic plasticity has been extensively studied in the context of natural selection, and can have various consequences for evolution over ecological time scales (Aitken et al. 2008; Nicotra et al. 2010). Much less is known about the phenotypic plasticity of high-yielding varieties in the context of breeding programs, which involve selection by humans. Ghalambor et al. (2007) distinguished between adaptive plasticity under stabilizing selection and adaptive plasticity under directional selection. This second type of adaptive plasticity is the most common and is likely to result in faster adaptive genetic differentiation. Maritime pine displays high levels of population differentiation and plasticity for growth across its natural geographic distribution (Alia et al. 1995). Artificial

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D. Bert
didier.bert@inrae.fr

[1] INRAE, Univ. Bordeaux, BIOGECO, 33610 Cestas, France

[2] Univ. Bordeaux, INRAE, BIOGECO, 33615 Pessac, France

[3] CIRAD, UMR AGAP, TA A-108/03, Avenue Agropolis, 34398 Montpellier, France

selection in maritime pine breeding can be seen as a type of directional selection (Bouffier et al. 2008), but little is known about the magnitude of phenotypic plasticity in genotypes selected principally on the basis of their growth characteristics.

Maritime pine (*Pinus pinaster* Ait.) has been undergoing domestication over the last few decades, through breeding programs to increase both its growth and the straightness of its stems (Bouffier et al. 2008, 2009a; Mullin et al. 2011; Vidal et al. 2017). Maritime pine is a major high-yielding forest tree species, covering four million hectares in south-western Europe. Most recent plantations of maritime pine in France were established with improved varieties from seed orchards (Trontin et al. 2016). The selection of maritime pine trees in the breeding program has increased the mean breeding value of the population in terms of growth traits, and has also decreased the associated genetic variance by 30% (Bouffier et al. 2008). Wood density also seems to have been slightly affected by selection for growth, as shown by comparisons between the improved population from the breeding program and the natural population (Bouffier et al. 2009b). In maritime pine, selection for growth is performed when the trees are 10–12 years old (Bartholomé et al. 2016), after repeated exposure of the juvenile trees to environmental heterogeneity with the changing seasons. The phenotypic plasticity of improved varieties in the face of environmental heterogeneity remains a major issue for breeders because, in the current context of climate change, the increasing frequency of periods of drought could lead to greater drought-induced forest mortality (Choat et al. 2012) and reduce the productivity of improved varieties.

It has been suggested that physiological, anatomic and morphological plasticities play different roles in plant adaptation to environmental heterogeneity (Gratani et al. 2006; Gratani 2014). In conifers, the needles are the key organs for both carbon assimilation and transpiration. Structural plasticity has been observed in response to environment (Grill et al. 2004; Gebauer et al. 2015) and can result in a greater proportion of mesophyll and a higher photosynthetic capacity in several species (Niinemets et al. 1999, 2007). In maritime pine, the cuticular wax content of the needles of maritime pine seedlings was modified by water stress. More rapidly growing progeny were found to display a stronger plastic response in terms of cuticular wax content, suggesting better adaptation to drought stress (Le Provost et al. 2013). More compartments in the needles of maritime pine might show some plasticity as they depend on two major processes under environmental influences: meresis (cell division to generate additional cells) and auxesis (the expansion of existing cells). In pine species, needle primordia are initiated in buds (through meresis) one year before the growth of new needles (through auxesis) (Kremer and Larson 1982; Kremer 1984; Hover et al. 2017).

The wood tissue also plays a major role in carbon and water fluxes within the tree. Most of the anatomic traits of the xylem vary with the environment, e.g. intra-annual fluctuations of wood density (Vieira et al. 2015; Kurz-Besson et al. 2016; Anjy et al. 2019) or wood tracheid enlargement at lower elevations during droughts (Castagneri et al. 2015). Such anatomical plasticity of key organs should modify embolism resistance, hydraulic conductance, and, by extension, water-use efficiency (WUE). Xylem hydraulic features directly limit drought tolerance in conifers (Larter et al.; 2017) and the ability of the tree to resist hydraulic failure during drought events is under strong genetic control (Lamy et al. 2011, 2014; Plomion et al. 2017).

Early stomatal closure maintains needle water potential homeostasis in maritime pine (Ripullone et al. 2007). This homeostasis is independent of tree age and height (Delzon et al. 2004) but is dependent on the relationship between stomatal conductance and leaf water potential (Klein, 2014). In drought conditions, the maintenance of tree growth depends on WUE, which can be defined as the ratio of biomass accumulation to water loss. In 15-year-old maritime pine, a low G × E interaction was detected for WUE, with moderate broad-sense heritability (0.29), and higher WUE values were not associated with slower growth (Marguerit et al. 2014). At the leaf level, intrinsic WUE can be defined as the ratio of CO_2 assimilation (A) to stomatal conductance (g_s) linked to carbon isotopic composition ($\delta^{13}C$, Ehleringer et al. 1993). Intrinsic WUE may therefore be modified by variations of A, g_s or both, and assessed with stable-carbon isotopes measurements. The phenotypic variation of $\delta^{13}C$ in needles of two-year-old maritime pines has been shown to depend on environment, needle position and season (Marguerit et al. 2014). Studies of the genetic architecture of two adaptive traits, water-use efficiency and vulnerability to embolism, showed that both adaptive traits are under genetic control (Plomion et al. 2017). However, the plasticity of these functional traits, which determine plant responses and genetic adaptation, remains largely unknown for high-yielding maritime pine, particularly as concerns the G × E component.

In this study, we assessed the phenotypic plasticity of major functional traits for carbon source (needles) and sink (wood) organs: vulnerability to embolism, the carbon isotope composition of needles and wood, and needle anatomy. We aimed to decipher the mechanisms involved in the environmental response and water-use efficiency of high-yielding genetic material. We compared the responses to environmental variation of two maritime pine families with contrasting growth rates, over a two-year period and three contrasting water regimes. We analyzed phenotypic plasticity for each trait, at family level, by classical analysis of variance, using phenotypic indices to compare the traits studied. Our findings concerning the phenotypic plasticity of maritime pines will contribute to assessments of the effects of selection for growth in breeding programs on the adaptive potential of improved varieties.

Materials and methods

Genetic material

Within the maritime pine breeding program, the breeding value for growth was estimated for a large number of genotypes from different generations (G0, G1…; Bouffier pers. com.). Two polycross families from Atlantic provenances of *Pinus pinaster* Ait. were selected for study, based on the distribution of the breeding values of 2,216 polycross families for volume at the age of 35 years (Fig. 1). One polycross family displayed low vigor (G0 mother tree 3802 crossed with 30 G0 males), i.e. it had a low breeding value for volume at 35 years ("V− family"). The other polycross family (the "V+ family") was generated by crossing a G1 elite mother tree (1301-5) with 30 G1 elite males. The parents of the V+ family were, therefore, obtained from a first selection cycle for growth (G1 population).

Experimental design

300 individuals from each family were sown in turf plugs in May 2007 and planted in the experimental greenhouse in February 2008. The greenhouse with gutters was 58 m long, 9 m wide, and had four open sides to exclude water supply by rain and keep strong aeration capacities to limit greenhouse effects. It was divided in three areas along its long axis to apply three water regimes (WR). Each of the three areas were sub-divided in three replications allowing to remove seedlings at three different dates within one year. In the replications, plants from the V+ and V− families alternated within the lines and rows, and occupied a 40 × 40 cm square area. The studied areas were surrounded by a buffer of saplings grown from commercially available maritime pine seeds. The buffer zone was 1.6 m wide of the sides of the greenhouse and 3 m wide between water treatments. The soil of in the greenhouse was the local hydromorphic sandy spodosol developed from Quaternary coarse aeolian deposits. The pH is about 4–6 and C/N ratio about 20–30. Particule-size distribution is fairly homogenous with a predominance of coarse sand. As a consequence, forest stands often suffer from excess water in winter and water shortage in summer.

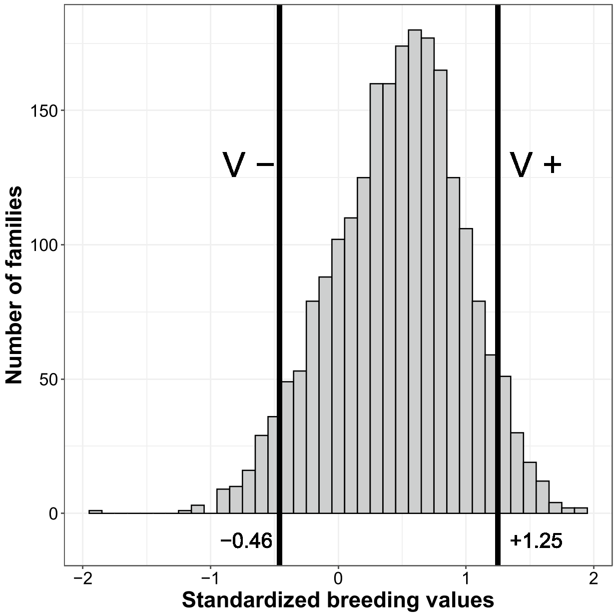

Fig. 1 Distribution of standardized breeding values of 2216 polycross families for volume at 35 years (Bouffier pers. com.), showing the position of the two families (V+, V−) selected for this study

Three water regimes (WR) were applied in an open-sided greenhouse. In the first, the plants were not irrigated (NI) in 2008 and 2009; in the second, they were irrigated with aerial sprinklers over both growing seasons (I), and in the third, they were not irrigated in 2008 and irrigated in 2009 (NI/I). For the I treatment, 560 mm of water were supplied over six months, an amount of water similar to the natural rainfall in the Aquitaine region. Soil water content (SWC, expressed as a %) was monitored with PR2 probes (Delta-T Devices Ltd, Cambridge, UK). Manual measurements were made at depths of 10, 20, 30, 40, 60 and 100 cm. Greenhouse air temperature and relative humidity were also monitored, with four weatherproof data loggers (Hobo H08-032–08, Onset Computer Corporation Co, Bourne, USA) located within the three treatment plots and outside the greenhouse. An overview of the trial is provided, in pictures, in additional file 5-Figure S4 in Le Provost et al. 2013.

Measurement of traits

In addition to the classical growth traits described by Le Provost et al. (2013), we measured 38 variables corresponding to the anatomic and chemical traits of the carbon source organ (the needles) and integrative functional traits of both the carbon source and the carbon sink (wood) (Table 1).

Needle anatomy

Pinus pinaster is a two-needled pine, which means that the needles are semi-circular in cross-section, with a flat adaxial side (internal side), and a convex abaxial side (external side) (Fig. 2). Each needle is surrounded by a cuticle secreted by the epidermis and punctuated by stomata, on both sides. The cuticle provides the needle with physical protection and prevents excessive water loss. The epidermis consists of two

Table 1 List of the studied variables: ID for each trait, definition and unit

Trait category	Trait ID	Explanation	Unit
Growth	H	Total tree height at the end of the second year of experiment	cm
	D	Basal tree diameter at the end of the second year of experiment	mm
	V	Volume at the end of the second year of experiment	cm^3
	B	Total biomass at the end of the second year of experiment	g
Anatomy	$\mathrm{St_n}$	Stomata number in a 1280 µm × 960 µm needle area	counts
	N_w	Needle cross-section width	µm
	N_t	Needle cross-section thickness	µm
	N_a	Total cross-section area	mm^2
	$\mathrm{Ep_{\%}}$	Proportion of the upper and lower epidermis	%
	$\mathrm{Ep_n}$	Number of cells in epidermis	counts
	$\mathrm{En_{\%}}$	Proportion of the upper and lower endodermis	%
	$\mathrm{En_n}$	Number of cells in endodermis	counts
	$\mathrm{CC_a}$	Central cylinder area	µm^2
	$T_{\%}$	Proportion of the transfusion tissue	%
	A_a	Assimilating tissue area	µm^2
	$\mathrm{Pa_{\%}}$	Proportion of the upper and lower palisade parenchyma	%
	$\mathrm{Sp_{\%}}$	Proportion of the upper and lower spongy parenchyma	%
	Pa/Sp	Ratio between palisade and spongy parenchyma	–
	$\mathrm{VB_a}$	Vascular bundles total area	µm^2
	$\mathrm{VB_{\%}}$	Proportion of the vascular bundles in the central cylinder	%
	$\mathrm{Ph_a}$	Total phloem area	µm^2
	$\mathrm{Ph_{\%CC}}$	Proportion of phloem in the central cylinder	%
	$\mathrm{Ph_{\%VB}}$	Proportion of phloem in the vascular bundles	%
	$\mathrm{Xy_a}$	Total xylem area	µm^2
	$\mathrm{Xy_{\%CC}}$	Proportion of xylem in the central cylinder	%
	$\mathrm{Xy_{\%VB}}$	Proportion of xylem in the vascular bundles	%
	V/A	Ratio between vascular and assimilating areas	–
	$\mathrm{Xy_n}$	Number of xylem cells	counts
	$\mathrm{Xy_{ca}}$	Average area of a xylem cell	µm^2
Chemicals	Chl *a*	Chlorophyll *a* concentration (in fresh weight)	µg g^{-1}
	Chl *b*	Chlorophyll *b* concentration (in fresh weight)	µg g^{-1}
	Chl *a*/Chl *b*	Ratio between Chlorophyll a and Chlorophyll b	–
	ChT	Chlorophyll a + Chlorophyll b concentration (in g of fresh weight)	µg g^{-1}
	Car	Carotenoids concentration (in fresh weight)	µg g^{-1}
	FS	Free sugar concentration (in dry weight)	mg g^{-1}
	ST	Starch concentration (in dry weight)	mg g^{-1}
Functional traits	Ks	Xylem-specific hydraulic conductivity	m^2 MPa^{-1} s^{-1}
	$\mathrm{P_{50}}$	Xylem pressure inducing a 50% loss of hydraulic conductivity	MPa
	$\mathrm{P_{88}}$	Xylem pressure inducing a 88% loss of hydraulic conductivity	MPa
	$\mathrm{W\delta^{13}C}$	Carbon isotopic composition in holocellulose extracted from wood	‰
	$\mathrm{N\delta^{13}C}$	Carbon isotopic composition in needles	‰
	N	Total nitrogen content (in percentage of dry mass)	%

or three layers of cells with some sclerenchyma. The mesophyll lies under the epidermis and consists of two layers, the palisade parenchyma, which is responsible for most of the photosynthesis occurring in the needle, and the spongy parenchyma, which exchanges gases with the intercellular spaces. Two or more resin ducts are embedded in the spongy parenchyma. The endodermis consists of large cells with many pit fields on the tangential walls for the control of water flux from the xylem to the stomata against an osmotic gradient. The transfusion tissue surrounds the vascular bundles and consists of dead tracheids and living parenchyma cells. Two fluxes operate in opposite directions across the

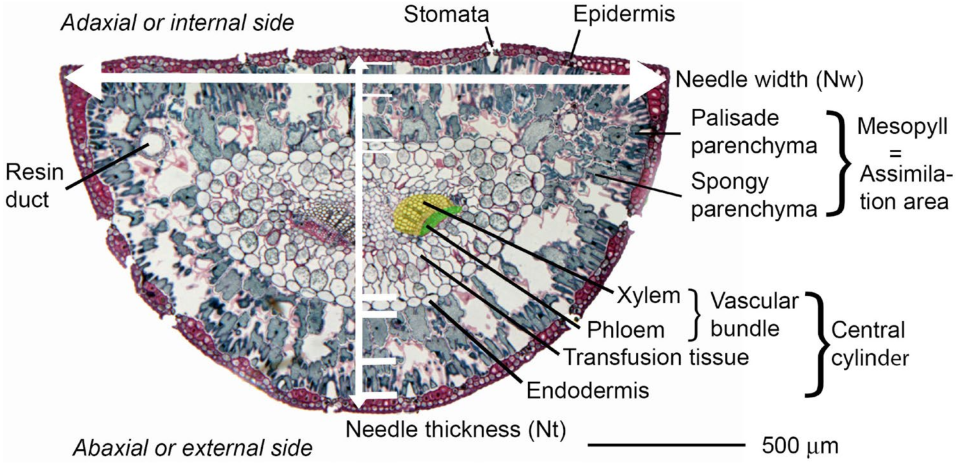

Fig. 2 Transverse section of a pine needle stained with PAS-NBB. The white arrows indicate needle width (N_w) and needle thickness (N_t). The white features along the thickness axis indicate the measurements made to determine the thickness of each tissue, on both the internal (flat) and external (convex) sides. On this figure, the xylem has been shaded in yellow and the phloem in green for the right vascular bundle

transfusion tissue: water moves out of the xylem tracheids to supply the palisade and for the purposes of transpiration, and sugars enter the phloem from the parenchyma cells. In the vascular bundles, the fusiform cambium differentiates into xylem towards the adaxial (flat) side of the needle, and phloem on the other side. In summary, there are two meresis processes in the needle related to primary and secondary meristems: (1) the meresis of the primary meristem done within buds one year before the elongation of the needle, (2) the meresis of the secondary meristem (cambium) initiated during the year of growth. If the cambium layer divides into xylem and phloem the year *n*, the primary meristem divided into extravascular components the year *n*−1.

Anatomic and histological measurements were made on needles sampled in September, 2009. We sampled first year needles from 10 seedlings per family and per treatment. We determined the number of stomata on a first set of fresh needles, corresponding to three needles per seedling and eight seedlings per family for two treatments (NI and I). We used transparent nail varnish and tape to obtain impressions of the adaxial surfaces of the needles, as described by Ceulemans et al. (1995). These impressions were observed with an Olympus BM2 RFCA light microscope (Olympus America Inc., Melville, New York, USA), at 100× magnification, corresponding to a needle area of 1229 mm^2 (1280 µm × 960 µm). We calculated the mean number of stomata for each seedling and used this value for further analyses. We placed a second set of fresh needles in hardening fixer (FAA glutaraldehyde-paraformaldehyde-caffeine). The needles were dehydrated with ethanol followed by butanol, impregnated with resin and cut into 3.5 µm-thick sections with a rotating microtome (RM2255 Leica Biosystems, Nussloch, Germany). We used Periodic acid-Schiff and naphthol blue black staining to dye the polysaccharides pink, and the proteins and nuclei blue. Sections cut from the middle portion of the needle with a microtome were examined under a light microscope (DM4500B Leica Biosystems) and photographed (Retiga 2000R QImaging Corp., Burnaby, Canada). We analyzed the images for one needle per seedling with ImageJ software (National Institutes of Health, Bethesda, USA), at a magnification of 50×–200×. For each transverse needle section, we measured the thickness of each tissue along an axis running from the adaxial side to the abaxial side of the needle (Table 1; Fig. 2). Total needle area was estimated from needle thickness and needle width, assuming a semi-circular shape. For determination of the proportions of the various tissues, the absolute dimensions on the thickness axis were converted into percentages of needle thickness, to take the differences in size between needles into account. The area of the cross-section used for assimilation (A_a) was calculated as the area of the needle below the epidermis minus the area of the central cylinder (CCa). *V*/*A* is the relationship between supply and assimilation tissues (Grill et al. 2004).

Needle chemical content

Chlorophyll and carotenoid contents Sampling occurred in September 2009 for each treatment. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoid (Car) contents were quantified for three biological replicates. Each biological replicate was obtained by bulking needles harvested from three individuals per treatment, as described by Le Provost et al. (2013). Chlorophyll and carotenoid contents were then assessed as described by Minocha et al. (2009). Briefly, needles were cut into 0.3 cm-long pieces with a scalpel blade, and were then incubated overnight in 15 ml dimethyl formamide at 4 °C. The liquid around the needles was then mixed with a magnetic stirrer to ensure an even distribution of compounds. The Chl *a*, Chl *b* and Car contents of the liquid were determined spectrophotometrically, as described by Minocha et al. (2009). The Chl *a*/Chl *b* ratio and total chlorophyll content (ChT) were also analyzed.

Non-structural carbohydrates (NSC) In total, 32 needle samples were used for the determination of free soluble sugars (FS) and starch (ST) for each family, for the I and NI treatments, in September 2009. The needles were collected at 2 p.m., frozen in liquid nitrogen and stored at − 80 °C. They were freeze-dried for six days and then ground into a fine powder (GenoGrinder model 2010). A first aliquot of this powder was then dried by heating at 103 °C in an oven for 24 h, to estimate needle dry weight as a proportion of the powdered samples. Another powder sample (weighing about 50 mg) was subjected to three successive extractions in ethanol/water (ratios of 80/20, 50/50 and 20/80) at 80 °C for 30 min each, followed by centrifugation to obtain a pellet and a supernatant. The supernatant was removed with a pipette, dried under vacuum (Speedvac Thermo SPD111V) and dissolved in 1 ml distilled water. The starch of the pellet was hydrolyzed by incubation with 1 ml 0.02 N NaOH at 90 °C for 30 min. The hydrolysate was dried (Speedvac) and dissolved in 0.5 ml water. The concentrations of both soluble sugars and sugars obtained from starch were measured by spectrocolorimetry (Spectro Libra S22 at 560 nm) after reaction with anthrone in sulfuric acid at 80 °C for 20 min (method adapted from Bachelier and Gavinelli 1966). Pure sucrose at concentrations of 0.25 to 2 g l^{-1} was used for calibration. FS and ST concentrations are expressed in mg g^{-1} dried needle.

Stem xylem vulnerability to embolism

Measurements were performed on the main stems of five to seven seedlings per family and per treatment during the fall of 2009. Each stem sample comprised two rings. Transpiration losses were prevented by removing the needles immediately after sampling and wrapping the segment in moist paper to keep it damp. The segments were stored at 5 °C until the measurement of embolism resistance. Susceptibility to drought-induced embolism was determined by the Cavitron technique (see Cochard et al. 2005 for technical details). The bark was removed to prevent resin contamination, and all stem segments were cut with a razor blade, under water, to a standard length of 0.27 m. The stem segments were then inserted into a custom-built honeycomb rotor attached to a centrifuge (see Delzon et al. 2010 for protocol details). The rotor was first spun at low xylem pressure ($P = -0.8$ MPa), and the speed was then gradually increased to expose the sample to a more negative xylem pressure (P_i). Hydraulic conductance (K_i, m^2 MPa^{-1} s^{-1}) was determined by injecting the reference ionic solution of 10 mM KCl and 1 mM $CaCl_2$ in deionized ultrapure water. Vulnerability curves show the percentage loss of xylem conductance as a function of xylem pressure. For each stem, the relationship between PLC and xylem pressure was fitted with the following sigmoidal equation (Pammenter and Vander Willigen 1998):

$$\mathrm{PLC} = \frac{100}{1 + \exp\left(\frac{S}{25(P_i - P_{50})}\right)}$$

where P_{50} (MPa) is the xylem pressure inducing a 50% loss of conductivity and S (% MPa^{-1}) is the slope of the vulnerability curve at the inflection point. The mean embolism vulnerability parameters (P_{50} and S) correspond to the mean values for three to seven samples per family and per treatment. The xylem-specific hydraulic conductivity (K_s, m^2 MPa^{-1} s^{-1}) was calculated by dividing the maximum hydraulic conductivity at low speed by the xylem area of the sample. We also estimated the pressure resulting in an 88% loss of hydraulic conductivity (P_{88}), as described by Urli et al. (2013).

Carbon stable isotopic composition

The stable carbon isotope composition was assessed in two different plant compartments: the mature needles, as a source of plant carbon, and the holocellulose from wood, as a plant carbon sink. Isotopic composition was determined on 10 seedlings per family and per treatment sampled in September 2009. For each seedling, a 3 cm-long piece of the 2009 stem growth unit was sampled, together with several needles. The pith of the wood cross-sections was removed with a 3.5 mm-diameter drill, and the bark was peeled off. The remaining wood was ground to a powder (particles of 0.5 mm diameter; Retsch ZM100 mill) and some of the wood powder was treated for the chemical purification of holocellulose (Leavitt and Danzer 1993). The needles were also ground (GenoGrinder model 2010). Finally, three replicates of 1 mg for wood holocellulose and needle powder were placed in a tin capsule for mass spectrometry. The ratio of stable carbon isotopes and the total nitrogen content (% of dry mass) were then measured with an elementary analyzer (Eurovector EA2000) and a mass spectrometer (Isoprime Elementar). Carbon isotope composition, expressed as $\delta^{13}C$ in ‰ (Craig 1957), was then converted into carbon discrimination $\Delta^{13}C$ in ‰ (Farquhar and Richards 1984). $\Delta^{13}C$ is related to the ratio between CO_2 assimilation (A) and stomatal conductance (g_s), also known as intrinsic water-use efficiency (iWUE) (Ehleringer et al. 1993). The iWUE increases with increasing $\delta^{13}C$.

Statistical analysis

The data for each trait were analyzed with the following fixed-effects model:

$$y_{ijk} = \mu + G_i + \mathrm{WR}_j + G_i \times \mathrm{WR}_j + \varepsilon_{ijk}$$

where μ is the overall mean of the variable, y_{ijk} is the value of the variable for the kth seedling within the ith family

(V− and V+) and the jth water treatment (water regimes NI, I, and NI/I), G_i is the effect of the ith family, WR_j is the effect of the jth water treatment, $G_i \times WR_j$ is the interaction between family and water treatment, and $\varepsilon_{\mathrm{ijk}}$ is the residual. Multiple comparisons of means were performed, with Bonferroni correction and a global significance threshold of 5%. Two plasticity indices (PI_1 and PI_2) were calculated separately for the V− and V+ families:

$$\mathrm{PI}_1(\%) = 100 \times \frac{\overline{y}_{\mathrm{NI}} - \overline{y}_{\mathrm{I}}}{\overline{y}_{\mathrm{I}}}$$

and

$$\mathrm{PI}_2(\%) = 100 \times \frac{\overline{y}_{\mathrm{NI/I}} - \overline{y}_{\mathrm{NI}}}{\overline{y}_{\mathrm{NI}}}$$

where $\overline{y}_{\mathrm{I}}$ is the mean of the variable y for the I treatment, $\overline{y}_{\mathrm{NI}}$ is the mean for the NI treatment and $\overline{y}_{\mathrm{NI/I}}$ is the mean for the NI/I treatment. PI values express the relative plasticity due to water supply for each trait and each family. A negative value for PI indicates that the variable had smaller values in the NI or NI/I treatments for PI_1 or PI_2, respectively. The data were analyzed with XLSTAT 19.01 software (Addinsoft, New York, USA).

Results

All the variables presented in this section are summarized in Table 1.

Effect of drought on growth

As expected, SWC values were highest for the I treatment throughout the study period. SWC values were similar for the NI and NI/I treatments in 2008 and were higher for NI/I than for NI in 2009 (Fig. 3). The SWC values were generally in the usual range for a sandy spodosol of this type, with a permanent wilting point at 5%, field capacity at 15%, and a saturation point at 30% (Saxton et al. 1986). The accuracy of water treatments was checked by determining predawn water potential (as described by Le Provost et al. 2013). The non-irrigated treatment was found to be subject to moderate water stress. Under the I treatment, predawn water potential remained above − 0.4 MPa, in both study years. By contrast, the seedlings subjected to the NI treatment had significantly lower values of predawn water potential during the summer, ranging from − 0.7 to − 1 MPa in September 2008 and from − 0.9 to − 1.5 MPa in September 2009.

The plants of the V+ family were significantly taller (164 cm) than those of the V− family (134 cm), and their biomass production was higher (616 g for V+ and 507 g for V−) (Table 2). A significant genetic effect was detected for primary growth (H) and biomass production (B). The WR effect was significant for all growth variables. Higher plasticity indices were obtained for the V + family than for the V− family, reflecting the better performance of the V+ family in the absence of limitations (I and NI/I).

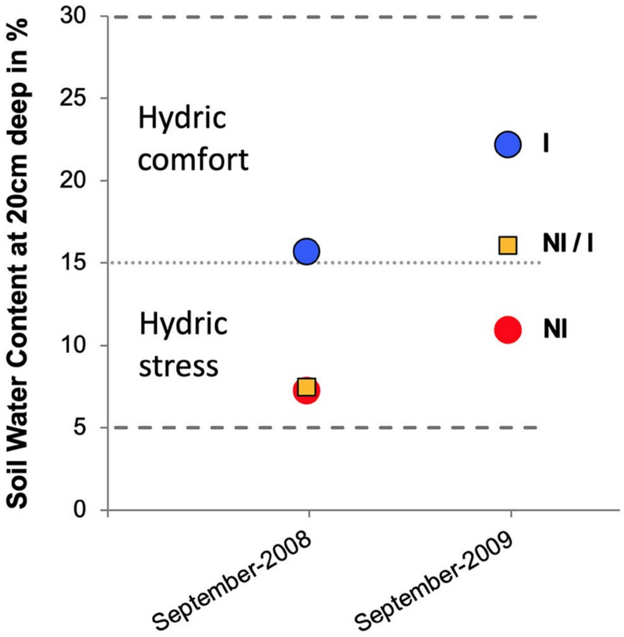

Fig. 3 Soil water content at a depth of 20 cm (%) in the three irrigation regimes (I, NI and NI/I) at the end of September 2008 and September 2009. The dotted lines show the saturation point of the soil (30%), the field capacity (15%), and the permanent wilting point (5%) in a sandy soil

Needle morphology and anatomy

The width (N_w), thickness (N_t) and total area (N_a) of the needle cross-section were all significantly greater for the I and NI/I treatments than for the NI treatment for both the V + and V− families (Table 3). Differences in needle size were associated with a significant effect of treatment on the number of epidermal cells (Ep_n). Mean Ep_n was lowest for NI (540 cells) and NI/I (596 cells), and highest for I (713 cells). This result suggested an effect of the first year of water deficit (NI and NI/I) on the number of epidermal cells generated by meresis (cell multiplication).

The assimilation area (A_a) of the needle section (i.e. the mesophyll tissue) also displayed a WR effect, covering a much larger surface in I (476,893 μm^2) and NI/I (442,217 μm^2) plants than in NI (278,638 μm^2) plants. The similar A_a values for the I and NI/I treatments, for both families (V+ an V−), may be due to the similar watering regimes applied during the second year. A significant WR effect was also observed for the percentage of spongy parenchyma ($Sp_{\%}$), with a higher $Sp_{\%}$ in NI (32%) and NI/I (30%) plants than in I (25%) plants, demonstrating an effect of the first year

Table 2 Results of the two-way variance analysis for growth and functional traits

	Growth				Vulnerability to embolism			iWUE		
Parameter	*H*	*D*	*V*	*B*	Ks	P$_{50}$	P$_{88}$	Wδ^{13}C	Nδ^{13}C	*N*
Genetic unit (G)										
V+	164	2.6	370	616	6.30E−04	− 3.870	− 4.612	− 26.57	− 27.70	0.886
V−	134	2.6	307	507	6.37E−04	− 3.764	− 4.484	− 26.98	− 27.86	0.887
Watering regime (WR)										
I	159	2.9	432	676	6.20E−04	− 3.752	− 4.479	− 27.38	− 28.88	0.899
NI	143	2.4	274	424	5.68E−04	− 4.053	− 4.842	− 26.05	− 26.06	0.932
NI/I	145	2.5	310	584	7.14E−04	− 3.645	− 4.323	− 26.89	− 28.40	0.828
G × WR interaction										
V+										
I	184	2.9	506	780	6.77E−04	− 3.740	− 4.434	− 27.08	− 29.04	0.871
NI	149	2.2	254	415	5.46E−04	− 4.241	− 5.131	− 25.77	− 25.43	0.996
NI/I	160	2.6	351	653	6.68E−04	− 3.627	− 4.273	− 26.86	− 28.63	0.792
V−										
I	134	2.9	358	572	5.63E−04	− 3.763	− 4.525	− 27.68	− 28.72	0.928
NI	137	2.5	294	433	5.90E−04	− 3.866	− 4.553	− 26.33	− 26.68	0.868
NI/I	131	2.5	270	516	7.59E−04	− 3.664	− 4.373	− 26.92	− 28.17	0.864
PI$_1$ (I− > NI)										
V+	− 19	− 23	− 50	− 47		− 13	− 16	5	12	14
V−	2	− 11	− 18	− 24		− 3	− 1	5	7	− 6
PI$_2$ (NI− > NI/I)										
V+	7	17	38	57		14	17	− 4	− 13	− 20
V−	− 4	− 2	− 8	19		5	4	− 2	− 6	0
Two way ANOVA (*p* values)										
Model	**< 0.0001**	**0.009**	**0.000**	**< 0.0001**	0.506	**0.002**	**0.003**	**< 0.0001**	**< 0.0001**	**0.021**
G	**< 0.0001**	0.642	0.063	**0.004**		0.241	0.297	**< 0.0001**	0.108	0.999
WR	**0.015**	**0.002**	**0.001**	**< 0.0001**		**0.001**	**0.003**	**< 0.0001**	**< 0.0001**	**0.025**
G × WR	**0.010**	0.250	0.072	**0.045**		0.100	**0.038**	**0.004**	**< 0.0001**	**0.032**

Bold in the tables indicates a probability lower than 0.05, i.e. a significant effect of the parameter at the 5% threshold

The mean value of the two main factors (family G, and watering regime WR = environmental part E) and their interaction (G × E) are given, as well as the two plasticity indexes (Pl$_1$ and Pl$_2$)

of water deficit on Sp$_{\%}$. This trend was more pronounced in the V+ family (PI$_1$ = 38% and PI$_2$ = -10%) than in the V− family (PI$_1$ = 16% and PI$_2$ = 1%). A significant genetic effect was observed for the percentage palisade parenchyma (Pa$_{\%}$), in which was lower in the V+ family (13%) than in the V− family (16%). A significant G × WR interaction was also detected for Pa$_{\%}$, with opposite trends for the two plasticity indices: from I to NI (PI$_1$ = − 12% in V+, and PI$_1$ = 12% in V−) and from NI to NI/I (PI$_2$ = 38% in V+, and PI$_2$ = − 7% in V−). Finally, the Pa/Sp ratio revealed the existence of a significant genetic effect, mostly due to the higher plasticity indices of the V+ family (PI$_1$ = − 38% and PI$_2$ = 55%) than the V− family (PI$_1$ = − 6% and PI$_2$ = − 7%). Thus, the V+ family had the highest plasticity indices (PI$_1$) for both the percentage of spongy parenchyma (38%) and Pa/Sp ratio (-38%). Surprisingly, no effect was detected for the number of endodermal cells (En$_n$) or for the proportion of endodermal cells (En$_{\%}$) (Table 3), despite the significant WR effect on the transfusion tissue ($T_{\%}$), the proportion of which was higher for the I treatment (44%) than for the other two treatments (37% for both NI and NI/I). We also detected a WR effect on the area of the central cylinder (CC$_a$), which was large for the I treatment (503,612 µm^2), intermediate for the NI/I treatment (351,374 µm^2) and small for the NI treatment (225,924 µm^2), for both families.

Overall, these results suggest that water deprivation during the first year (NI and NI/I treatments) altered needle anatomy by affecting meresis, whereas water supply in the second year (I and NI/I) mostly affected auxesis.

Needle vascular bundles

Within the central cylinder (Table 4), a significant effect of WR on the total area of the two vascular bundles (VB$_a$) was

Table 3 Results of the two-way variance analysis for the traits related to needle anatomy

Parameter	St_n	Needle anatomy												
		N_w	N_t	N_a	$Ep_{\%}$	Ep_n	A_a	$Pa_{\%}$	$Sp_{\%}$	Pa/Sp	$En_{\%}$	En_n	CC_a	$T_{\%}$
Genetic unit (G)														
V+	40	1638	999	1.084	10	621	391,910	13	29	0.45	7	44	376,697	41
V−	40	1694	998	1.142	10	611	406,589	16	28	0.58	7	42	343,910	38
Watering regime (WR)														
I	39	1789	1085	1.270	10	713	476,893	14	25	0.57	7	43	503,612	44
NI	42	1423	848	0.812	10	540	278,638	14	32	0.45	8	44	225,924	37
NI/I	40	1786	1062	1.257	10	596	442,217	15	30	0.52	7	43	351,374	37
G × WR interaction														
V+														
I	40	1806	1097	1.291	10	729	486,033	12	24	0.53	7	45	545,300	47
NI	41	1328	832	0.714	9	540	258,602	11	34	0.33	8	44	232,619	38
NI/I	40	1782	1069	1.248	11	595	431,095	15	30	0.50	7	44	352,174	37
V−														
I	39	1773	1074	1.249	10	696	467,754	15	26	0.62	7	41	461,925	42
NI	42	1518	864	0.911	11	540	298,673	17	30	0.58	7	43	219,229	36
NI/I	40	1791	1056	1.266	10	597	453,339	16	30	0.54	7	42	350,575	37
PI_1 (I− > NI)														
V+		− 26	− 24	− 45		− 26	− 47	− 12	38	− 38			− 57	− 18
V−		− 14	− 20	− 27		− 22	− 36	12	16	− 6			− 53	− 15
PI_2 (NI− > NI/I)														
V+		34	28	75		10	67	38	− 10	55			51	− 4
V−		18	22	39		10	52	− 7	1	− 7			60	5
Two way ANOVA (*p* values)														
Model	0.121	**0.000**	**< 0.0001**	**0.001**	0.847	**0.001**	**0.000**	**0.000**	**0.014**	**0.028**	0.680	0.876	**< 0.0001**	**0.000**
G		0.351	0.970	0.449		0.728	0.774	**< 0.0001**	0.504	**0.008**			0.347	0.110
WR		**< 0.0001**	**< 0.0001**	**< 0.0001**		**0.000**	**< 0.0001**	0.078	**0.002**	0.100			**< 0.0001**	**0.000**
G × WR		0.283	0.729	0.419		0.857	0.445	**0.009**	0.330	0.135			0.559	0.298

Bold in the tables indicates a probability lower than 0.05, i.e. a significant effect of the parameter at the 5% threshold

The mean value of the two main factors (family G, and watering regime WR) and their interaction (G × WR) are given, as well as the two plasticity indexes (Pl_1 and Pl_2)

Table 4 Results of the two-way variance analysis for the trait related to conducting tissues

	Conducting tissue										
Parameter	VB_a	$VB_{\%}$	Ph_a	$Ph_{\%CC}$	$Ph_{\%VB}$	Xy_a	$Xy_{\%CC}$	$Xy_{\%VB}$	Xy_n	Xy_{Ca}	*V/A*
Genetic unit (G)											
V+	34,993	9.2	11,031	3.0	33	23,962	6.2	67	160	148	0.09
V−	29,833	8.6	10,718	3.2	37	19,115	5.4	63	154	120	0.07
Watering regime (WR)											
I	45,755	8.8	14,599	2.8	33	31,156	6.0	67	201	158	0.10
NI	20,814	9.2	8286	3.7	40	12,529	5.5	60	114	110	0.07
NI/I	30,671	8.8	9740	2.8	32	20,931	6.0	68	158	134	0.07
G × WR interaction											
V+											
I	51,383	9.4	15,305	2.8	30	36,078	6.6	70	218	176	0.11
NI	21,353	9.1	7828	3.3	36	13,525	5.8	64	113	121	0.08
NI/I	32,244	9.2	9961	2.8	32	22,283	6.3	68	150	147	0.07
V−											
I	40,127	8.2	13,892	2.9	36	26,235	5.3	64	183	140	0.09
NI	20,276	9.3	8744	4.0	44	11,533	5.3	56	114	99	0.07
NI/I	29,097	8.4	9519	2.7	33	19,578	5.6	67	165	122	0.07
PI_1 (I− > NI)											
V+	− 58		− 49	19	21	− 63		− 9	− 48	− 31	
V−	− 49		− 37	41	24	− 56		− 13	− 37	− 29	
PI_2 (NI- > NI/I)											
V+	51		27	− 15	− 13	65		7	33	22	
V−	44		9	− 32	− 26	70		21	45	23	
Two way ANOVA (*p* values)											
Model	**0.003**	0.858	**0.031**	**0.034**	**< 0.0001**	**0.001**	0.538	**< 0.0001**	**0.018**	**0.014**	0.260
G	0.268		0.832	0.419	**0.004**	0.139		**0.004**	0.741	**0.032**	
WR	**0.000**		**0.003**	**0.018**	**0.000**	**0.000**		**0.000**	**0.003**	**0.011**	
G × WR	0.618		0.809	0.446	0.216	0.525		0.216	0.499	0.877	

Bold in the tables indicates a probability lower than 0.05, i.e. a significant effect of the parameter at the 5% threshold

The mean value of the two main factors (family G, and watering regime WR) and their interaction (G × WR) are given, as well as the two plasticity indexes (Pl_1 and Pl_2)

detected (45,755 μm^2 in I, 30,671 μm^2 in NI/I, and 20,814 μm^2 in NI). The pattern was consistent with that for the CC_a, phloem (Ph_a) and xylem (Xy_a) areas. Interestingly, no significant effect was observed for the proportion of the central cylinder occupied by the vascular bundle ($VB_{\%}$). However, we observed genetic and WR effects for the proportions of both phloem ($Ph_{\%VB}$) and xylem ($Xy_{\%VB}$) in the vascular bundle, with $Xy_{\%VB}$ slightly higher in the V+ family (67%) than in the V− family (63%), and in the I (67%) and NI/I (68%) water regimes than for NI (60%). A WR effect was also observed on the number of xylem cells (Xy_n), with more cells in I (201 cells), than in NI/I (158) and NI (114). Finally, genetic and WR effects were significant for the mean xylem cell area (Xy_{Ca}), which was higher in the V + family (148 μm^2) than the V− family (120 μm^2), and for the I treatment (158 μm^2) than for the NI treatment (110 μm^2). In summary, the vascular bundles had more xylem cells and the cells were larger in the V + family than in the V− family, for the I and NI water regimes, suggesting that water conductivity was higher in the needles of the V + family than in those of the V− family.

Needle chemical contents

A significant WR effect was observed for Chl *a*, Chl *b* and Chl *a*/Chl *b* ratio (Table 5). Chl *a* and Chl *b* were lower for the NI/I treatment than for the I and NI treatments, for both families. There was no significant difference between the I and NI treatments. A similar trend was observed for Car, with lower concentrations for the NI/I treatment than for the other two treatments. The significant G × WR interaction for Car can be accounted for by the highest Car PI_1 value in the V− family (25%) and the lowest PI_2 in the V+ family (− 54%).

Table 5 Results of the two-way variance analysis for needle chemicals

	Needle chemicals						
Parameter	Chl *a*	Chl *b*	Chl *a*/Chl *b*	ChT	Car	FS	ST
Genetic unit (G)							
V+	277	73	3.81	350	70	42.9	21.8
V−	251	65	3.93	316	62	46.3	23.5
Watering regime (WR)							
I	296	77	3.88	373	74	43.1	21.7
NI	310	84	3.70	393	79	46.2	23.5
NI/I	186	46	4.02	232	45		
G × WR interaction							
V+							
I	342	89	3.86	431	88	38.8	21.1
NI	325	88	3.69	414	83	47.0	22.5
NI/I	163	42	3.88	205	38		
V−							
I	250	65	3.90	314	60	47.3	22.3
NI	294	79	3.70	373	75	45.3	24.6
NI/I	209	50	4.17	259	52		
PI$_1$ (I−>NI)							
V+	− 5	− 1	− 4	− 4	− 6	21	7
V−	18	23	− 5	19	25	− 4	10
PI$_2$ (NI−>NI/I)							
V+	− 50	− 52	5	− 51	− 54		
V−	− 29	− 37	13	− 31	− 31		
Two way ANOVA (*p* values)							
Model	**0.003**	**0.004**	**0.013**	**0.003**	**0.000**	0.058	**0.002**
G	0.233	0.209	0.104	0.227	0.129		**0.014**
WR	**0.001**	**0.001**	**0.005**	**0.001**	**< 0.0001**		**0.008**
G × WR	0.055	0.150	0.203	0.070	**0.010**		0.532

Bold in the tables indicates a probability lower than 0.05, i.e. a significant effect of the parameter at the 5% threshold

The mean value of the two main factors (family G, and watering regime WR) and their interaction (G × WR) are given, as well as the two plasticity indexes (Pl$_1$ and Pl$_2$)

No significant effect was detected for free sugar (FS), although concentrations were lower in the V+ family for the I treatment (38.8 mg g^{-1}) for the other family x treatment combinations (V+ NI = 47.0 mg g^{-1}, V− I = 47.3 mg g^{-1}, and V− NI = 45.3 mg g^{-1}). Both family and treatment significantly affected starch content (ST). In V + family needles, mean ST concentrations were 21.1 mg g^{-1} for I and 22.5 mg g^{-1} for NI. In V− family needles, ST concentrations were higher, at 22.3 mg g^{-1} (I) and 24.6 mg g^{-1} (NI). The WR effect was similar in the two families, with ST values lowest in the absence of water restriction. The observed trends for both FS and ST suggest higher levels of non-structural carbohydrate (FS + ST) mobilization in the carbon source (needles) in the V+ family than in the V− family, particularly in the I environment, which was the most favorable for growth (Fig. 4).

Vulnerability to embolism and iWUE

A significant effect of treatment (WR) was detected for vulnerability to embolism ($P_{50;}$ Fig. 5), mostly due to the higher embolism resistance (low P_{50} value) of the V+ family under the NI treatment ($P_{50} = -4.241$ MPa) and the pressure at which 88% of hydraulic conductivity was lost ($P_{88} = -5.131$ MPa). Higher plasticity indices were obtained for the V + family for P_{50} (PI$_1$ = − 13% and PI$_2$ = 14%) and P_{88} (PI$_1$ = − 16% and PI$_2$ = 17%), than for the V− family for P_{50} (PI$_1$ = − 3% and PI$_2$ = 5%) and P_{88} (PI$_1$ = − 1% and PI$_2$ = 4%). No difference in xylem-specific hydraulic conductivity (K_s) was observed between water regimes (Table 2). The more negative P_{50} values for the V+ family in drought conditions cannot therefore result from a bias due to native embolism induced by the drought treatment. Indeed, the

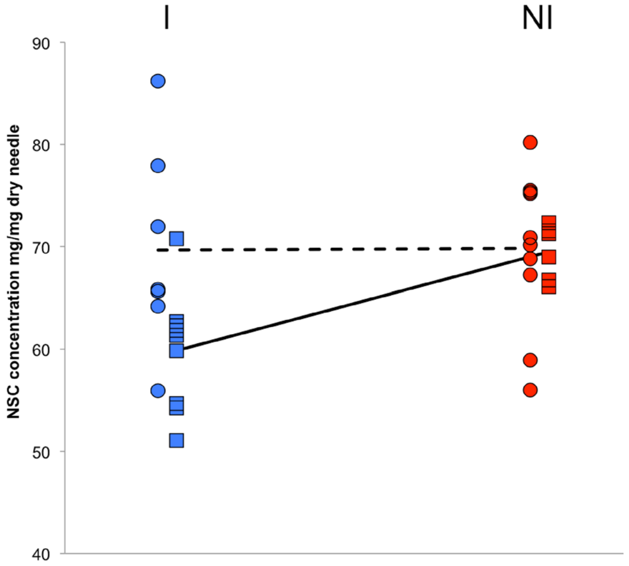

Fig. 4 Non-structural carbohydrate (NSC) concentration (FS + ST) in the needles. Each data point is the sum of the concentrations of free sugars and starch for the same needle. The circles and dotted line are for the V− family and the squares and solid line are for the V+ family. The lines join the mean values for each group

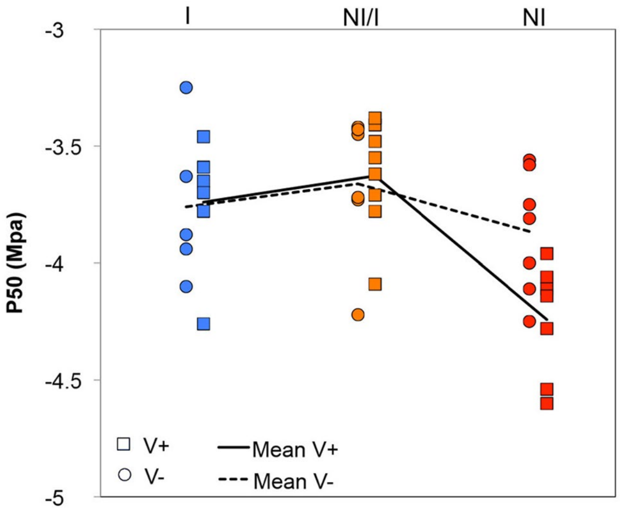

Fig. 5 Resistance to cavitation, as measured by P_{50}. P_{50} is the xylem pressure corresponding to a 50% loss of hydraulic conductivity, i.e. cavitation resistance. Individual measurements (dots) and means (lines) for the three water treatments (I, NI/I, NI) and two levels of vigor (V− and V+)

presence of native embolism would have reduced hydraulic conductivity and biased our vulnerability curves assessment, which is clearly not the case in this study.

Wood holocellulose δ^{13}C (Wδ^{13}C) differed significantly between families and water treatments, and there was a significant G × WR interaction (Table 2; Fig. 6a, b), with slightly higher values for the V+ family (Wδ^{13}C = − 26.57‰) than for the V− family (Wδ^{13}C = − 26.98‰). The G × WR effect was explained by the high Wδ^{13}C values observed for V+ in NI conditions (− 25.77‰) together with the higher Wδ^{13}C PI_2 for the V+ family (− 4%) than for the V− family (− 2%). For the NI/I treatment, mean Wδ^{13}C was close to that for the I treatment as the wood sampled was exclusively from the last year of the study (corresponding to irrigated water regimes for I and NI/I). Interestingly, needle Nδ^{13}C displayed significant treatment and G × WR effects, but no genetic effect. The G × WR effect for Nδ^{13}C was conditioned by the NI treatment and, more precisely, by the higher plasticity indices of the V+ family (PI_1 = 12%, and PI_2 = − 13%) than for the V− family (PI_1 = 7% and PI_2 = − 6%). These differences in iWUE between families (i.e. higher δ^{13}C plasticity of the V + family for both wood and needles) were not due to a difference in stomatal density (St_n), as St_n did not differ significantly between families or water regimes.

Both WR and G × WR had significant effects on total nitrogen content (*N*). These effects were mostly due to the N content of the V + family under the NI water regime, revealing a possible higher photosynthetic capacity potentially related to the observed higher iWUE.

Discussion

We describe here a multi-scale study of phenotypic plasticity in two maritime pine families with different growth performances. We found that broad-sense phenotypic plasticity was higher in the V+ family than in the V− family at the age of three years, suggesting a better ability to cope with environmental heterogeneity before the age at which selection took place. An anatomic investigation of needles from the two families highlighted differences in the plasticity of functional traits relating to water use of relevance for growth performance.

In forest trees, phenotypic plasticity is generally investigated in seedlings growing in small containers (Aranda et al. 2010; Sanchez-Gomez et al. 2010; de la Mata et al. 2014), making it difficult to generalize the results. The experimental design of this study made it possible to study the effects of water restriction on pine seedlings growing in their natural environment (ambient atmosphere, natural soil and light) for two growing seasons. As reported by Le Provost et al. (2013), height growth was affected by the end of the experiment, particularly in the V+ family, which displayed the greatest primary growth regardless of water regime. Moreover, the significant effect of water regime on the needle cuticle suggested that this more vigorous family had better drought tolerance. In the NI/I regime, in which water was limited during the first year but the trees were irrigated in the second, we were able to take the temporal heterogeneity

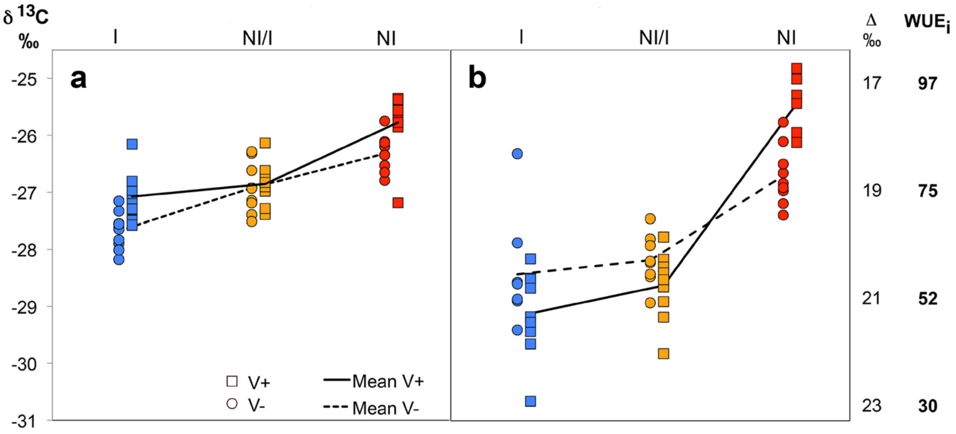

Fig. 6 Carbon isotope composition of wood (**a**) and needles (**b**) from 2009. Each data point represents the mean of three replicates and gives the δ^{13}C for a single seedling. The circles and dotted line are for the V− family, and the squares and solid line are for the V+ family. The lines join the mean values for each group. On the right, the *y* axis is labeled with two different linear-transformed units: the plant discrimination Δ (‰), the intrinsic water-use efficiency *WUE$_i$* in mmol CO_2/mol H_2O. These variables are for both graphs, a and b. The abscissa for each treatment has been shifted slightly to improve the visualization of the data points

of the environment into account and to highlight the roles of two major processes in needle organogenesis: meresis and auxesis. After two years of experimentation, the pine seedlings had different sizes depending on the treatment and the family. For some anatomical variables, these differences were taken into account by standardizing their value according to the size of the needle studied (see the section on "Materials and methods"). The concentration variables of the compounds analyzed (NSC, chlorophyll, carbon isotopes, nitrogen, etc.) were expressed as a percentage of dry weight. Finally, the embolism resistance variables take into account the dimensions of each sample analyzed at the time of measurement. These precautions greatly limited any possible effect of plant size on the analyses. Therefore, this study provides new insight by considering the anatomic plasticity of needles together with more integrative traits of both needles and stems, such as biomass production, mechanistic traits relating to drought resistance, and water use. The fastest growing of the two families displayed a higher degree of anatomic plasticity, for both needles and stems, and the greatest functional plasticity for water-related traits.

Anatomic plasticity of needles and iWUE

One of the classic effects of drought in pines is a decrease in total needle length, as shown in cohorts of *Pinus canariensis* (Grill et al. 2004). This quantitative plasticity has also been detected in mature *Pinus laricio,* with needle lengths 30, 19 and 29% shorter in trees subjected to drought than in control trees over a period of three years (Cinnirella et al. 2002). Plasticity for needle thickness has been less widely documented, but follows a pattern similar to that for needle length (Grill et al. 2004; Wahid et al. 2006). These changes in the needle morphology of maritime pine seem to be related to variation of needle histology and anatomy, in terms of tissue organization and proportions, and cell number and size (Fig. 7). The study of the anatomy of pine needles requires many steps that limit the number of individuals studied. In our study, the initial 10 individuals per treatment, year and family provided usable measurements on 4–10 individuals, and more often 7–9. This number of individuals therefore made it possible to show only the most significant effects and interactions. In particular, the division of the mesophyll into palisade and spongy parenchyma in the needles studied here suggested: (1) differences in the structural organization of needles in response to water regime during both needle initiation (meresis, year 1) and needle elongation (auxesis, year 2), and (2) a higher efficiency of the photosynthetic parenchyma (Pa) in the most vigorous family (V+). The hydraulic resistance of the extravascular compartment is similar to that of the vascular compartment, although this relationship varies between species and environmental conditions (Zwieniecki et al. 2002; Sack et al. 2005; Nardini and Salleo 2006). Following a decline in leaf water status, leaf hydraulic conductance (K_{leaf}) decreases, mostly due to a decrease in extravascular leaf hydraulic conductance ($K_{\text{extravascular}}$) (Charra-Vaskou et al. 2012; Scoffoni et al. 2015; Bouche et al. 2016) rather than a decrease in leaf vascular hydraulic conductance (K_{vascular}). The plasticity of the mesophyll and transfusion tissue thickness (i.e. the distance from the vascular conduits to the stomatal subcavity) may determine $K_{\text{extravascular}}$ (Aasamaa et al. 2001; Sack et al. 2003; Brodribb et al. 2007), as variation of the spongy parenchyma may modify g_m, leading to changes in K_{leaf}. In *Pinus taeda*, Domec et al. (2015) reported that high CO_2 levels decreased the number of tracheids per needle and increased

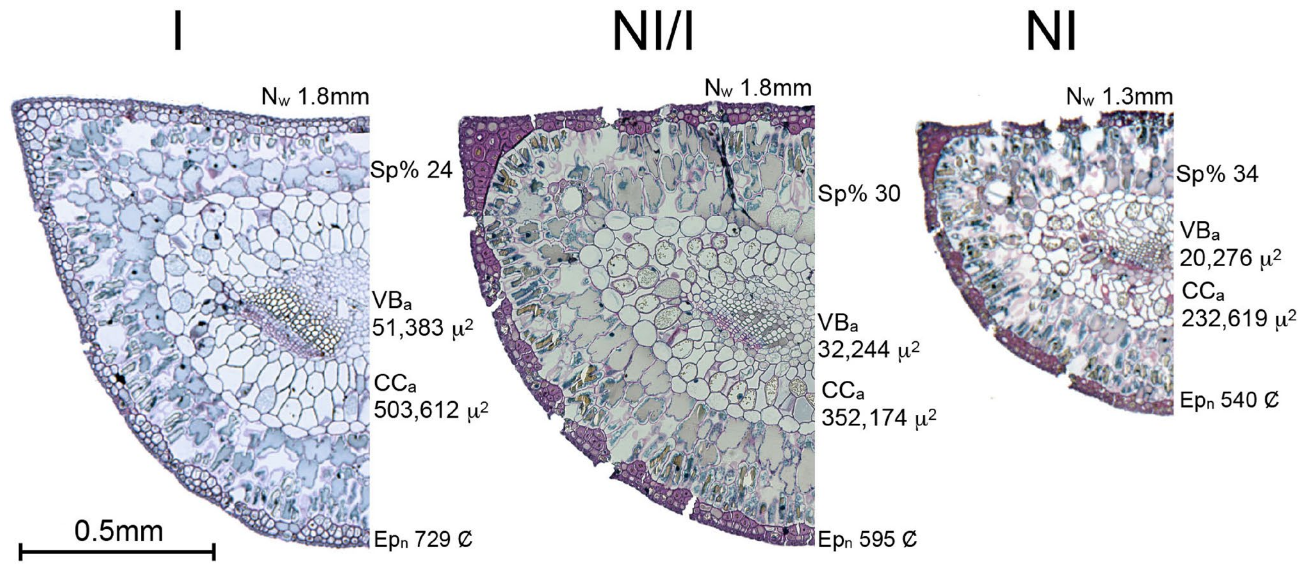

Fig. 7 Needle anatomy of the V+ family, with the mean values of some variables (see Table 1 for variable description) for three water regimes applied over 2 years: I (irrigation in both years), NI/I (no water during the first year, irrigation in the second), NI (no irrigation during both years). Periodic acid-Schiff and naphtol blue-black staining (polysaccharides in pink and proteins and nuclei in blue)

the distance from the vascular bundle to the stomatal cavities, thereby altering the leaf hydraulic system and putatively increasing resistance to xylem collapse. Hydraulic conductance outside the xylem has been shown to be strongly influenced by the proximity of the central cylinder to the lower epidermis, and by spongy mesophyll anatomy (Buckley et al. 2015). In the conditions of water restriction studied here, the increase in the proportion of spongy parenchyma, together with a smaller central cylinder area, probably affected K_{leaf}, and may also have contributed to the highest needle iWUE (Nδ^{13}C) in family V+ during two years of water restriction. Furthermore, the higher percentage of nitrogen found in the leaves of plants from the V+ family in these conditions suggests a higher photosynthetic capacity that may improve iWUE by increasing A. The iWUE depends on the variation of both A and g_s (Ehleringer et al. 1993), which are closely linked to mesophyll CO$_2$ conductance (g_m) in diverse species (Flexas et al. 2013). Our results reveal a high degree of plasticity in the proportion of spongy parenchyma in the V+ family, suggesting a major role of the extravascular compartment (mesophyll, endodermis and transfusion tissue) in the iWUE plasticity of needles.

In both families, a comparison of wood and needles revealed the expected higher level of Wδ^{13}C (holocellulose) than of Nδ^{13}C (Leavitt and Long 1986). This difference results mostly from discrimination during respiration, releasing 30–50% of the carbon fixed by photosynthesis (Ryan 1991). The V+ family had a higher iWUE for wood, and for needles, in dry conditions. These differences in iWUE between the two families can be explained partly by NSC production and NSC consumption. We hypothesized that NSC might accumulate in needles due to lower consumption rates during drought (Fig. 4), consistent with the lower biomass production. The range of NSC concentrations in the needles during the drought period was consistent with the results of previous studies on pine seedlings (Guehl et al. 1993) and mature pines (Gruber et al. 2012). As Nδ^{13}C corresponds to a mixture of structural and rapid-turnover carbon (Brendel 2001; Ogée et al. 2009), its value would be expected to change if NSC accumulated. A previous study showed that the sugars in the sap of maritime pine have δ^{13}C values close to $-$ 26.5‰ (Devaux et al. 2009), consistent with the Nδ^{13}C values presented here (Fig. 6). NSC concentration decreased in the needles of the V+ family during the growing period, due to higher rates of NSC consumption. This higher NSC consumption is consistent with the higher levels of biomass production of this family and its lower iWUE in needles under well-watered conditions.

Anatomic plasticity of needles and embolism resistance

Hydraulic conductivity is determined by the vascular compartment of the needles. We found that the number of xylem cells was highest in the absence of water limitation over 2 years (I). Irrigation during the second year increased the percentage of xylem in the vascular bundle similarly for the I and NI/I treatments. Thus, water supply determined the properties of the vascular compartment by influencing both meresis and auxesis in the secondary meristem (cambium) over the two years of this study. Water limitation over the two years of the study (NI) affected the secondary meristem similarly in the two families, resulting in a smaller vascular bundles area, a smaller number of xylem cells and a smaller xylem cell size. These trends observed for the vascular compartment in both families suggest greater resistance to drought, because xylem tubes of smaller diameter are less likely to collapse when water potential reaches very low levels in the needles (McCulloh et al. 2014). Moreover, the change in xylem cell size may partly counterbalance the loss of area, because xylem efficiency scales to the fourth power of conduit diameter, according to the Hagen-Poiseuille law (Tyree and Zimmerman 2002; Corcuera et al. 2011). The proportion of the vascular cylinder occupied by xylem was larger in the V+ family, in which the xylem cells were also larger, suggesting that xylem efficiency was higher in this family than in the V− family. Embolism resistance, estimated by determining the pressure inducing a 50% loss of xylem hydraulic conductivity (P_{50}), is strongly associated with drought stress resistance in conifers (Brodribb and Cochard 2009; Brodribb et al. 2010). In our study, stem xylem resistance to embolism was highest (− 4.2 MPa) for the V+ family in dry conditions. Phenotypic plasticity, albeit of only small magnitude (less than 0.3 MPs) has already been reported for embolism resistance in natural populations of maritime pine (Lamy et al. 2014. The observed P_{50} of − 4.2 MPa for the V+ family is the highest value reported to date for *Pinus pinaster*. The hypothesis of vulnerability segmentation between the needle xylem and the stem xylem does not seem to apply to maritime pine, because P_{50} values are similar in these two organs (Bouche et al. 2016). This suggests that the P_{50} values of needle xylem in the V+ family are likely to be similar to those of the stem xylem, resulting in better resistance to embolism in dry conditions. Susceptibility to embolism is driven purely by pit membrane structure and function (Delzon et al. 2010; Pittermann et al. 2010; Bouche et al. 2014), suggesting that the pit membrane may undergo anatomic changes in dry conditions in the V+ family. Similar findings have been reported for several provenances of *Pinus halepensis,* with an overlap between torus and pit aperture sizes (David-Schwartz et al. 2016). In *Pinus pinaster*, the plasticity of both the vascular and extravascular compartments of the needles may play a major role in hydraulic failure, as the collapse of needle tracheids may prevent or delay xylem cavitation (Cochard et al. 2004; McCulloh et al. 2014).

The anatomic plasticity of needles links resistance to embolism and iWUE

Conifer stems are generally more resistant to embolism than those of angiosperms. P_{50} values vary widely within coniferous taxa (− 2.1 to − 18.8 MPa; Maherali et al. 2004; Delzon et al. 2010; Pittermann et al. 2010; Larter et al. 2017). The highest xylem resistance to embolism observed in this study (− 4.2 MPa) was associated with the highest iWUE value, for both the needles (Nδ^{13}C) and wood (Wδ^{13}C), in the V+ family subjected to two years of water restriction. A positive relationship between xylem resistance to embolism and WUE has already been reported in populations of *Pinus sylvestris* L. (Martínez-Vilalta et al. 2009). However, a negative relationship has also been observed in a natural population of maritime pine (Lamy et al. 2011), suggesting that susceptibility to stem embolism and iWUE may evolve independently in response to environmental changes (Guet et al. 2015). A trade-off between xylem resistance to embolism and xylem hydraulic efficiency has been reported (Pockman and Sperry 2000; Martínez-Vilalta et al. 2002; Guet et al. 2015), but remains controversial (Fan et al. 2011). It cannot, therefore, be generalized to different species of woody plants (Maherali et al. 2004; Lopez et al. 2013; Gleason et al. 2016). Intraspecific comparisons of populations with different evolutionary drivers can also make functional interpretation difficult (Kavanagh et al. 1999; Maherali and DeLucia 2000; Martínez-Vilalta et al. 2009; Corcuera et al. 2011; Lamy et al. 2012; Sterck et al. 2012). In our study, no plasticity was observed for xylem conductivity in the stem (K_s), and xylem resistance to embolism was higher in dry conditions only in the V + family. Xylem hydraulic conductivity (or xylem efficiency) is determined by the number and size of tracheids in the wood of conifer trees (Domec et al. 2009), and by the characteristics of bordered pit membranes (Domec and Gartner 2002; Pittermann et al. 2010; Jansen et al. 2012), whereas susceptibility to embolism (xylem safety) is driven exclusively by the structure and function of the pit membrane (Delzon et al. 2010; Pittermann et al. 2010; Bouche et al. 2014). In addition, water transport outside the leaf xylem is strongly determined by different components of the extravascular compartment (Buckley et al. 2015). Our results for maritime pine suggest that anatomic plasticity trends observed in both the needle compartments (vascular and extravascular) in response to drought may account for the observed positive relationship between water-use efficiency and resistance to embolism.

Growth performance and embolism resistance have been reported to be unrelated in natural pine populations (Martínez-Vilalta et al. 2009; Lamy et al. 2011; Sterck et al. 2012; Lopez et al. 2013), but, to our knowledge, no such analysis has been performed specifically for high-yielding maritime pines. This study overcomes this gap, by showing that the most vigorous of the two families considered was more resistant to embolism under dry conditions (lower P_{50} and P_{88}), thanks to a higher phenotypic plasticity. A positive relationship between growth and embolism resistance has already been reported for poplar hybrids subjected to different water regimes (Fichot et al. 2010). Our results showed that mean embolism resistance was higher only for the regime in which the saplings experienced water limitation during two consecutive years. Similar findings have been reported for interspecific comparisons (Maherali et al 2004; Larter et al. 2017; Pockman and Sperry 2000; Choat et al. 2012). Indeed, P_{50} trends follow the minimum seasonal xylem water potential experienced in situ (Ψ_{xmin}), so species experiencing a low Ψ_{xmin} are generally more resistant to embolism (Choat et al. 2012; Urli et al. 2013; Brodribb et al. 2010). Lopez et al. (2016) reported similar results for natural populations of *Pinus canariensis,* for which plasticity in terms of susceptibility to embolism was observed only in the driest provenance trials. The phenotypic plasticity of the V+ family in terms of the anatomic traits of the vascular and extravascular compartments of needles potentially underlying the variation of water-use functional traits, may enable fast-growing maritime pines to cope with the environmental heterogeneity of successive periods of water availability and limitation.

From the anatomic plasticity of needles to growth performance

In this study, the recovery treatment (NI/I) revealed an effect of the temporal heterogeneity of the environment on the anatomic plasticity of needles. We can hypothesize that the environmental conditions of the two years affected needle plasticity differently in the two families, changing the growth kinetics of needles during their initiation and elongation phases. Cellular plasticity may be affected by two major processes: (1) cell multiplication (meresis), and (2) cell enlargement (auxesis). In maritime pine, ontogenetic and environmental effects affect the growth cycle (Kremer and Roussel 1982). The growth cycle begins with the initiation of needles within buds. This initiation phase involves both meresis and auxesis and occurs after bud flush, but before needle elongation (Kremer and Roussel 1982). The environment has two successive effects on the vascular and extravascular compartments of the needle, affecting: (1) the establishment of cell types in the needle through meresis, and (2) elongation through auxesis. Finally, the anatomic plasticity of the needles in the most vigorous family was characterized by a greater sensitivity of meresis and auxesis to environmental conditions or a greater ability of each phase to adjust to a specific environment.

Conclusion

Our findings suggest that growth performance and phenotypic plasticity are related in maritime pine. This analysis of the anatomic plasticity of needles sheds light on the variations of functional traits related to plant hydraulics, and their relationships. In conditions of water limitation, the V+ family display a greater degree of needle anatomic plasticity, associated with a higher water-use efficiency and greater resistance to embolism. Both the extravascular and vascular compartments are involved in the response to water deficit, through: (1) changes to the proportions of spongy and assimilation parenchyma, modifying mesophyll conductance and, by extension, the water-use efficiency of needles; (2) changes to the vascular compartment (xylem in the needles and stem) rendering the embolism more resistant. Our observations of the anatomic responses of needles to drought revealed different effects on meresis and auxesis during needle development. This finding is particularly relevant to the initial formation of needles within the buds of maritime pine. The ability to develop organs with anatomic properties combining better water use efficiency with embolism resistance may be a major advantage, enabling genotypes to achieve better growth performances mainly under favorable conditions.

This phenotypic plasticity study on maritime pine could be extended as follows: (1) enlargement of the analysis to a larger number of families within each breeding population generation, (2) monitoring of phenotypic plasticity until the age at which selection takes place (12 years old), (3) improvements to the characterization of the anatomy of needles and wood (e.g. cell size, cell walls, pits). The results of this study are encouraging, suggesting that high-yielding maritime pine families should be able to cope with water limitation during the growing period, thanks to anatomic plasticity. Anatomic approaches could be used to decipher the responses of maritime pines to the environment and to improve the prediction of adaptation for the products of breeding programs.

Author contribution statement JMG and DB initiated the study. DB collected and prepared the samples, and analyzed the data. DB and JMG wrote the manuscript. SD, GLP and CP revised and contributed to the manuscript.

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Data availability The datasets generated and analyzed during the current study are available in the DATAINRA repository. https://data.inrae.fr/dataset.xhtml?persistentId=doi:10.15454/N8GLMY

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval The authors fully follow the guidelines of the journal Trees.

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