

Phenotypic plasticity in *Pinus pinaster* $\delta^{13}\text{C}$: environment modulates genetic variation

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Abstract

- Carbon isotope composition ($\delta^{13}\text{C}$) is a complex trait involved in acclimation, adaptive processes and related to water use efficiency (WUE) and/or productivity.
- To estimate the genetic variation in $\delta^{13}\text{C}$ and growth (h), their relationship, and the genotype by environment interaction effect in both variables, we analyzed three *Pinus pinaster* populations and six to ten families per population, in two experimental trial sites and in two consecutive years.
- $\delta^{13}\text{C}$ increased in the drier site (from -27.15 ± 0.18 to -24.53 ± 0.22) and was more affected by environment (62% of variance) than by genotype.
- All populations and families exhibited a high phenotypic plasticity in $\delta^{13}\text{C}$ and increased WUE in the xeric site.
- As expected, significant height differences between sites, years, populations and families were displayed. Smaller trees were associated to higher water use efficiency ($\delta^{13}\text{C}$) in both, mesic and xeric trial sites.
- Aridity and continentality enhanced population differences in $\delta^{13}\text{C}$, therefore, in afforestation programs of arid and continental zones, the selection at the population level would be more efficient than at the family level. In the mesic site, the presence of a high genetic variation in $\delta^{13}\text{C}$ and h between families allows the possibility of a selection for growth and water use efficiency within populations in sub-humid sites.

1. INTRODUCTION

The Mediterranean conifer *Pinus pinaster* Ait. grows under contrasting water availabilities: from oceanic (south France) to arid conditions (south Spain and north Africa) and expands over more than 4 million ha. The species displays a high level of population differentiation and plasticity throughout its natural geographical distribution in growth (Alia et al., 1995) and physiological parameters related to drought resistance (Fernandez et al., 1999).

Plant drought responses involve many drought-related traits. Stomatal regulation plays a key role when water vapor pressure increases, but results in lower photosynthetic rates. Intrinsic water-use efficiency (WUE) or the ratio of total dry matter production to the total amount of transpiration during a certain carbon fixation period (Fischer and Turner, 1978), is a complex trait sometimes associated to the superior performance of genotypes in drought-prone environments (Sun et al., 1996), although there are evidences of the contrary (Guehl et al., 1995, Lauteri et al., 1997). WUE in maritime pine may vary with both, the environment (Nguyen-Queyrens

et al., 1998) and the genetics of the species (Brendel et al., 2002).

Measurements of WUE have been simplified by the discovery of a strong and positive correlation between WUE and stable carbon isotope composition ($\delta^{13}\text{C}$). $\delta^{13}\text{C}$ shows discrimination against the heavy isotope of CO_2 (C^{13}O_2) during photosynthesis, and is negatively correlated with intercellular CO_2 concentration (C_i) and stable carbon isotope discrimination (δ , Farquhar et al., 1989). δ is the ratio $(\delta_a - \delta_p) / (1 - \delta_p)$, where δ_p is the isotopic composition of the plant material and δ_a is that of the air. In *P. pinaster* it was validated that $\delta^{13}\text{C}$ may be used as a surrogate for WUE (Fernandez et al., 2006), is linked to other hydraulic traits in *Pinaceae* (Martinez-Villalta et al., 2004) and might have higher heritability than other measures of WUE (McKay et al., 2003). On the other hand, the trait shows a remarkable environmental influence, and consequently needs to be tested in a wide range of environments with a rigorous environmental control, and the complexity of the trait, which involve several functional candidate gene types, might complicate validation of genotype/phenotype associations for WUE (Gonzalez et al., 2008).

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Adaptive responses to environmental changes depend on species (Zhang and Clegg, 1996). A lack of population differentiation in physiological traits like net photosynthesis (A), stomatal conductance (g) and δ indicates genetic stability in these characters and a high level of plasticity of gas exchange (Zhang and Marshall, 1995). It supports physiological plasticity as the primary mechanism that allows species to grow in a range of environments. However, population differences in δ provided evidence of genotypic differences between populations in *P. pinaster* (Nguyen-Queyrens et al., 1998). In seasonally dry climates, *P. pinaster* foliar $\delta^{13}\text{C}$ can be used as a drought stress indicator due to its strong correlation with predawn water potential (Warren et al., 2001). For the application of carbon isotope indexes in selection programs, it would be appropriate to obtain a relationship between variation in $\delta^{13}\text{C}$ and growth on the field, in order to use $\delta^{13}\text{C}$ as a surrogate for WUE and improve WUE or/and productivity. Intraspecific variability in growth traits (Harfouche, 2003), $\delta^{13}\text{C}$, and their relationship (Aranda et al., 2010) have been previously reported in *Pinus pinaster*.

A high WUE is the result of the stomatal conductance regulation via a high net photosynthesis, a low transpiration, or both (Faquhar et al., 1989). Then, growth and $\delta^{13}\text{C}$ may be positively, negatively, or no correlated, depending on the main physiological process regulating stomatal performance. If variation in discrimination is primarily controlled by differences in carboxylation efficiency rather than stomatal conductance (Flanagan and Johnsen, 1995), a strong and negative genetic correlation between δ and growth indicates that photosynthetic differences are the main factor of genetic variation in δ .

Johnson et al. (1999) observed a high δ heritability and lower environmental sensitiveness than growth and suggested δ as a suitable trait for indirect selection in *Picea mariana* families. However, in *P. pinaster*, a significant but moderate heritability was found for $\delta^{13}\text{C}$ and ring width and the phenotypic correlation between them was attributable to environmental components (Brendel et al., 2002). This was corroborated by Aranda et al. (2010), who also found phenotypic plasticity in δ in response to different drought-stress levels in *P. pinaster* seedlings at the population and family level. In addition, the relationship between $\delta^{13}\text{C}$ and growth depends on environmental conditions. A negative correlation between δ and growth was found in *Picea mariana* families on a dry site, while no significant correlation was observed on sites where water availability was higher (Flanagan and Johnsen, 1995). In *P. pinaster* provenance trials with high precipitation regimes, no correlation (Nguyen-Queyrens et al., 1998) or a negative correlation between growth performances and δ values were found for all provenances in one of several trial sites (Tognetti et al., 2000). In a single trial site, the relationship between $\delta^{13}\text{C}$ and growth showed different patterns depending on the origin of the maritime pine populations. Low $\delta^{13}\text{C}$ values were associated to low height in a Mediterranean population and to high height in the Atlantic populations (Correia et al., 2008).

Previous studies in *P. pinaster* obtained $\delta^{13}\text{C}$ variation at a population level (Correia et al., 2008), family level (Fernandez et al., 2006), and both, population and family level (Aranda

et al., 2010). We analyzed the intra-specific variability of $\delta^{13}\text{C}$ at the inter- and intra- population levels. As environmental heterogeneity is attributed to differences in place and time, various locations and periods are necessary to get an estimate of spatial and temporal phenotypic plasticity and adaptive genetic variation in WUE (estimated by $\delta^{13}\text{C}$) and growth. We examined the environmental and genetic variation in several *P. pinaster* families from three populations, in two provenance-progeny trials that vary in productivity due to contrasting altitudes and precipitation regimes, during two consecutive years. Our objectives were: (i) to assess the variability in growth and $\delta^{13}\text{C}$ at a population and family level, (ii) to investigate the relationship between $\delta^{13}\text{C}$ and growth and (iii) to determine whether variation in $\delta^{13}\text{C}$ was associated to the geographic variation in water availability of the seed sources (adaptation), (iv) or to the water availability in the trial sites (plasticity).

2. MATERIALS AND METHODS

2.1. Study site and plant material

The study was conducted during late summer of 2005 (2-year old plants) and 2006 (3-year old plants) in two maritime pine provenance-progeny tests. The trials were established using seedlings grown in nursery from open-pollinated seed collected in natural stands of maritime pine. Provenance progeny trials were located in Parderrubias, NW Spain, at a low elevation, near the Atlantic ocean, exposed to wet and mild winters (hereinafter, mesic site, *Me*) and Calcena, NE Spain, at an interior mid-high elevation, with a continental climate and colder and drier winters (hereinafter, xeric site, *Xe*). Both sites undergo summer drought (Fig. 1). Besides drought, other environmental factors could have influenced $\delta^{13}\text{C}$ in maritime pine, like nutrient availability (Fernández et al., 2006). However, soil fertility analysis revealed no differences between sites.

In site *Me* two-year old seedlings were planted in 2005 at a spacing of 3 m \times 2 m in a randomized complete block design with 4 replications of 71 blocks, 225 families, 4 plants per experimental unit (a total of 16 plants per family). In site *Xe* one-year old seedlings were planted in 2004 at a spacing of 2 m \times 3 m in an α -lattice incomplete block design with 3 replications of 65 blocks, 8 families by block and 4 plants by experimental unit. Both trials were designed in a nested structure of families within populations. The three populations were selected in order to cover a wide range of temperature, precipitation and altitude (Arenas de San Pedro (A), interior, central Spain; Oria (O), south-east coast of Spain; and Mimizan (M), south-west coast of France, Landes). Location and climatic data for the progeny trials and seed sources are presented in Table I.

Sampling: selection of populations, number of families per population and individuals per family were performed depending on the availability of plant material at *Xe* site, due to low survival. Number of families: A (10), O (8) and M (6). In *Me*, due to the high percentage of survival (close to 100%), we selected 10 families per population at random, having preference for the families sampled at *Xe*. From 4 to 8 individuals within families were measured in both sites.

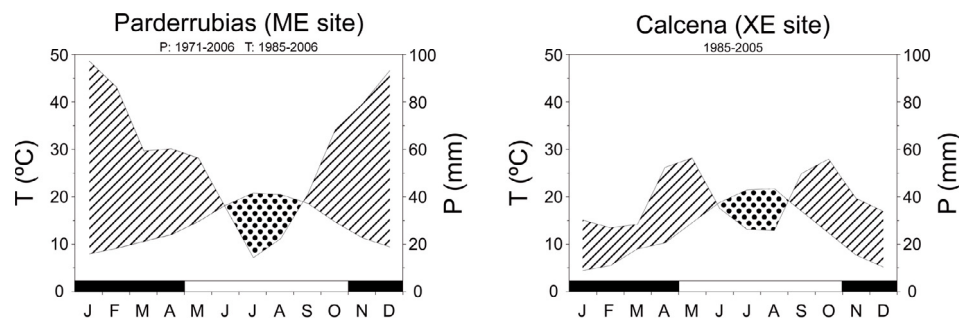


Figure 1. Climate in the study area according to the ombrothermogram of the nearby Allariz (A) and Aranda de Moncayo (B) meteorological stations. Precipitation information: dotted area, precipitation < 2 temperature (dry period); striped area, precipitation > 2 temperature (humid season). Temperature information at bottom: lower black block, period with probable frost (absolute monthly minimum temperature ≤ 0 °C and mean minimum temperature of the coldest month ≥ 0 °C); lower white block, frost-free months, i.e. mean minimum temperature of the coldest month ≥ 0 °C.

Table I. Location and climatic data of the progeny trials and seed sources. P: Mean annual precipitation; T: Mean annual temperature; TM: Mean of maximum/mean temperature, in month with highest mean; Tm: Mean of minimum/mean temperature, in month with lowest mean. Xe: xeric, Me: mesic.

| | M | A | O | Site Me | Site Xe |
|---------------|-----------|----------------------|-----------|-----------------|--------------------------------|
| Locality | Mimizan | Arenas de San Pedro | Oria | Parderrubias | Calcena |
| Latitude (N) | 44° 8' | 40° 30' | 37° 30' | 42° 14' | 41° 37' |
| Longitude (W) | 1° 10' | 4° 24' | 2° 20' | 7° 56' | 1° 44' |
| Elevation (m) | 37 | 1 359 | 1 232 | 460 | 1 017 |
| P (mm) | 995 | 957 | 348 | 722 | 461 |
| T (°C) | 13 | 14.6 | 14.4 | 14.4 | 12.3 |
| TM (°C) | 25.0/21.2 | 34.2/24.4 | 30.0/22.9 | 27.8/20.5 | 28.6/22.8 |
| Tm (°C) | 3.0/6.3 | 0.28/4.8 | 3.0/3.9 | 1.5/5.9 | 1.1/4.3 |
| Soil | Sandy | Leached on sandstone | Limestone | Sandy siliceous | Siliceous, over slate outcrops |

2.2. Carbon isotope discrimination

Needle samples were collected at both provenance-progeny trials at the end of September 2005 (1st year of measurements) and September 2006 (2nd year of measurements) from the top one-third of the crown on each of the trees used for growth measurements. The samples were oven-dried at 70 °C for 72 h and finely ground to pass a 40-mesh screen. The abundance of stable isotopes of carbon in combusted samples were performed using a mass spectrometer at the Stable Isotope Lab, INSTAAR, University of Colorado, Boulder. Stable carbon isotope ratios were calculated as $\delta^{13}\text{C} = R_{\text{sample}}/R_{\text{standard}} - 1$, where R is the molar ratio of heavy to light isotope ($^{13}\text{C}/^{12}\text{C}$). All values were reported in per mil and relative to PDB, Pee Dee Belemnite limestone, the international standard (Craig, 1957).

2.3. Statistical analysis

Following the experimental design and assuming that the residuals were independent and normally distributed (0, V_e),

the following model was established for both variables (h and $\delta^{13}\text{C}$):

$$y_{ijklrnb} = \mu + p_i + f_{j(i)} + y_k + s_l + \eta_{(rxl)} + \beta_{b(rxl)} + \varepsilon_{ijklrnb}$$

where,

$y_{ijklrnb}$ is the value of the variable for the n th seedling from the j th family within i th population, measured the k th year at the l th site located in m th block within the n th replication;

μ is the overall mean of the variable;

p_i is the effect of the i th population ($i = 1-3$);

$f_{j(i)}$ is the effect of the j th family ($j = 1-10$ max.) within the i th population;

y_k is the effect of the k th year ($k = 1-2$);

s_l is the effect of the l th population ($l = 1-3$);

$\eta_{(rxl)}$ is the effect of the m th-replication ($k = 1-3,4$);

$\beta_{b(rxl)}$ is the effect of n th block ($b = 1-65$ max) within m th replication; and

$\varepsilon_{ijklrnb}$ is the residual ($n = 1-4,16$ max).

The model was analyzed as a mixed model with fixed (population, family, year and site) and random (block, replication and error) effects, where the components of variance were obtained by restricted maximum likelihood (REML). The best linear unbiased estimators and predictors (BLUE and BLUP) for

fixed and random factors, respectively, were obtained (SAS, 1999).

Pooled heritabilities (narrow sense) over populations, h^2 , were calculated for both variables after removing the population component.

$$h^2 = \frac{V_A}{V_P} = \frac{4V_{fam}}{V_{fam} + V_{rep} + V_{bl} + V_e}$$

where: V_P is the phenotypic variance, V_A is the additive variance, V_{fam} is the family variance, V_{rep} is the replication variance, V_{bl} is the block variance and V_e is the error variance.

In order to plot the $\delta^{13}C$ population expression in the four different environments tested in the experiment (2 sites \times 2 years), we constructed a dummy index based on climatic data, absorbing as much variability as possible. The climatic variables used were precipitation (mean annual and monthly values) and temperature (mean annual values and mean of average, maximum and minimum monthly values), summing up 52 climatic variables. This climatic index was the first component obtained from a principal component analysis (PCA), which accounted for a 52.45% of the total variation (SAS, 1999).

A canonical discriminate analysis for height, diameter (d) and $\delta^{13}C$, was performed (proc CANDISC. SAS, 1999) and all the observations (means by population and family as grouping factors) on the two first canonical variables were obtained.

Pearson correlation coefficients were conducted to analyse the relationship between $\delta^{13}C$ and growth.

Phenotypic variability was displayed by means of the coefficient of variation obtained from the phenotypic values. In the same way, for genetic variability, the coefficient of additive variance (defined as the ratio between additive variance and mean) was considered (Houle, 1992).

Genetic correlations could not be calculated accurately because the model was quite unbalanced, without enough degrees of freedom for useful estimations.

3. RESULTS

3.1. Height

h varied significantly between sites, years, populations and families ($P < 0.0001$, Tab. IIA). Height differences between the populations from mesic (M and A) and xeric origins (O), were expressed in site *Me*, where M and A presented higher growth. In *Xe*, only A had a significant superior growth than O the 1st year (Tab. IIB, Figs. 2A, 2B). Population and most family means had higher h values in *Me* (Figs. 4B1, 4B2; published on line) and height differences between sites were higher for the mesic populations, M and A (Figs. 2A, 2B). Furthermore, height differences between *Xe* and *Me* in O were only displayed the 2nd year. The interaction Fam \times site ($P < 0.0001$) was significant (Tab. IIA). Environmental conditions (site \times year) absorbed a higher percentage of the variance than genotype (Tab. III). Population and family variation in h were higher in site *Me* (16.86–94.40 cm) than in *Xe* (14.00–44.29 cm; Tab. IV). In addition, population variation in *Xe* was

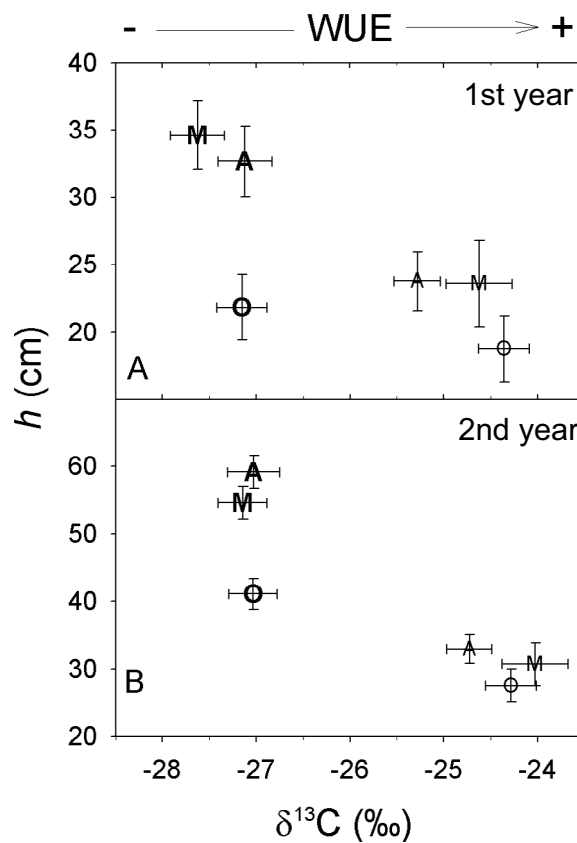


Figure 2. Representation of the carbon isotope composition ($\delta^{13}C$) vs. height (h). Population LS means the 1st (A) and 2nd years (B). Populations: Arenas (A), Oriá (O) and Mimizan (M). Families per population in the xeric trial site, *Xe*: A (10), O (8) and M (6), and 10 families in the mesic trial site, *Me* (bold letters). Data are means \pm standard errors. Higher values (less negative) are associated to higher water use efficiency.

only expressed the 1st year (Tab. IV). As a result, significant population differences in h were displayed the 1st year in *Xe* (Tab. IIB). Individual heritability pooled over population was 1.14 ± 0.27 . This high value is due to the extraction of the population effect.

3.2. Carbon isotope composition

Significant differences in $\delta^{13}C$ by sites ($2.63\text{‰} \pm 0.12$, $P < 0.0001$) were higher than by years ($0.32\text{‰} \pm 0.12$, $P < 0.0001$). Similar $\delta^{13}C$ values were displayed in *Me* both years (difference of $0.26\text{‰} \pm 0.19$ between years) and slightly different in *Xe* (difference of $0.63\text{‰} \pm 0.22$ between years). Trees had higher water use efficiency in *Xe* both years. The $G \times E$ interaction was significant at a population (Pop \times site) and family level (Fam (Pop) \times site, Tab. IIA). Environmental conditions (site \times year) absorbed the highest proportion of variance for $\delta^{13}C$ (62%, Tab. III). There was higher $\delta^{13}C$ variation at the family level than at the population level (Tab. III). Genetic variation in $\delta^{13}C$ was displayed at the population level

Table II.A. Summary of ANOVA significances. $\delta^{13}\text{C}$: isotope carbon composition; *h*: height. Num DF: numerator of degrees of freedom. Den DF: denominator of degrees of freedom. *P*: probability. *F*: *F*-values. Pop: population. Fam: family. Significant values in bold.

| Source of variation | $\delta^{13}\text{C}$ | | | | <i>h</i> | | |
|---------------------------------------|-----------------------|--------|-----------------|---------------------|----------|-----------------|---------------------|
| | Num DF | Den DF | <i>F</i> -Value | <i>P</i> > <i>F</i> | Den DF | <i>F</i> -Value | <i>P</i> > <i>F</i> |
| Year (1st/2nd) | 1 | 365 | 21.25 | < 0.0001 | 374 | 167.65 | < 0.0001 |
| Site (<i>Me/Xe</i>) | 1 | 365 | 258.94 | < 0.0001 | 374 | 23.85 | < 0.0001 |
| Site \times year | 1 | 365 | 0.62 | 0.4334 | 374 | 5.45 | 0.0201 |
| Pop | 2 | 365 | 1.37 | 0.2548 | 374 | 32.4 | < 0.0001 |
| Pop \times year | 2 | 365 | 0.17 | 0.8427 | 374 | 1.17 | 0.3118 |
| Pop \times site | 2 | 365 | 8.62 | 0.0002 | 374 | 0.25 | 0.7771 |
| Pop \times site \times year | 2 | 365 | 0.74 | 0.4799 | 374 | 0.15 | 0.8565 |
| Fam (Pop) | 41 | 365 | 3.76 | < 0.0001 | 374 | 5.41 | < 0.0001 |
| Fam (Pop) \times year | 41 | 365 | 1.27 | 0.1308 | 374 | 0.74 | 0.8755 |
| Fam (Pop) \times site | 6 | 365 | 3.15 | 0.0050 | 374 | 6.53 | < 0.0001 |
| Fam (Pop) \times site \times year | 6 | 365 | 0.45 | 0.843 | 374 | 1.36 | 0.2279 |

Table II.B. Summary of ANOVA significances by site and year (*Xe/Me*; 1st/2nd year). Abbreviations like in Table II.A.

| Source of variation | Genetic level | $\delta^{13}\text{C}$ | | | | <i>h</i> | | |
|---------------------|---------------|-----------------------|--------|-----------------|---------------------|----------|-----------------|---------------------|
| | | Num DF | Den DF | <i>F</i> -Value | <i>P</i> > <i>F</i> | Den DF | <i>F</i> -Value | <i>P</i> > <i>F</i> |
| Xeric, 1st year | Pop | 2 | 90 | 6.99 | 0.0015 | 93 | 3.88 | 0.0241 |
| | Fam (Pop) | 21 | 90 | 1.29 | 0.2060 | 93 | 2.44 | 0.0018 |
| Xeric, 2nd year | Pop | 2 | 92 | 6.16 | 0.0031 | 94 | 1.69 | 0.1902 |
| | Fam (Pop) | 21 | 92 | 1.10 | 0.3645 | 94 | 2.34 | 0.0029 |
| Mesic, 1st year | Pop | 2 | 84 | 1.98 | 0.1446 | 87 | 18.20 | < 0.0001 |
| | Fam (Pop) | 26 | 84 | 2.63 | 0.0005 | 87 | 3.41 | < 0.0001 |
| Mesic, 2nd year | Pop | 2 | 99 | 0.30 | 0.7379 | 100 | 12.59 | < 0.0001 |
| | Fam (Pop) | 26 | 99 | 3.02 | < 0.0001 | 100 | 3.34 | < 0.0001 |

Table III. Percentages of variance. Abbreviations like in Table II.

| Variance | $\delta^{13}\text{C}$ | <i>h</i> |
|---------------------------------------|-----------------------|----------|
| Site \times year | 62% | 37% |
| Pop | 0% | 8% |
| Pop \times site \times year | 1% | 0% |
| Fam (Pop) | 6% | 16% |
| Fam (Pop) \times site \times year | 4% | 3% |
| Error | 27% | 36% |

Table IV. Percentages of variance by site and year. Abbreviations like in Tables I and II.

| | Variance | 1st year | | 2nd year | |
|-----------------------|-----------|----------------|----------------|----------------|----------------|
| | | Site <i>Xe</i> | Site <i>Me</i> | Site <i>Xe</i> | Site <i>Me</i> |
| $\delta^{13}\text{C}$ | Pop | 16% | 0% | 12% | 0% |
| | Fam (Pop) | 5% | 30% | 4% | 30% |
| | Residual | 78% | 70% | 84% | 70% |
| <i>h</i> | Pop | 4% | 20% | 0% | 13% |
| | Fam (Pop) | 22% | 30% | 22% | 30% |
| | Residual | 74% | 50% | 78% | 57% |

in *Xe* and at the family level in *Me* (Tab. II.B). As a result, $\delta^{13}\text{C}$ family variation was higher in site *Me* and there was no population variation in *Me* (Tab. IV). Individual heritability pooled over population was 0.66 ± 0.25 .

The three populations displayed higher $\delta^{13}\text{C}$ values (less negative, higher WUE) in site *Xe* (Fig. 3). Population variation was $1.26\text{‰} \pm 0.30$ in *Xe* and $0.60\text{‰} \pm 0.28$ in *Me* (Fig. 2A, 2B). Families varied $3.95\text{‰} \pm 0.92$ in *Me* and $1.85\text{‰} \pm 0.66$ in *Xe* (data not shown). There was a significant population/family by site interaction (Tab. II.A). In *Xe*, A showed lower WUE than M and O the 1st year and lower than M the 2nd year (Fig. 2) and differences between families emerged in *Me* (Tab. II.B). There was a high, positive and significant correlation among $\delta^{13}\text{C}$ family values in consecutive years in both sites ($R^2 = 0.79$, $P < 0.05$; data not shown).

$\delta^{13}\text{C}$ mean population and mean family values from a subsample of the families present in both progeny trials were plotted in a bi-directional graph (*Me* vs. *Xe*, Figs. 4A1, 4A2; published on line). Populations and most families showed a high level of plasticity (away from the diagonal line) and displayed higher $\delta^{13}\text{C}$ values in *Xe*. Populations showed genetic differentiation for plasticity and a $G \times E$ interaction (Fig. 3). The climatic index of the PCA arranged environmental conditions in a way that climate close to the origin of the X axis was more extreme (in terms of temperatures, i.e., continentality) and drier and became milder and wetter along the axis. Population differentiation in $\delta^{13}\text{C}$ increased with aridity and continentality.

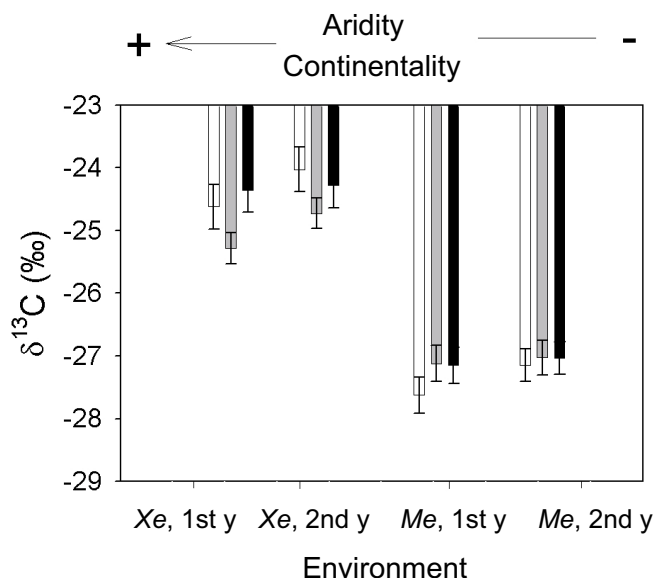


Figure 3. Representation of the $\delta^{13}\text{C}$ population means and standard errors across environments (*Me*: mesic trial site, *Xe*: xeric trial site, 1st y: first year, 2nd y: second year). M: white bars, A: grey bars, O: black bars. Division of X axis obtained from a PCA integrating precipitation and temperature values. Climate close to the origin of the X axis is drier, with more extreme maximum and minimum temperatures and becomes milder and wetter along the axis. Precipitation and mean of maximum and minimum temperature values of the hottest and coldest month, respectively: *Xe*, 1st year: 323 mm, 32.03 °C, -1.11 °C; *Xe*, 2nd year: 488 mm, 30.9 °C, 0.41 °C; *Me*, 1st year: 704 mm, 28.5 °C, 1.04 °C; *Me*, 2nd year: 969 mm, 26.7 °C, 2.1 °C.

3.3. Relationship between $\delta^{13}\text{C}$ and growth

When environmental effects were removed, i.e., studying the relationship between h and $\delta^{13}\text{C}$ corrected by their environment, no correlation appeared ($R^2 = -0.03$, $P = 0.426$). In a global model including the 4 conditions (2 sites, 2 years), there was a negative phenotypic correlation between h and $\delta^{13}\text{C}$ from all the individual values ($R^2 = -0.23$, $P < 0.0001$), individual values by year of measurements (1st year: $R^2 = -0.32$, $P < 0.0001$; 2nd year: $R^2 = -0.28$, $P < 0.0001$), individual values from each population (A: $R^2 = -0.24$, $P = 0.0013$; O: $R^2 = -0.18$, $P = 0.0175$; M: $R^2 = -0.31$, $P = 0.0003$), mean family values (1st year: $R^2 = -0.26$, $P < 0.01$; 2nd year: $R^2 = -0.46$, $P < 0.01$) and mean population values ($R^2 = -0.65$, $P < 0.01$; excluding the O population growing at *Me*, data not shown). h and d displayed a strong and positive correlation ($R^2 = 0.8076$, $P < 0.0001$), then, there was a trend in higher and more vigorous plants having lower $\delta^{13}\text{C}$ values (more negative, lower WUE) and vice versa. However, in populations or families growing under the same environmental conditions, that is, in every site and year, there was no trend. In general, taller populations or families, did not showed lower $\delta^{13}\text{C}$ values, except for the A and O populations in the xeric site (Fig. 2A), A families in the mesic site ($R^2 = -0.63$, $P = 0.0061$; data not shown) and M families in the xeric site ($R^2 = -0.81$, $P = 0.0058$; data not shown).

A canonical discriminant analysis based on height, diameter (d) and $\delta^{13}\text{C}$, and population and family as grouping factors, showed that in *Xe* the variance split among $\delta^{13}\text{C}$ and growth the 1st year (drier). $\delta^{13}\text{C}$ absorbed the 66% of the variation in axis 1 ($R^2 = 0.12$, $P = 0.0003$) and h and d the 34% in axis 2 ($R^2 = 0.07$, $P = 0.0061$). Only in this case, in the drier situation, $\delta^{13}\text{C}$ had a greater effect on population and family variation than growing characters. The 2nd year, the variation of physiological and growth parameters was displayed in a single axis in the xeric site (90% of variation, $R^2 = 0.15$, $P = 0.0005$). In *Me*, $\delta^{13}\text{C}$, h , and d accounted for the 96% ($R^2 = 0.26$, $P < 0.0001$) and 98% ($R^2 = 0.15$, $P = 0.0004$) of the variation in a single axis, both years (data not shown).

4. DISCUSSION

4.1. Growth

In our study, population differences in height were related to drought adaptation. In maritime pine low growth is associated to populations from dry climates and vice versa (Nguyen-Queyrens et al., 1998). The superior height of the A population and a population from the French Landes versus O was previously reported in seedlings (Fernandez et al., 1999). Natural selection might have favored trees which limit water loss in drier habitats and fast growing and more competitive trees in rainy areas. The limiting conditions for growth in the xeric site shaped only slight height differences between the A and O populations the 1st year.

The site had a significant effect on growth. Trees grown in the mesic trial site had higher height and diameter. Growth restrictions in response to soil drought were presumed and previously reported in *P. pinaster* (Nguyen-Queyrens et al., 2002). Populations from more mesic origins (M and A) displayed plasticity in growth and increased height in more favorable environmental conditions, i.e., in the mesic site. However, O, original from South Spain, with high xericity, showed small growth differences between the mesic and xeric trial sites. This agrees with Guyon and Kremer (1982), who observed that a provenance from Morocco showed a reduced physiological activity and high growth stability in all the locations tested and the Atlantic populations reacted quickly to the interannual and spatial climatic variability. As a result of the growth reduction by the mesic populations there seems to be a convergence in size in the xeric site.

There was no interaction between genotype and ambient (year or site) at the population level in h . Thus, the ranking of the provenances was similar in both sites and years. The lack of population \times year interaction is in accordance with Danjon (1994) who found a stable ranking of the *P. pinaster* populations for height across years. The absence of a population \times site interaction agrees with Chambel et al. (2007) who found a non significant population \times treatment interaction in *P. pinaster* seedlings in response to drought stress.

We also found significant differences in h between families and a family \times site interaction, indicating adaptation and plasticity to water availability within populations. This results

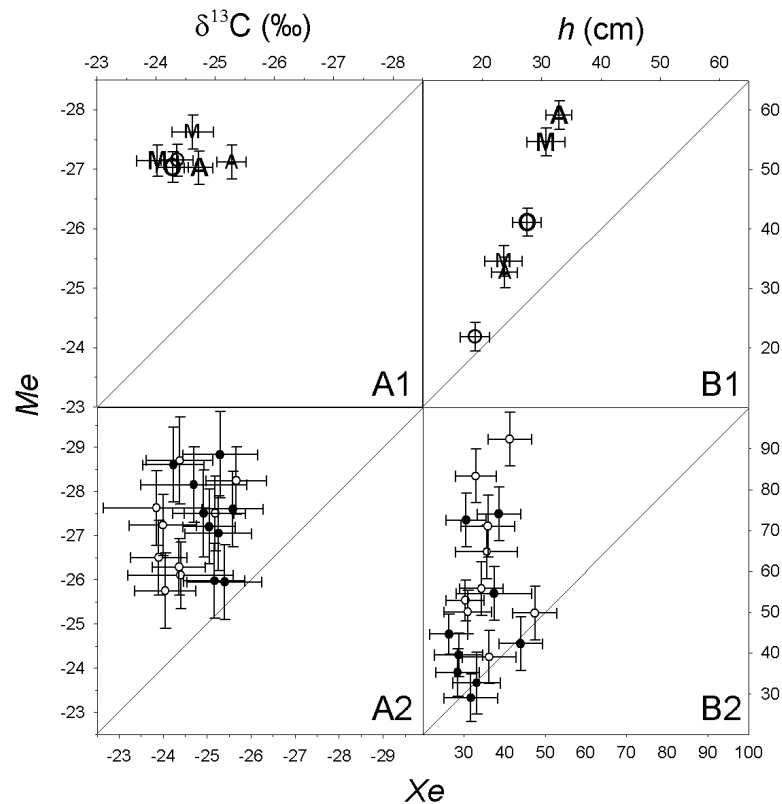


Figure 4. Bi-directional population LS means \pm standard errors for carbon isotope composition ($\delta^{13}\text{C}$, A1) and height (h , B1) in the 2 trial sites (axis X: X_e , axis Y: M_e). Symbols: Arenas (A), Oria (O) and Mimizan (M) the 1st year. Bold letters the 2nd year. Bi-directional LS means \pm standard errors for $\delta^{13}\text{C}$ (A2) and h (B2) in a subsample of families present in both progeny trial sites. Symbols: ●, 1st year; ○, 2nd year. Diagonal lines represent equal values in both sites.

agree with Zas et al. (2004) and Zas and Fernandez-Lopez (2005) who found a family \times site interaction in growth of *P. pinaster* adult trees and a family \times water availability interaction in *P. pinaster* seedlings, respectively.

4.2. Carbon isotope composition

4.2.1. Inter- and intra-population variation in $\delta^{13}\text{C}$

$\delta^{13}\text{C}$ variation between our populations was 1.26‰ in the xeric site and 0.60‰ in the mesic site, which is in the range of previous *P. pinaster* observations (Correia et al., 2008). As a result, we only found $\delta^{13}\text{C}$ differentiation of populations in the xeric site (Tab. IIB), which suggests that the WUE was affected by both, external and internal factors, like water availability and gas exchange performance, or by their interaction. Higher $\delta^{13}\text{C}$ variability in the drier trial site was also observed by Voltas et al. (2008) in *Pinus halepensis* populations.

In X_e , the A population presented lower WUE than M and O the 1st year and lower WUE than M the 2nd year. This result could be expected for O, from a dry origin. However, M, from the French Landes, has proved to be as much drought resistant as O in the mesic trial site and has shown a great plasticity, been even more drought resistant than O in the xeric

trial site, from the results obtained in the vulnerability curves to cavitation (unpublished data). Despite the mean annual precipitation of the geographic origin of M is high, the location is very windy, which could cause high vapor pressure deficit around the needles, and the soils are sandy with low water retention. These environmental factors could have played a role in the drought resistance displayed by M. Furthermore, in a previous study, the population from the French Landes exhibited the highest $\delta^{13}\text{C}$ among one Mediterranean and six Atlantic *P. pinaster* populations (Correia et al., 2008). They explained the higher WUE of the Landes population by its adaptive evolution. It originates in the northern range of the species, exposed to cold winters, less severe summer droughts, and shorter growing seasons and could initiate stomatal closure at lower water deficits, leading to inferior discrimination and higher $\delta^{13}\text{C}$.

The ranking of the populations A and M in $\delta^{13}\text{C}$ was maintained from the 1st to the 2nd year, but this was not the case for O, which showed intermediate values among the other populations the 2nd year. It is not surprising that despite the high heritability of $\delta^{13}\text{C}$ the ranking of populations differed through years. By definition, heritability is a ratio between variances affected by the environmental conditions. Aridity and continentality changed from year 1 to year 2 (Fig. 3), and therefore, the ranking of populations might vary.

The lack of $\delta^{13}\text{C}$ population differentiation in the mesic site is in agreement with Tognetti et al. (2000) who tested *P. pinaster* populations in experimental trials of high annual precipitation and different altitudes. Correia et al. (2008) observed minor but significant $\delta^{13}\text{C}$ differences between populations in a subhumid trial site. However, the populations from the driest and more humid origins displayed equal $\delta^{13}\text{C}$ values, which is in agreement with our results in the mesic trial site. Absence or small population variation might indicate population convergence when these populations grow in a stress-free environment. Populations from drier and lower latitudes did not exhibit higher water use efficiency compared to northern and humid latitudes. This could be related to and absence of differences in gas exchange rates (A and g), which was reported for the A and O populations and another population from the French Landes, in two water treatments, by Fernandez et al. (2000). In any case, $\delta^{13}\text{C}$ is a physiological response affected by many others characters than stomatal control and photosynthetic capacity, like leaf area, nitrogen content, phenology, carbon allocation, root characteristics and hydraulic properties. Then, variations in other species-specific traits could have balanced to keep WUE at a constant rank.

In the mesic site, *P. pinaster* $\delta^{13}\text{C}$ variation was partitioned within populations, which is in accordance with Fernandez et al. (2006) who found a larger *P. pinaster* family variation in the more favorable water treatment. The significant genetic variation at the family level was consistent with the results of Brendel et al. (2002) and suggests the existence of genetic adaptive variation upon which the process of natural selection could act and the achievement of additional gains in $\delta^{13}\text{C}$ by family selection. Fernandez et al. (2006) did not obtained $\delta^{13}\text{C}$ differences among *P. pinaster* families grown in a greenhouse, which was associated to the low water stress reached at the experiment (60% and 50% of field capacity in control and water stress treatments, respectively). Phenotypic response in $\delta^{13}\text{C}$ is largely influenced by environmental conditions (62% of variance, Tab. IV, Brendel et al., 2002). Nevertheless, we obtained significant $\delta^{13}\text{C}$ differences, in both control and water stress treatments, in *P. pinaster* populations and families grown in a greenhouse (unpublished data).

The more favorable environmental conditions in the mesic site allowed the differentiation of $\delta^{13}\text{C}$ at a family level but the extreme environmental conditions in the xeric site enhanced minor $\delta^{13}\text{C}$ population divergences. This could be interpreted as a consequence of the low- and high- differentiation between- and within- populations, respectively, found with nuclear and cytoplasmic markers in maritime pine (Ribeiro et al., 2001).

4.2.2. Relationship between $\delta^{13}\text{C}$ and environmental conditions

In general, populations and families presented much higher $\delta^{13}\text{C}$ mean values in the xeric site. The higher water use efficiency of trees in the more xeric trial site is in agreement with the well-known effect that, in a global scale, $\delta^{13}\text{C}$ increases with increasing evaporative demand/water limitation

(Ehleringer et al., 2002). Particularly, in the case of *P. pinaster*, an increase in $\delta^{13}\text{C}$ with water stress has already been reported by several authors at the species (Warren et al., 2001) and population level (Nguyen-Queyrens et al., 1998). On the contrary, Porte and Lostau (2001) found an inverse relationship between latewood $\delta^{13}\text{C}$ and soil water availability. The positive relationship between WUE and water stress in *P. pinaster* is associated to a high stomata control in detriment of net carbon assimilation rate under drought conditions. Decreasing water potential caused the ratio A/g to increase (Picon et al., 1996), such that a reduction in soil water potential from -0.4 to -0.8 MPa produced an 80% loss of stomatal conductance in maritime pine (Loustau et al., 1990).

There was a clear environmental influence on carbon isotope composition, as demonstrated by the significant differences between sites and years for the $\delta^{13}\text{C}$ values. This is in agreement with Tognetti et al. (2000) who studied five *P. pinaster* populations in four trial sites. We found important site differences in $\delta^{13}\text{C}$ ($2.63\text{‰} \pm 0.11$), higher than the ones observed by Aranda et al. (2010, $0.82\text{‰} \pm 1.00$) in *P. pinaster* seedlings subjected to a water stress treatment in which the substrate was kept at 40% of the saturation level. In the present study, the location of the trial site had a greater influence on $\delta^{13}\text{C}$ than the year of measurements, i.e., differences by sites were more important than the interannual variation of climatic factors. This across-year stability of $\delta^{13}\text{C}$ despite the between-year variation in environmental conditions is in agreement with previous works and indicates that the ranking of individuals in $\delta^{13}\text{C}$ remained almost constant between years (Pennington et al., 1999). In maritime pine the relationship between $\delta^{13}\text{C}$ values in consecutive years could be related to the use of carbon stocks from the previous year as a carbon source in spring (Porte and Lostau, 2001).

Despite the precipitation in the mesic site the drier year (704 mm), approached the rainfall in the xeric site the wetter year (488 mm), there was not a differentiation between populations in the mesic conditions. This indicates that, in addition to precipitation, other parameters like water availability in the soil (influenced by the soil type/structure) and/or the air relative humidity (R.H.) could play an important role in stomatal closure (66–67% in *Me* and 54–55% in *Xe*, mean R.H. during the growing season, spring+summer, in the 1st and 2nd year, respectively).

4.2.3. G \times E interaction

We tested the genotype by environment interaction under two approaches (site and year) and two levels (population and family). While year had a significant effect on $\delta^{13}\text{C}$, the genotype \times year interaction was non significant. This is consistent with the results of Cregg et al. (2000) and Flanagan and Johnsen (1995), who found non significant interactions between genotypes and years of contrasting rainfall in *Pinus ponderosa* populations and *Picea mariana* families, respectively, and was interpreted as a lack of $G \times$ water stress interaction. In contrast to the absence of genotype \times year interaction, we found a significant genotype \times site interaction, that

is, Pop \times site and Fam (Pop) \times site interactions, which implies differences in plasticity among populations and families. This was formerly noticed in *P. pinaster* populations (Tognetti et al., 2000) and families (Aranda et al., 2010; Fernandez et al., 2006). In our study all populations and families presented higher WUE in the drier site.

The significant, but minor, differences in WUE between populations in *Xe* could be related to the high stomatal sensitivity to water stress of the species. Fan et al. (2008) observed that $\delta^{13}\text{C}$ is highly dependant on the environmental conditions and obtained different rankings of redcedar populations under different environments and none relationship between WUE and origin of populations. There are also evidences of the contrary, $\delta^{13}\text{C}$ decreased with summer dryness of the seed sources between ten *Pinus contorta* populations (Guy and Holowachuck, 2001).

Besides aridity, other environmental factors could have affected $\delta^{13}\text{C}$. Correia et al. (2008) found no association between $\delta^{13}\text{C}$ and the precipitation of the place of origin in *Pinus pinaster* populations. However, $\delta^{13}\text{C}$ variation was negatively correlated with the difference between the mean maximal temperature of the hottest month and the mean minimal temperature of the coldest month, that is, the continentality. Populations originating from colder climates could have adapted to shorter vegetative periods and showed earlier stomatal closure and less discrimination against ^{13}C during the growing period, which led to higher WUE. We did not find evidences of that, as A originates from a continental site with colder winters and high differences between maximum and minimum temperatures and showed the lowest WUE in *Xe*.

We found high $\delta^{13}\text{C}$ phenotypic plasticity and adaptation to the environmental conditions of the growing place, independently of the origin of populations. We did not found a lower responsiveness of $\delta^{13}\text{C}$ to drought in O, the southern population from a dry origin, which disagree with Aranda et al. (2010), who noticed a different behavior among populations from wet and dry sites. Most of the families from wet provenances decreased δ in response to water stress, while the Moroccan population showed the same values and O a lower reduction in δ than the populations from mesic origins. In this work, all populations and families showed a higher WUE in the xeric site (Fig. 4, online). However, the genetic differentiation for plasticity was only found in the drier and continental location and the discrimination of the populations increased from the wettest and mildest to the driest and more continental situation along the X axis (Fig. 3). In fact, populations' $\delta^{13}\text{C}$ kept stable and non differentiated (canalization) in the two mild and wetter situations, but as conditions became more extreme, in terms of precipitation and temperatures (continentality), more differences were found between populations (plasticity).

4.3. Relationship between $\delta^{13}\text{C}$ and growth

Heritabilities for height and $\delta^{13}\text{C}$ were significant, comparable with other values found in the literature for maritime pine (Danjon, 1994; Aranda et al., 2010) and imply the presence of

considerable additive genetic variation and adequate control of environmental variation. We found a significant negative correlation between $\delta^{13}\text{C}$ and h , i.e., smaller plants having higher WUE, which is consistent with previous works in *P. pinaster* populations (Correia et al., 2008). The opposite, a positive correlation between $\delta^{13}\text{C}$ and growth, was observed in *P. pinaster* populations only in one site (Tognetti et al., 1997) and none relationship at all in other trial sites (Tognetti et al., 2000) or in *P. pinaster* families (Brendel et al., 2002). In the mesic site, growth differences between populations (higher growth in the mesic populations) did not lead to different $\delta^{13}\text{C}$. However, in the xeric site, small differences in h between A and O, led to significant differences in $\delta^{13}\text{C}$ the 1st year (drier, Fig. 2A). On the contrary, A and M, having similar growth, displayed different $\delta^{13}\text{C}$ values both years (Figs. 2A, 2B). At the family level, variation in growth was not accompanied by variation in $\delta^{13}\text{C}$ in the xeric site, which agrees with Rowell et al. (2009) and suggests that growth differences were influenced by other mechanisms to cope with drought stress, instead of WUE. Nguyen-Queyrens et al. (1998) did not find a reliable correlation between $\delta^{13}\text{C}$ and h in *P. pinaster* either. Higher trees displayed higher values of $\delta^{13}\text{C}$ in 26 year-old trees but none relationship in 7 year-old trees, and the correlation at a population level was low ($R^2 = 0.08-0.16$). In our case, the unclear relationship between $\delta^{13}\text{C}$ and h at a population level could be related to the absence or low $\delta^{13}\text{C}$ population variation displayed in the mesic and xeric site, respectively, and to the small number of populations tested. When combining the mean population values in the two sites a significant relation arose.

Differing relationship patterns between $\delta^{13}\text{C}$ and growth have been explained as differences in the primary source for genetic differentiation in WUE. Genetic variation in $\delta^{13}\text{C}$ is influenced by changes in photosynthetic capacity and/or stomatal conductance and is species-specific (Farquhar et al. 1989). In the case of *P. pinaster*, the species presents a drought avoiding strategy, with very high sensitivity of stomatal conductance to decreases in water potential, higher than the sensitivity of CO_2 assimilation rate (Picon et al., 1996). If variation in carbon isotopic composition, and then, in WUE, is mainly caused by changes in stomatal conductance, higher $\delta^{13}\text{C}$ and WUE values would lead to lower growth. This is consistent with the negative relationship between height and $\delta^{13}\text{C}$ observed. The high stomatal sensitivity is an advantage under water stress but limits net carbon assimilation rate and biomass production. However, the lower WUE observed in trees from the more mesic trial site is advantageous for young establishing plants in natural stands where rainfall is relatively high as it was associated to higher growth. In addition to gas exchange performance, genetic differences in carbon allocation patterns should be taken into account when studying plant adaptations to water availability. On dry sites, with long periods of high evapotranspirative demand, a high stomatal control in order to reduce transpiration water losses and a greater carbon allocation to root growth in detriment of aboveground biomass, would lead to higher WUE in smaller trees. In fact, the seedlings of the O population showed a significant greater carbon allocation to roots than the other

populations (unpublished data), which agrees with Guyon and Kremer (1982) and Aranda et al. (2010) who noticed that *P. pinaster* drought adapted ecotypes produced higher root to shoot biomass ratios.

5. CONCLUSIONS

Both, h and $\delta^{13}\text{C}$, were significantly influenced by the environment (site). The inter- and intra-population phenotypic plasticity observed in $\delta^{13}\text{C}$ and h was mainly attributable to the environmental influence (62% and 37% of variance, respectively). *P. pinaster* populations and families of contrasting origins adapted to drought showed higher WUE (indicated by $\delta^{13}\text{C}$) and lower growth in the xeric site, confirming the adaptive potential of the species.

Phenotypic correlations appeared between tree growth and $\delta^{13}\text{C}$. There was a trend of smaller trees showing higher water use efficiencies, indicating a higher influence of stomatal control, rather than photosynthetic capacity, over $\delta^{13}\text{C}$ and growth.

Population differences in h suggest adaptation to the environment of origin. Significant genetic variation in tree growth was also displayed at the family level. However, in the case of $\delta^{13}\text{C}$, aridity and continentality enhanced population differences and more favorable environmental conditions promoted family variation. Consequently, environment drove the genetic variation in such way that under stressful conditions differences in WUE among populations showed up but in a milder background the standard family variation was expressed instead.

The high influence of environmental conditions on $\delta^{13}\text{C}$ and the minor population variation in stressful environments, point out an environmental control of $\delta^{13}\text{C}$ compared with the genetic one. There is an aptitude for selecting maritime genotypes with higher WUE, based on carbon isotopic discrimination, but we have to take into account the effect of environment on $\delta^{13}\text{C}$. The superior $\delta^{13}\text{C}$ variation found at a population level in the xeric site and at a family level in the mesic site, finger to a selection of populations in drier locations and families in sub-humid sites.

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