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# Genetic variation for carbon isotope composition in *Juglans regia* L.: relationships with growth, phenology and climate of origin

Neus ALETÀ<sup>1</sup>, Antoni VILANOVA<sup>1</sup>, Raquel DÍAZ<sup>2</sup>, Jordi VOLTAS<sup>3\*</sup>

<sup>1</sup> Institut de Recerca i Tecnologia Agroalimentàries (IRTA) Torre Marimon, 08140 Caldes de Montbui, Spain

<sup>2</sup> Centro de Investigaciones Ambientales de Lourizán, Apdo. 127, 36080 Pontevedra, Spain

<sup>3</sup> Department of Crop and Forest Sciences, University of Lleida, Rovira Roure 191, 25198 Lleida, Spain

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## Abstract

• Among the traits of breeding interest for the common walnut tree *Juglans regia* L., characteristics such as timing of budbreak and leaf fall, water-use efficiency and growth performance are regarded as being of utmost relevance in Mediterranean conditions.

• The authors evaluated intraspecific variation in  $\delta^{13}\text{C}$  (carbon isotope composition, surrogate of intrinsic water-use efficiency,  $\text{WUE}_i$ ) for 22 *J. regia* families grown in a progeny test under supplementary irrigation, and investigated whether such variation correlated with climatic indicators of native habitats. The genetic relationships between  $\delta^{13}\text{C}$ , growth and phenology were also assessed during two consecutive years.

• Overall, the most water-use-efficient families (i.e. with higher  $\delta^{13}\text{C}$ ), which originated mainly from drought-prone provenance regions which have a high vapour pressure deficit and low rainfall, exhibited less height growth and smaller DBH. Using a stepwise regression procedure,  $\delta^{13}\text{C}$  was included as the main explanatory variable of genotypic variation in growth traits, together with growing season duration (for DBH in both years) and flushing (for height in 2007).

• It was concluded that  $\text{WUE}_i$  is largely unconnected to phenology effects in the explanation of growth performance for *J. regia*, therefore suggesting the opportunity of simultaneously selecting for low  $\text{WUE}_i$  and extended growing period to maximise productivity in non-water-limited environments.

## Résumé – Variation génétique de la composition isotopique du carbone chez *Juglans regia* L. : relations avec la croissance, la phénologie et le climat d'origine.

• Parmi les traits d'intérêt pour la sélection de noyer commun *Juglans regia*, des caractéristiques telles que la précocité de débourrement et la date de chute des feuilles, l'efficience d'utilisation de l'eau et la performance de croissance sont considérées comme étant importantes dans les conditions méditerranéennes.

• Les auteurs ont évalué la variation intraspécifique de composition isotopique en  $^{13}\text{C}$  ( $\delta^{13}\text{C}$ , substitut de l'efficience intrinsèque d'utilisation de l'eau,  $\text{WUE}_i$ ) dans 22 familles de *Juglans regia* cultivées dans un essai de descendance avec une irrigation d'appoint, et ils ont examiné si la variation était corrélée avec les indicateurs du climat des habitats d'origine. Les relations génétiques entre  $\delta^{13}\text{C}$ , croissance et phénologie ont également été évaluées au cours de deux années consécutives.

• En général, les familles présentant les plus fortes valeurs de  $\text{WUE}_i$  (c'est-à-dire avec le  $\delta^{13}\text{C}$  le plus élevé), qui proviennent essentiellement des régions de provenance à sécheresse élevée et à fort déficit de pression de vapeur et faibles précipitations, présentent une croissance en hauteur moindre et un plus faible diamètre à 1,3 m. En utilisant une procédure de régression par étapes,  $\delta^{13}\text{C}$  a été inclus en tant que principale variable explicative de la variation génotypique des caractéristiques de croissance, de concert avec la durée de la saison de croissance (pour le diamètre à 1,3 m) et le débourrement (pour la hauteur en 2007).

• Il a été conclu que  $\text{WUE}_i$  est en grande partie sans rapport avec la phénologie dans l'explication de la performance de croissance pour *J. regia*, suggérant donc la possibilité de sélectionner simultanément pour de faibles niveaux de  $\text{WUE}_i$  et une période de croissance prolongée pour maximiser la productivité dans des environnements où l'eau n'est pas limitante.

\* Corresponding author: [jvoltas@pvcf.udl.es](mailto:jvoltas@pvcf.udl.es)

## 1. INTRODUCTION

In recent years, in the western Mediterranean there has been a rising interest in the cultivation of common walnut (*Juglans regia* L.), due to its high economic value as timber. In contrast, the scarcity of improved planting stock available for timber production obliges landowners to use nut-producing cultivars or seedlings from outstanding local trees (Fady et al., 2003). Some interspecific hybrid progenies resulting from *J. regia* and the American black walnut (*J. nigra* L.) (Becquey, 1997), but also wild populations, such as ‘Bleggiana’ or ‘Feltrina’ in Italy, ‘Charente’ in France, or ‘Nerpio’ in Spain, are still being planted specifically for timber production. Among the traits of breeding interest at early ages that are usually regarded as being of the utmost importance are adaptive characteristics such as flushing date and growth performance (Fady et al., 2003). An appropriate phenological adjustment to local conditions ensures a near-optimal exploitation of the growing season while avoiding the negative impact of both late spring and early autumn frosts on growth and survival, which have perhaps been the main issues that have been addressed in walnut cultivation (Hemery et al., 2005).

Current water scarcity and increasing water demands are expected to be limiting factors for plant productivity over southern Europe, as drought is becoming progressively more intense in the Mediterranean (IPCC, 2007). *J. regia* is known to severely reduce water use during drought by stomatal regulation (Rosati et al., 2006). This behaviour, triggered by changes in leaf water status, seems crucial in avoiding xylem cavitation in the walnut tree, indeed a major threat for plant functioning (Cochard et al., 2002). Thus, *J. regia* can be considered as a drought-avoider, adapted to some extent to effective water-saving. This realisation poses the question of whether intraspecific variability for drought avoidance in *J. regia* has developed under contrasting selective pressures in terms of water supply. A traditional approach to studying this issue is based on analysing carbon isotope composition ( $\delta^{13}\text{C}$ ) in plant tissues, which offers the possibility of getting an integrated record of the ratio of chloroplastic to atmospheric  $\text{CO}_2$  concentration ( $C_c/C_a$ ) during the period in which the carbon is fixed (Farquhar et al., 1989; Farquhar and Richards, 1984). Consequently, and assuming near-constancy of  $C_i-C_c$  (the difference between intercellular and chloroplastic  $\text{CO}_2$  concentration) at the intraspecific level (Warren and Adams, 2006),  $\delta^{13}\text{C}$  may be used to assess genetic variability in  $C_i/C_a$ , and thus in the ratio of net assimilation to stomatal conductance, or “intrinsic” water-use efficiency ( $\text{WUE}_i$ ) (Farquhar and Richards, 1984). In this regard, earlier studies have detected intraspecific variation in  $\delta^{13}\text{C}$  for temperate hardwoods such as *Castanea sativa* (Casasoli et al., 2004; Lauteri et al., 1997), *Populus* spp. (Bonhomme et al., 2008; Monclus et al., 2005) and *Quercus robur* (Casasoli et al. 2006). Variation in  $\delta^{13}\text{C}$  has also been linked to the distribution of genotypes across gradients in air humidity and soil water availability for a number of species (e.g. Cregg and Zhang, 2001; Pennington et al. 1999; Zhang and Marshall, 1995), suggesting that water availability acts as a selective force, leading to divergent intraspecific profiles for  $\text{WUE}_i$ .

Present breeding activities for timber production in north-eastern Spain focus on parental selection for seed orchards undertaken through a two-stage scheme in which early screening of open-pollinated progenies in the nursery is followed by extensive testing under conditions typical of operational plantations (Aletà et al., 2004). A five-year trial for on-farm testing was used in this study, and aimed to (i) assess genetic variation in leaf  $\delta^{13}\text{C}$  for a set of 22 open-pollinated progenies of common walnut originating mainly from seven provenance regions of Spain; (ii) determine whether such variation correlates with climatic indicators of native habitats; and (iii) report variance components and heritability estimates for  $\delta^{13}\text{C}$ , growth and phenology traits (flushing, leaf fall and growing season duration) and characterise their genetic relationships. The results are discussed in the framework of breeding activities for this species.

## 2. MATERIALS AND METHODS

### 2.1. Field test

In April 2003 a field test consisting of 22 *J. regia* progenies was established in Constantí in the province of Tarragona in north-eastern Spain (Tab. I). At the Constantí site the climate is sub-humid Mediterranean with temperate winters and a prolonged summer drought period (about 10% of total precipitation). The trial was planted on a flat area and tilled with a mouldboard plough before the seedlings were planted out. The soil type was a clay-loam typic xerofluvent with a depth of about 0.8 m and a pH above 8. In spring 2002 open-pollinated seeds were obtained from 22 mother trees and prior to sowing, the seeds were soaked in a 50 ppm solution of giberellic acid ( $\text{GA}_3$ ) for 48 h in order to break their physiological dormancy (Aletà, 1994). Eighteen seedlings per progeny were grown in nursery beds for one year (2002 growing season), and then planted in the field at spacings of 5 m  $\times$  5 m in three replicates consisting of rectangular plots of six trees following a randomised complete block design. The seedlings were planted directly into the soil and were drip-irrigated fortnightly from April to June and three times per month in July and August during the five-year study in order to minimise the impact of water stress on growth. The total volume of supplementary irrigation was equivalent to approximately 100 mm  $\text{y}^{-1}$ . As from the second growing season after the nursery seedlings were planted out, a corrective light pruning was carried out every year in late spring. This pruning was specifically aimed at eliminating multiple leaders and long branches that had grown too large in diameter, and sought to achieve an overall balanced tree growth, whilst avoiding any excessive interference in the development of individual differences in tree architecture (i.e. leaving about 2/3 of tree height as crown). Every year in mid-October, a solution of copper oxychloride with 3% Cu (w/v) was sprayed over the tree foliage to trigger bud cold hardening and therefore decrease the risk of late frost damage.

### 2.2. Plant material

Of the total of 22 progenies used in the field test (common garden), seeds of 21 progenies were obtained from open-pollinated mother trees grafted into two clonal banks, Mas de Bover – IRTA (16 mother trees) and Lourizán – CINAM (Pontevedra, north-west of Spain;

**Table 1.** Geographic origin (provenance region), habitat characteristics and leaf  $\delta^{13}\text{C}$  of 22 *Juglans regia* families, and details of the study site in Constantí.

Provenance region	Family or study site	Country	Latitude	Longitude	Elevation (m.a.s.l.)	Tmax <sub>an</sub> (°C)	Tmin <sub>an</sub> (°C)	P <sub>an</sub> (mm)	PET <sub>an</sub> (mm)	P <sub>an</sub> /PET <sub>an</sub>	VPD <sub>an</sub> (Pa)	$\delta^{13}\text{C}_{2006}$ (‰)	$\delta^{13}\text{C}_{2007}$ (‰)
Asturias Coast	AC1	Spain	43° 27' N	8° 22' W	50	16.4	8.6	1 001	811	1.23	515	-28.50	-26.52
"	AC2	"	42° 81' N	7° 11' W	490	17.6	6.8	1 389	996	1.39	655	-28.25	-27.00
"	AC3	"	42° 78' N	7° 01' W	720	16.5	5.9	1 431	959	1.49	644	-28.08	-26.44
Cazorla-Segura	GranJefe	"	38° 15' N	2° 30' W	1 600	20.7	5.6	432	1 306	0.33	1 139	-27.91	-26.80
Galician Coast	GC1	"	42° 41' N	8° 76' W	10	18.6	9.2	1 557	967	1.61	577	-28.49	-27.29
"	GC2	"	42° 82' N	9° 03' W	380	19.4	10.6	1 667	964	1.73	718	-28.57	-27.58
"	GC3	"	42° 79' N	8° 23' W	455	17.5	7.4	1 518	962	1.58	632	-27.97	-26.22
"	GC4	"	42° 80' N	8° 12' W	330	17.9	7.8	1 427	974	1.47	630	-28.76	-27.31
Inner Galicia	IG1	"	43° 36' N	8° 18' W	65	18.3	9.8	1 238	912	1.36	605	-28.70	-27.44
"	IG2	"	43° 30' N	8° 20' W	50	16.7	8.7	1 080	828	1.30	522	-28.28	-26.85
"	IG3	"	43° 48' N	8° 23' W	1	17.7	9.8	1 350	849	1.59	541	-28.65	-26.89
Levant	L1	"	40° 90' N	0° 05' E	40	21.8	11.2	491	1 161	0.42	931	-27.41	-26.14
"	L2	"	40° 60' N	0° 45' E	130	21.8	11.2	598	1 143	0.52	937	-28.52	-27.92
"	L3	"	40° 72' N	0° 50' E	75	21.8	11.2	648	1 154	0.56	921	-28.16	-26.74
"	L4	"	40° 90' N	0° 05' E	40	21.8	11.2	491	1 161	0.42	931	-27.65	-26.48
Northern Ebro	NE1	"	41° 30' N	1° 03' E	850	18.5	7.4	612	1 048	0.58	875	-27.45	-26.46
"	NE2	"	41° 27' N	1° 00' E	830	16.8	5.9	555	1 020	0.54	771	-27.41	-26.51
PrePyrenees	P1	"	41° 88' N	2° 52' E	630	16.3	5.8	854	959	0.89	703	-27.50	-26.98
"	P2	"	41° 87' N	0° 88' E	322	20.5	7.5	506	1 205	0.42	897	-27.72	-26.38
-	Lozeronne	France	-	-	-	-	-	-	-	-	-	-29.31	-27.90
-	ChaseD9	USA	-	-	-	-	-	-	-	-	-	-27.54	-26.84
-	Serr	"	-	-	-	-	-	-	-	-	-	-28.47	-26.57
-	Constantí	Spain	41° 09' N	1° 12' E	90	21.6	10.9	537	1 112	0.48	903		

Tmax<sub>an</sub> = mean annual maximum temperature; Tmin<sub>an</sub> = mean annual minimum temperature; P<sub>an</sub> = annual precipitation; PET<sub>an</sub> = annual potential evapotranspiration; P<sub>an</sub>/PET<sub>an</sub> = ratio of annual precipitation to annual potential evapotranspiration; VPD<sub>an</sub> = annual mean of daytime vapour pressure deficit.

5 mother trees) (Tab. I). At each clonal bank every mother tree was surrounded by 10 to 15 potential male genitors originating from similar ecological conditions, as grafted trees were allocated to the clonal bank layout according to geographic patterns of distribution at origin. Seeds of an additional progeny, 'Lozeronne', were obtained directly from a local nursery (Pépinières Payre, L'Albenc, France). 'Lozeronne' is a progeny native of Grenoble (Isère department, Rhône-Alpes region, France) and it is the most widely planted *J. regia* material for timber production in France.

Most progenies (18) tested in the common garden originated from wild individuals selected between 1988 and 1998 in Catalonia (north-eastern Spain) and Galicia (north-western Spain), which were later grafted into any of the abovementioned clonal blanks. The selection of the trees was based on their outstanding phenotype for timber production, taking into account traits such as stem form, apical dominance and branch type. Another progeny of Iberian origin ('Gran Jefe') had been previously selected by D. Frutos (IMIDA, Murcia, Spain) in a natural habitat of the province of Albacete (eastern Spain). These 19 Iberian progenies (designated in parentheses) were assigned to seven provenance regions of the distribution area for this species in Spain (García del Barrio et al., 2001): Asturias Coast (AC1 to AC3), Cazorla-Segura Range (GranJefe), Galician Coast (GC1 to GC4), Inner Galicia (IG1 to IG3), Levant (L1 to L4), Northern Ebro Depression (NE1, NE2) and PrePyrenees (P1, P2). The concept of a provenance region, which defines an adaptive unit in which phenotypically or genetically similar stands or seeds are found (European Council directive 1999/105/EC), represents a sensible way to classify individuals in the absence of clearly defined populations (i.e. useful for a species characterised by its scattered distribution, usually as isolated trees, and scarce presence and role in SW European forests). In addition, two more progenies from high-vigour commercial nut-producing cultivars, 'Chase D-9' (Oregon, USA) and 'Serr' (California, USA), along with the aforementioned 'Lozeronne', were also evaluated in the common garden for a total of 22 progenies.

For each progeny of Iberian origin (19), the means of monthly maximum (Tmax) and minimum (Tmin) temperature, and the means of total monthly precipitation (P) for the period 1950–1999 were obtained from the Digital Climatic Atlas of the Iberian Peninsula database (<http://opengis.uab.es/wms/iberia/index.htm>) Ninyerola et al., 2005) implemented in MiraMon-GIS with a spatial resolution of 200 m. Mean monthly potential evapotranspiration (PET following the Hargreaves method, Hargreaves and Samani, 1982) and mean monthly vapour pressure deficit (VPD following Ferrio and Voltas, 2005) were also estimated using these long-term temperature and precipitation records. Finally, the ratio of precipitation to PET (P/PET) was calculated on a monthly basis and was used as a drought index. Mean provenance values were then calculated from climate records of progenies of each provenance region.

### 2.3. Growth and phenology

Tree height (H) and diameter at breast height (DBH) of the six trees of each replicate plot were measured yearly (2003–07 period) at the end of each growing season. Initial total height after planting was also recorded. Increments in height (HI) and in diameter at breast height (DBHI) for 2006 and 2007 were generated by subtracting values at the end of each particular year from the values registered the previous year. Phenology traits were also collected on each individual tree in 2006 and 2007. Flushing, considered as the stage at which bud scales and leaf bracts open and the first leaves start to appear (herein

denoted  $C_f$  following the nomenclature of phenological stages described by Germain et al. (1999) for *J. regia*) and leaf fall (LF, the stage equivalent to 50% of fallen leaves) dates were recorded twice per week at the beginning (early spring) and end (late autumn) of each growing season. The growing season duration (GSD) was obtained by subtracting LF from  $C_f$  for each individual tree. Data on  $C_f$ , GSD and LF were recorded as Julian days computed from the 1st of January. The means of the meteorological records in Constantí were: 21.8 °C (Tmax), 11.2 (Tmin), 550 mm (P), 1128 mm (PET), 0.49 (P/PET) and 922 Pa (VPD) for 2006, and 22.6 °C (Tmax), 10.6 (Tmin), 337 mm (P), 1176 mm (PET), 0.29 (P/PET) and 967 Pa (VPD) for 2007.

### 2.4. Leaf carbon isotope composition ( $\delta^{13}\text{C}$ )

Foliage samples were collected in mid-October during 2006 and 2007 along a top-bottom transect within the south-facing part of each tree crown. This was done to account for (i) potential spatial variations in  $\delta^{13}\text{C}$  within the foliage owing to light gradients (Le Roux et al., 2001), and (ii) seasonal  $\delta^{13}\text{C}$  differences among leaves due to varying environmental conditions at the time of production of structural matter. Three leaves belonging to different shoots were sampled per tree and year (starting from a young leaf found in the top position to an old one at the bottom of the crown), and all leaves from each replicate plot (i.e. six trees  $\times$  three leaves) were pooled together and sealed in a plastic bag. The third leaflet starting from the outer end of each compound leaf was removed and the leaflets thus obtained were subsequently bulked (for a total of 66 samples per year, 22 progenies  $\times$  three replicate plots), oven-dried at 60 °C for 48 h and ground through a 0.5-mm screen in a Cyclotec 1093 sample mill (Tecator AB, Höganäs, Sweden). Leaflet  $\delta^{13}\text{C}$  was determined by mass spectrometry at Iso-Analytical (Sandbach, Cheshire, UK).

### 2.5. Statistical analysis

Growth and phenology traits were subjected to the following mixed model analysis of variance (ANOVA) at the individual-tree level for randomised complete block designs:

$$y_{ijk} = \mu + B_i + f_j + bf_{ij} + e_{ijk} \quad (1)$$

where  $y_{ijk}$  is the observation of the  $k$ th individual of the  $j$ th family in the  $i$ th block,  $\mu$  is the overall mean,  $B_i$  is the fixed effect of the  $i$ th block,  $f_j$  is the random effect of the  $j$ th family,  $bf_{ij}$  is the random residual effect of the  $j$ th family in the  $i$ th block (i.e. the family-by-block interaction), and  $e_{ijk}$  is the random tree effect of the  $k$ th individual in the  $ij$ th plot.

For  $\delta^{13}\text{C}$ , only a single value per replicate plot was available, so the model fitted was:

$$y_{ij} = \mu + B_i + f_j + bf_{ij} \quad (2)$$

with  $y_{ijk}$ ,  $B_i$ ,  $f_j$  and  $bf_{ij}$  as defined in (1).

To test for possible differences in  $\delta^{13}\text{C}$  among provenances, the term  $f_j$  in (2) was partitioned into two terms accounting for fixed provenance and between-family within-provenance effects.

The degree and similarity of the genetic control of all traits were evaluated by estimating family heritabilities as well as genetic correlations. For growth and phenology traits, family heritabilities for

**Table II.** Mixed model analysis of several traits evaluated in two consecutive years (2006 and 2007): estimates ( $\pm$  SE) of phenotypic and genetic variances and family heritabilities. Traits evaluated were carbon isotope composition ( $\delta^{13}\text{C}$ ), height increment (HI), diameter at breast height increment (DBHI), flushing date ( $C_f$ ), leaf fall date (LF) and growing season duration (GSD). Between brackets: probabilities associated with Wald Z-scores testing the null hypothesis of family variance component equal to zero. See text for explanation of variance component subscripts.

Source	Traits					
	$\delta^{13}\text{C}$	HI	DBHI	$C_f$	LF	GSD
2006						
$\sigma_f^2$	$0.23 \pm 0.087$ (0.003)	$109.2 \pm 56.66$ (0.021)	$1.40 \pm 0.684$ (0.021)	$37.2 \pm 11.94$ (< 0.001)	$29.7 \pm 14.58$ (0.021)	$65.1 \pm 26.54$ (0.007)
$\sigma_{\text{plot}}^2$	$0.13 \pm 0.029$	0	$0.22 \pm 0.516$	$1.1 \pm 0.89$	$24.4 \pm 11.15$	$33.3 \pm 13.51$
$\sigma_e^2$	–	$1214.6 \pm 94.05$	$11.15 \pm 0.918$	$14.9 \pm 1.27$	$125.4 \pm 10.77$	$122.8 \pm 10.90$
$h_f^2$	$0.84 \pm 0.060$	$0.62 \pm 0.126$	$0.67 \pm 0.132$	$0.97 \pm 0.012$	$0.66 \pm 0.132$	$0.78 \pm 0.086$
2007						
$\sigma_f^2$	$0.21 \pm 0.084$ (0.004)	$34.9 \pm 23.59$ (0.068)	$2.11 \pm 1.192$ (0.029)	$32.6 \pm 11.95$ (0.003)	$3.0 \pm 2.78$ (0.141)	$46.0 \pm 18.73$ (0.009)
$\sigma_{\text{plot}}^2$	$0.18 \pm 0.040$	$26.3 \pm 26.18$	$1.88 \pm 0.973$	$1.6 \pm 2.23$	$7.8 \pm 3.51$	$14.8 \pm 6.11$
$\sigma_e^2$	–	$442.6 \pm 37.94$	$12.73 \pm 1.067$	$41.2 \pm 3.55$	$42.3 \pm 3.51$	$61.9 \pm 5.35$
$h_f^2$	$0.77 \pm 0.086$	$0.51 \pm 0.198$	$0.61 \pm 0.159$	$0.92 \pm 0.034$	$0.38 \pm 0.246$	$0.85 \pm 0.066$

open-pollinated progenies were calculated from restricted maximum likelihood (REML) estimates of variance components as:

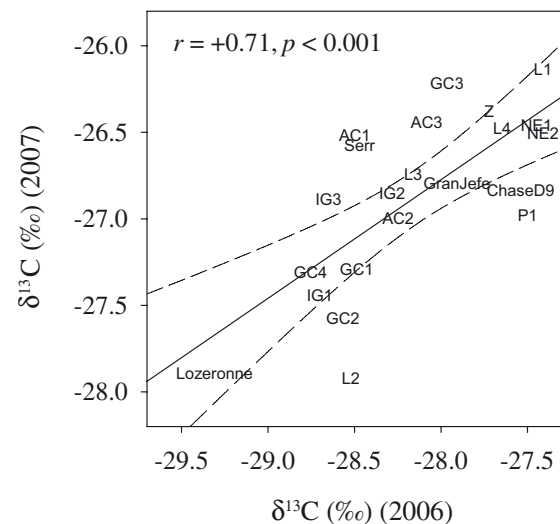
$$h_f^2 = \frac{0.25\sigma_A^2}{\sigma_P^2} = \frac{\sigma_f^2}{\sigma_f^2 + \sigma_{\text{bf}}^2/b + \sigma_e^2/bk} \quad (3)$$

where  $\sigma_A^2$  is the additive genetic variance,  $\sigma_P^2$  is the phenotypic variance of family means,  $\sigma_f^2$  is the family variance,  $\sigma_{\text{bf}}^2$  is the family-by-block interaction (or plot) variance,  $\sigma_e^2$  is the between-tree within-plot variance,  $b$  is the number of blocks and  $k$  is the number of trees per plot. For  $\delta^{13}\text{C}$ , family heritabilities for open-pollinated progenies were calculated by removing  $\sigma_e^2/bk$  from (3). Standard errors of family heritabilities were obtained according to Gilmour et al. (2002). The Wald Z-score was used as an approximate statistic to test whether the family variance component differed from zero. Genetic correlations and their standard errors were calculated from the variance-covariance component matrices obtained from bivariate analyses of variance involving the traits of interest (Gilmour et al., 2002). Because data at the individual-tree level were not available for  $\delta^{13}\text{C}$ , the plot means were employed for all correlations involving this trait. The general form of a correlation is expressed as:

$$r_{12} = \frac{\sigma_{12}}{\sqrt{\sigma_1^2 \times \sigma_2^2}} \quad (4)$$

where  $\sigma_{12}$  stands for the estimated family covariance component between traits 1 and 2;  $\sigma_1^2$  is the family variance component of trait 1; and  $\sigma_2^2$  is the family variance component of trait 2. Bivariate analyses were also performed to obtain correlations for particular traits measured in 2006 and 2007.

Relationships with climate of origin were investigated using product-moment correlations between provenance means of  $\delta^{13}\text{C}$  measurements and climatic data. To further test the association between growth increments (HI, DBHI) and both  $\delta^{13}\text{C}$  and phenology, linear stepwise models were built independently for 2006 and 2007 using the phenotypic means, with  $P = 0.15$  as the criterion for variables to either be included or removed from the model. Unless otherwise stated, variance components and correlation coefficients were considered statistically significant when  $P < 0.05$ . All analyses were carried out using the MIXED and REG procedures of SAS/STAT (SAS Institute Inc., Cary, NC).

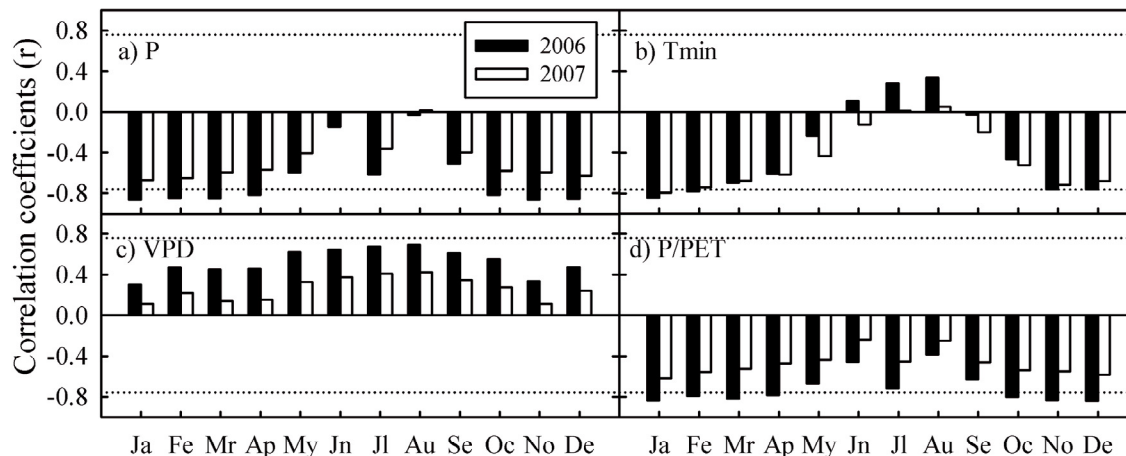


**Figure 1.** Relationship between family means of leaf  $\delta^{13}\text{C}$  evaluated in two consecutive years (2006 and 2007). Dashed lines indicate the 95% confidence interval.

### 3. RESULTS

#### 3.1. Variation in $\delta^{13}\text{C}$ and relationships with climate of native habitats

According to estimates of variance components, significant variability in  $\delta^{13}\text{C}$  was observed amongst the 22 half-sib progenies for both years, and the family heritabilities were high (Tab. II). The  $\delta^{13}\text{C}$  values varied from  $-29.3\text{‰}$  for Lozeronne to  $-27.4\text{‰}$  for L1 in 2006 and from  $-27.9\text{‰}$  for Lozeronne to  $-26.1\text{‰}$  for L1 in 2007. The mean  $\delta^{13}\text{C}$  values were lower in 2006 ( $-28.2\text{‰}$ ) than in 2007 ( $-26.9\text{‰}$ ). However, genotypic ranking for  $\delta^{13}\text{C}$  was stable across years (Fig. 1), as indicated by a high age-age genetic correlation ( $r = +0.87 \pm 0.140$  SE).



**Figure 2.** Correlation coefficients between leaf  $\delta^{13}\text{C}$  of provenances and either monthly (a) P, (b) P/PET ratios, (c) VPD or (d) Tmin at origin for two consecutive years (2006 and 2007). Dotted lines indicate the threshold value for significant correlations ( $P < 0.05$ ).

When the Iberian progenies were assigned to provenance regions, there were significant differences in  $\delta^{13}\text{C}$  among provenances for both years and lack of significant provenance $\times$ year interaction (results not shown). P and P/PET were climatic features associated (negatively) with provenance  $\delta^{13}\text{C}$  values across months (Figs. 2a and 2d). However, the strength of these relationships was higher (and only significant) in 2006. Similar results were obtained using annual values of P and P/PET, with significant correlations between provenance  $\delta^{13}\text{C}$  records averaged across years and either P ( $r = -0.77$ ,  $p < 0.05$ ) or P/PET ( $r = -0.77$ ,  $p < 0.05$ ). VPD was also associated (positively) with provenance  $\delta^{13}\text{C}$  values (Fig. 2c), especially in spring and summer of 2006. Other climatic factors associated with  $\delta^{13}\text{C}$  were Tmin, but only in autumn-winter (Fig. 2b), and to a lesser extent, Tmax for the same period (results not shown). For both years the PET was non-significantly related to  $\delta^{13}\text{C}$ .

### 3.2. Variation in growth and phenological traits

Significant or near-significant variation in HI and DBHI was detected among progenies for both years, with moderate to high family heritability estimates (Tab. II). HI ranged from 39.1 cm (NEI) to 86.2 cm (GC1) in 2006 and from 28.7 cm (ChaseD9) to 67.1 cm (L2) in 2007. DBHI ranged from 7.1 mm (Lozeronne) to 11.8 mm (Serr) in 2006 and from 5.2 mm (Lozeronne) to 11.2 mm (AC2) in 2007. The mean growth was higher in 2006 than in 2007 (57.4 cm versus 42.8 cm for HI; 9.4 mm versus 8.2 mm for DBHI). Differences in growth in the two years were related to contrasting climate conditions. For example, in 2007 rainfall was scarce (39% lower than in 2006) and, thus, P/PET was decreased by 41%. However, genotypic ranking for growth traits was rather stable as suggested by age-age genetic correlations ( $r = +0.76 \pm 0.318$  for HI;  $r = +0.68 \pm 0.235$  for DBHI). Significant variability in total H and DBH at age 5 was also observed among progenies, with values ranging from 197.5 cm (Lozeronne) to 416.9 cm (GC1) for H, and from 16.0 mm

(Lozeronne) to 53.5 mm (GC2) for DBH. Significant Spearman rank correlations were obtained between H (or DBH) at age 5 and HI (or DBHI) over the period 2006-07 ( $r = +0.58$  and  $r = +0.59$ , respectively). At age 5, Lozeronne had about 40%-reduced H and 59%-reduced DBH compared with the mean value of all other progenies, and 23%-reduced H and 41%-reduced DBH compared with the mean value of the second shortest progeny (IG1) and of the second thinnest progeny (AC3), respectively.

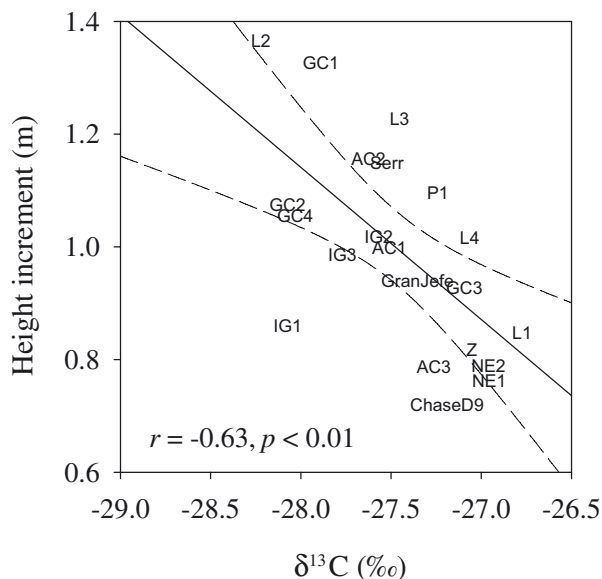
With the exception of LF in 2007, phenology traits showed significant variability among progenies for both years (Tab. II). Family heritabilities showed high values for GSD and LF in 2006 and very high values for  $C_f$ . Flushing (days after 1 January) ranged from 89 d (L1) to 115 d (Lozeronne) in 2006 and from 95 d (L1) to 122 d (Lozeronne) in 2007. The mean  $C_f$  was lower in 2006 (94 d) than in 2007 (105 d) due to the low spring temperatures in 2007. LF ranged from 324 d (L2) to 349 d (GC2) in 2006, and from 304 d (AC3) to 317 d (P1) in 2007, and the mean LF value was higher in 2006 (335 d) than in 2007 (312 d). In this regard, a weather event that was crucial was the autumn frost which occurred on the 11th of November 2007 when the minimum air temperature fell to  $-5.2^\circ\text{C}$ , and in most of the progenies this frost resulted in a sudden end of the growing season. Age-age genetic correlations for  $C_f$  ( $r = +0.92 \pm 0.046$ ) and LF ( $r = +0.21 \pm 0.286$ ) suggested that the genotypic performance was stable across years for  $C_f$ , but not for LF. Significant variability in GSD was also observed among progenies for both years, with high family heritability estimates (Tab. II). GSD varied from 219 d (ChaseD9) to 254 d (GC2) in 2006 and from 180 d (Lozeronne) to 220 d (Serr) in 2007. The mean GSD was higher in 2006 (242 d) than in 2007 (207 d), with an intermediate age-age genetic correlation ( $r = +0.52 \pm 0.184$ ).

### 3.3. Relationships among traits

The data from Lozeronne showed a high degree of deviation, giving simultaneously the poorest growth, the lowest

**Table III.** Genetic correlations ( $\pm$ SE) between several traits evaluated in two consecutive years (2006 and 2007). Traits evaluated were carbon isotope composition ( $\delta^{13}\text{C}$ ), height increment (HI), diameter at breast height increment (DBHI), flushing date ( $C_f$ ), leaf fall date (LF) and growing season duration (GSD).

	2006				
	HI	DBHI	$C_f$	LF	GSD
$\delta^{13}\text{C}$	$-0.83 \pm 0.189$	$-0.99 \pm 0.348$	$-0.01 \pm 0.265$	$-0.73 \pm 0.203$	$-0.58 \pm 0.226$
HI		$1.06 \pm 0.092$	$-0.14 \pm 0.295$	$0.46 \pm 0.271$	$0.44 \pm 0.268$
DBHI			$-0.34 \pm 0.251$	$0.60 \pm 0.208$	$0.64 \pm 0.188$
$C_f$				$-0.13 \pm 0.243$	$-0.74 \pm 0.115$
LF					$0.85 \pm 0.071$
	2007				
	HI	DBHI	$C_f$	LF	GSD
$\delta^{13}\text{C}$	$-0.91 \pm 0.410$	$-0.23 \pm 0.532$	$-0.64 \pm 0.162$	$-0.12 \pm 0.451$	$0.64 \pm 0.161$
HI		$0.90 \pm 0.151$	$-0.30 \pm 0.274$	$0.29 \pm 0.326$	$0.38 \pm 0.267$
DBHI			$-0.49 \pm 0.229$	$0.64 \pm 0.233$	$0.70 \pm 0.183$
$C_f$				$-0.19 \pm 0.277$	$-0.94 \pm 0.035$
LF					$0.51 \pm 0.211$

**Figure 3.** Relationship between family means of leaf  $\delta^{13}\text{C}$  and height increment across two consecutive years (2006 and 2007). Dashed lines indicate the 95% confidence interval.

$\delta^{13}\text{C}$  and highest  $C_f$ , and this suggested that this atypical progeny could greatly influence the outcome of a correlation analysis. This view was confirmed after having calculated the genetic correlations with and without Lozeronne in the dataset, and so Lozeronne was thereafter excluded from the analysis (Tab. III). Low- $\delta^{13}\text{C}$  progenies exhibited a vigorous growth in height (in both years) and a larger DBHI (in 2006). This held true for the association between progeny means of  $\delta^{13}\text{C}$  and accumulated height growth across two consecutive years (2006-07 period; Fig. 3). Genetic relationships between  $\delta^{13}\text{C}$  and phenology traits were strongly dependent on the year; in 2006, low- $\delta^{13}\text{C}$  genotypes had late LF and prolonged GSD, whereas in 2007 low- $\delta^{13}\text{C}$  genotypes exhibited late  $C_f$  and reduced GSD. As expected, GSD was negatively associated

with  $C_f$  and positively associated with LF in both years, but  $C_f$  and LF were genetically unrelated. HI and DBHI tended to be negatively related to  $C_f$  and positively related to LF, but the strength of these relationships was moderate to low. Accordingly, genotypes with prolonged GSD showed higher growth in both years, especially for DBHI.

The complementarity of  $\delta^{13}\text{C}$  and phenology traits in explaining tree growth variability (HI and DBHI) was investigated by fitting stepwise linear regressions using progeny means for each year separately (2006 and 2007) (Tab. IV). Initially, only phenological data were used; in most cases the only trait included in the stepwise process was GSD and this resulted in a low or very low model adjustment ( $R^2$ ). In contrast, when  $\delta^{13}\text{C}$  and phenological traits were combined, the best subset of predictors always included  $\delta^{13}\text{C}$  as contributing to explaining variability in HI and DBHI. In addition, phenology traits such as GSD (for DBHI) or  $C_f$  (for HI in 2007) were also selected in the stepwise process, and the initial model adjustment was largely improved.

## 4. DISCUSSION

### 4.1. Intraspecific variation in $\text{WUE}_i$ and associations with climate of origin

Our data show consistent genetic variation in leaf  $\delta^{13}\text{C}$ , with a sizeable range of mean progeny values (about 1.9‰ in both years). This finding suggests that there is intraspecific variability in  $\text{WUE}_i$  for *J. regia*. However,  $\delta^{13}\text{C}$  depends not only on net assimilation and stomatal conductance for  $\text{CO}_2$  (components of  $\text{WUE}_i$ ), but also on the resistance to the movement of  $\text{CO}_2$  from substomatal cavities to sites of carboxylation (i.e. internal conductance,  $g_i$ ), which is known to modify relationships of  $\text{WUE}_i$  with carbon isotope ratios (Seibt et al., 2008). This is indeed crucial for interpreting genetic variability in  $\delta^{13}\text{C}$  among and within species (Warren, 2008; Warren and Adams, 2006). The effects of  $g_i$  on the economics of water were recently addressed by Warren and Adams (2006). These



**Table IV.** Multiple linear stepwise regressions to explain growth increments (HI and DBHI) from phenology traits (C<sub>f</sub>, LF, GSD) and carbon isotope composition ( $\delta^{13}\text{C}$ , obtained from family mean values) in two consecutive years (2006 and 2007).

Trait	Initial variable	Initial $R^2$	Initial MSE	Final stepwise model	Final $R^2$	Final MSE
HI (2006)	phenology (C <sub>f</sub> , LF, GSD)					
	LF	0.12	0.0167	$H = -1.71 + 0.007 \text{ LF}$	0.12	0.0167
	$\delta^{13}\text{C}$ + phenology (C <sub>f</sub> , LF, GSD)					
	$\delta^{13}\text{C}$	0.35***	0.0123	$H = -4.18 - 0.17 \delta^{13}\text{C}$	0.35***	0.0123
DBHI (2006)	phenology (C <sub>f</sub> , LF, GSD)					
	GSD	0.30**	$0.15 \times 10^{-3}$	$DBH = -0.143 + 0.001 \text{ GSD}$	0.30**	$0.15 \times 10^{-3}$
	$\delta^{13}\text{C}$ + phenology (C <sub>f</sub> , LF, GSD)					
	$\delta^{13}\text{C}$	0.35***	$0.14 \times 10^{-3}$	$DBH = -0.423 - 0.013 \delta^{13}\text{C} + 0.006 \text{ GSD}$	0.45***	$0.13 \times 10^{-3}$
HI (2007)	phenology (C <sub>f</sub> , LF, GSD)					
	GSD	0.11	0.0068	$H = -0.42 + 0.004 \text{ GSD}$	0.11	0.0068
	$\delta^{13}\text{C}$ + phenology (C <sub>f</sub> , LF, GSD)					
	$\delta^{13}\text{C}$	0.15	0.0065	$H = -2.40 - 0.16 \delta^{13}\text{C} - 0.01 \text{ C}_f$	0.50***	0.0040
DBHI (2007)	phenology (C <sub>f</sub> , LF, GSD)					
	GSD	0.36***	$0.21 \times 10^{-3}$	$DBH = -0.237 + 0.0015 \text{ GSD}$	0.36***	$0.21 \times 10^{-3}$
	$\delta^{13}\text{C}$ + phenology (C <sub>f</sub> , LF, GSD)					
	$\delta^{13}\text{C}$	0.36***	$0.21 \times 10^{-3}$	$DBH = -0.836 + 0.002 \text{ GSD} - 0.018 \delta^{13}\text{C}$	0.54***	$0.15 \times 10^{-3}$

can be summarised as (i) the imposition of large limitations on photosynthesis (draw-down from C<sub>i</sub> to C<sub>c</sub>, or C<sub>i</sub>-C<sub>c</sub>); (ii) the existence of genetic variation in C<sub>i</sub>-C<sub>c</sub>; and (iii) the presence of systematic variation in C<sub>i</sub>-C<sub>c</sub> as a function of  $g_i$ , with an imperfect scaling of  $g_i$  with net CO<sub>2</sub> assimilation. To our knowledge there is only one published study on variation in internal conductance for *Juglans* spp. (including *J. regia*) (Piel et al., 2002), which reported a strong correlation between  $g_i$  and net assimilation and a relatively small range of phenotypic variation in C<sub>i</sub>-C<sub>c</sub> ( $86\text{--}108 \mu\text{mol mol}^{-1}$ ). These results suggest the adequacy of  $\delta^{13}\text{C}$  records to infer intraspecific changes in WUE<sub>i</sub> for *J. regia*, although additional measurements on intraspecific variation in  $g_i$  would be necessary to shed more light on this issue.

Earlier studies have also reported intraspecific variation in  $\delta^{13}\text{C}$  for a number of Mediterranean forest trees (Lauteri et al., 2004; Tognetti et al., 2000; Voltas et al., 2008). The high heritabilities for *J. regia* are within the range reported for forest trees (Johnsen et al., 1999; Prasolova et al., 2000; 2001), but the higher value in 2006 suggests a greater consistency of genotypic differences as compared with 2007. This result is probably the consequence of more favourable growing conditions, as substantiated by the lower  $\delta^{13}\text{C}$  values for this year (cf. Fig. 1). In comparison, the high  $\delta^{13}\text{C}$  value for 2007 suggests that in that year there was greater stomatal limitation to photosynthesis, which was most likely caused by an unusually low precipitation (Warren et al., 2001).

To design appropriate breeding strategies it is helpful to understand the selection forces at work in evolutionary adjustments. In our study, the most water-use-efficient *J. regia* provenances originated mainly from drought-prone areas (i.e. eastern Spain) which have a low rainfall and P/PET, and also a high VPD. Lauteri et al. (2004) also found differences in  $\delta^{13}\text{C}$  among families of *Castanea sativa* Miller, a species that thrives under meso-Mediterranean conditions similar to those of *J. regia*, but they reported a negative association be-

tween WUE<sub>i</sub> and drought impact on native habitats. This contrasting behaviour suggests different strategies for reaching optimum fitness under comparable selective roles of climate variables. For *J. regia*, climate-mediated divergence in transpiration rates, hydraulic architecture or vulnerability to cavitation (Cochard et al., 2002; Rosati et al., 2006) are probably involved in genotypic changes in patterns of water use, as suggested by the  $\delta^{13}\text{C}$  records. Minimum temperature (for autumn-winter) was another climate variable that was also found to trigger divergence in  $\delta^{13}\text{C}$ , pointing to possible intraspecific differences in growth rhythms, as discussed below. In this context, a more extensive dataset broadening information on finely-tuned patterns of climate adaptation at the geographic level would reinforce the hypothesis of an early spread and diversification of *J. regia* after ice retreat in the western Mediterranean, as already suggested by palynological, charcoal and genetic analyses (Carrión, 2002; Figueiral and Bettencourt, 2004; Fornari et al., 1999).

## 4.2. Genetic variability in growth and phenology

For *J. regia*, there is little information available on progeny evaluation under field conditions (Díaz and Fernández-López, 2005; Fady et al., 2003). According to these studies, the Constantí field site can be considered a medium-potential location for walnut growth, with a mean annual increment in height of  $60 \text{ cm}\cdot\text{y}^{-1}$  after five growing seasons. Under these field conditions, the genetic control of growth traits was moderate to high considering both family variances and heritability estimates, the latter being similar to those reported for *Juglans* spp. (0.5–0.7) (Díaz and Fernández-López, 2005; Rink and Kung, 1995). As for  $\delta^{13}\text{C}$ , the higher heritabilities in 2006 are in agreement with better weather conditions in that year that would favour the expression of genetic mechanisms controlling growth. In principle, since the Constantí site is representative of coastal areas of Mediterranean Spain, one could expect

a higher growth rate with progenies such as L2, L3 or L1 that originate from similar ecological areas. However, the results of the trial did not always support this premise, and progenies of other origins showed either outstanding or poor performance as well (cf. Fig. 3). In this regard, the clinal pattern for reduced height increment with increasing distance of origin from planting which was described by Hemery et al. (2005) in the UK was not observed here under more xeric conditions. This suggests that sources from compatible environments may perform satisfactorily on fairly good sites without water shortage. In contrast, the poor performance of Lozeronne agrees with results from a multi-site network across Europe (Fady et al., 2003), and indicates that it is poorly adapted to Mediterranean conditions.

In most cases, the genetic control of phenology traits was high to very high ( $h^2 > 0.6$ ), thereby confirming previous results for *Juglans* spp. devoted to nut production (Germain et al., 1999; Hansche et al., 1972; McGranahan et al., 1988). The exception was LF, which showed a low heritability in 2007 owing to autumn frost damage that affected progenies from sites that have a prolonged growing season (e.g. those from milder areas of A Coruña and Tarragona). The highest heritabilities were achieved for  $C_f$ , with Lozeronne showing very late flushing as would be expected considering its colder, Alpine origin (Hemery et al., 2005). Apart from this case, ranking of progenies based on  $C_f$  could not be related to climate of origin, although both a late LF and extended GSD in 2006 were significantly associated with high autumn Tmin ( $r = +0.59$ ,  $p < 0.01$  for LF, and  $r = +0.54$ ,  $p < 0.05$  for GSD). These results emphasise the possibility of selecting for contrasting growth rhythms in *J. regia* and substantiate the key role of phenology in adaptedness to contrasting sites (Fady et al., 2003; Hemery et al., 2005).

#### 4.3. Phenology and the relationship between $\delta^{13}\text{C}$ and growth

Our data indicate that progenies with a prodigal water use (i.e. lower  $\delta^{13}\text{C}$ ) grow faster as compared to water-saving ones. The strong negative genetic link between productivity and  $\text{WUE}_i$  agrees with other studies involving a number of forest trees such as *Acacia senegal* (Raddad and Lukkanen, 2006), *Eucalyptus microtheca* F. Muell. (Li et al., 2000), *Pinus halepensis* Mill. (Voltas et al., 2008) and *Pinus sylvestris* L. (Cregg and Zhang, 2001), among others. However, links between productivity traits and  $\delta^{13}\text{C}$  are known to differ among plant species, with instances of either positive (e.g. *Castanea sativa*; Lauteri et al., 2004) or non-existing (e.g. *Populus × euramericana*; Monclus et al., 2005) relationships reported as well. In this regard, the particular performance of the common walnut suggests the existence of a trade-off between growth and drought tolerance for this species, as reported elsewhere (Boltz et al., 1986; Cregg and Zhang, 2001). Current results also show a tendency of genotypes with prolonged GSD to outperform those with a shorter vegetative period. An optimal exploitation of the available growing season through budbreak adjustment is recognised as crucial for *J. regia* (e.g. Fady et al.,

2003), but the authors were unaware of previous works characterising the role of GSD on growth. Remarkably,  $C_f$  and LF were genetically unrelated, as was previously shown by Díaz and Fernández-López (2005), which suggests the suitability of performing simultaneous selection on both traits to tailor phenology to specific ecological requirements.

The changing pattern observed in the genetic relationships between phenology and  $\delta^{13}\text{C}$  (Tab. III) may at least in part reflect the specific environmental conditions in which leaf dry matter was synthesised in each of the years. For example, under the more humid conditions of 2006 the expected decrease in  $\text{WUE}_i$  with a lower evaporative demand was probably accentuated for genotypes with delayed LF and, presumably, this prolonged carbon gain in autumn. However, the drier conditions and the autumn frost of 2007 made  $\delta^{13}\text{C}$  dependent upon variation in budbreak, with early-flushing genotypes showing a high  $\text{WUE}_i$ . Put simply,  $\delta^{13}\text{C}$  variation in *J. regia* appears to be partially driven by genotypic changes in the timing of leaf fall, but it also points to a drought-avoidance strategy by which stomatal closure in high- $\text{WUE}_i$ , early-flushing genotypes considerably reduces water expenditure (Cochard et al., 2002; Rosati et al., 2006). This realisation raises the question whether the negative associations between growth traits and  $\delta^{13}\text{C}$  were actually indirectly sustained by an effect of phenology on both elements. Because the phenology-based stepwise regressions largely and systematically improved with the inclusion of  $\delta^{13}\text{C}$  in the models, it can be concluded that  $\text{WUE}_i$  is not linked to phenology in the explanation of growth performance. Nevertheless, most models included GSD as an additional factor explaining either HI or DBHI, which suggests the opportunity of simultaneously selecting for low  $\text{WUE}_i$  and extended growing period to maximise productivity in non-water-limited environments. This finding requires further testing under a range of conditions encompassing the potential cultivation area for this species, and especially where a suboptimal water availability may become a critical factor for the establishment of commercial plantations. If more extensive trials support this premise, then the development of breeding activities would be faced with the critical challenge of reconciling this strategy with minimising the chance of frost injury either in late spring or early autumn. Because  $\delta^{13}\text{C}$  is a highly heritable trait, it might be used either as a selection criterion of parents retained in clonal banks, in which inferences on growth potential in grafted trees are unreliable, or to screen individuals obtained from open-pollinated or controlled crosses at the nursery stage, since nursery-field correlations for productivity traits in forest trees are usually low, as reported elsewhere (e.g. Adams et al., 2001). In the latter case, a two-stage independent culling selection strategy (Hallauer and Miranda, 1988), in which low- $\delta^{13}\text{C}$  seedlings are selected, subject to a threshold probability of suffering frost injury, could be a valuable early-testing approach to identify highly productive genotypes, matching phenology to regional environments.

## 5. CONCLUSION

This study demonstrates the presence of intraspecific variability for  $\text{WUE}_i$  of *J. regia* under common-garden conditions,

with the most water-use-efficient provenances originating mainly from drought-prone areas which have high VPD together with low rainfall and P/PET ratios. The results also indicate that progenies showing a prodigal water use (i.e. lower  $\delta^{13}\text{C}$ ) tend to grow faster than water-saving progenies, which suggests that for *J. regia* there is a trade-off between productivity and drought tolerance. The lack of a genetic link between  $C_f$  and LF indicates the suitability of simultaneously selecting for both traits to tailor phenology to specific ecogeographical units. Although variation in  $\delta^{13}\text{C}$  appears to be partially determined by genotypic changes in the timing of leaf fall and budbreak, we conclude that  $\text{WUE}_i$  is largely unconnected to phenology effects in explaining growth performance.

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