

# Are budburst dates, dormancy and cold acclimation in walnut trees (*Juglans regia* L.) under mainly genotypic or environmental control?

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**Abstract** As observed for most stresses, tree frost resistance can be split into two main processes: avoidance and tolerance. Avoidance of freezing is achieved by introducing species only in the climatic context in which the probability of freezing events is very low for the sensitive stages of buds or stems; i.e., when good synchronism exists between the annual cycle and the critical climatic periods. Buds become able to grow only after chilling requirements have been satisfied (endodormancy released) during winter; they subsequently break after heat requirements have been completed (end of ecodormancy) in early spring. Actually, this period is often subject to more or less severe freezing events. Trees are also able to adjust their freezing tolerance by increasing their capacity of extracellular freezing and decreasing the possibility of intracellular freezing through the process of frost acclimation. Both freezing resistance processes (avoidance and tolerance) are environmentally driven (by photoperiod and temperature), but there are also genotypic effects among species or cultivars. Here, we evaluated the degree to which differences in dormancy release and frost acclimation were related to environmental and genetic influences by comparing trees growing in common garden conditions. This investigation was carried out for two winters in lowland and

mountain locations on different walnut genotypes differing significantly for budburst dates. Chilling requirement for endodormancy release and heat requirement during ecodormancy were evaluated in all situations. In addition, frost acclimation was assessed by the electrolyte leakage method on stems from the same trees before leaf fall through budburst. No significant differences were observed in chilling requirements among genotypes. Moreover, frost acclimation dynamics were similar between genotypes or locations when expressed depending on chilling units accumulated since 15 September as a time basis instead of Julian day. The only exception was for maximal frost hardiness observed during winter with the timber-oriented being significantly more resistant than fruit-oriented genotypes. Heat requirement was significantly different among genotypes. Thus, growth was significantly faster in fruit-oriented than in wood-oriented genotypes. Furthermore, among wood-oriented genotypes, differences in growth rate were observed only at cold temperatures. Frost acclimation changes differed significantly between fruit- and wood- walnuts from January through budburst. In conclusion, from September through January, the acclimation dynamic was driven mainly by environmental factors whereas from January through budburst a significant genotype effect was identified in both frost tolerance and avoidance processes.

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## Abbreviations

CU Chilling units  
LT<sub>50</sub> Lethal temperature for 50% of cells  
MTB Mean time until bud break

## Introduction

Annual cycles of tree development are temperature- and genotype-dependent. In the last decade, phenology has been studied extensively for many living forms. It has been shown clearly that climate change (mostly through temperature effects) has an impact on life cycles and, in perennial plants, growing season is getting longer with earlier budburst and delayed senescence of leaves (Parmesan and Yohe 2003; Menzel et al. 2006). Phenological synchronization with environmental conditions is a mechanism of freezing resistance, i.e., avoidance of freezing temperatures. Thus, in relation to phenology (e.g., timing of senescence, endodormancy release, or budburst), the probability of freezing damage may alter with climate change.

During the leafless period in deciduous trees, bud growth is blocked through a dormancy stage driven by several environmental factors (Perry 1971; Lang et al. 1987), i.e., mainly the shortening photoperiod at the end of the summer until leaf fall (Welling et al. 1997), or cold nights (Heide and Prestrud 2005). During dormancy, mitotic activity is blocked in meristems and cell nuclei remain in G1 stage of the cell cycle (Larcher 1995). The resulting state of endodormancy, defined as a temporary suspension of growth in every part containing a meristem (Lang et al. 1987), can be released by chilling exposure (Weinberger 1950; Landsberg 1974; Richardson et al. 1974; Sarvas 1974). A chilling requirement for endodormancy release prevents trees from initiating growth during transient warm events. Thereafter, within buds in the ecodormancy stage (a dormant state that is limited only by environmental factors), the rate of ontogenic development increases with increasing temperature (“heat requirement”; Lang et al. 1987), and for some species longer photoperiod (Heide 1993a, 1993b). Budburst is observed when growth of young leaves reaches a defined threshold (in most cases leaf unfolding), characterized on the BBCH scale (Meier 2001).

Differences in chilling and heat requirements are genetic adaptations to environmental conditions. Thus, genotypes with low chilling requirements would be able to flush earlier in cold environmental conditions when risks of frost damages are still high (Scorza and Okie 1990). On the other hand, genotypes with a high chilling requirement will be exposed, in warm environmental conditions, to insufficient chilling, which triggers later or even erratic budburst limiting the length of the photosynthetic period (Dennis 1987, 1994; Topp et al. 2008).

In order to limit freezing damage, another mechanism is tolerance to freezing in tissues, which is modulated during the annual cycle. During winter, aboveground parts of trees also develop resistance to freezing temperatures by an acclimation process. Environmental signals involved in the acclimation process are short

photoperiod in first stages and then low temperatures (Aronsson 1975; Christersson 1978). When environmental conditions become warmer, trees are deacclimated in response to warm temperatures (Kalberer et al. 2006). The relationship between deacclimation kinetics and ecodormancy heat requirements have often been described (Campbell and Sugano 1975; Thomson and Moncrieff 1982). Furthermore, Larcher and Mair (1968) observed, in *Fraxinus* sp., differences in frost acclimation or deacclimation rates among environmental conditions whereas frost resistances were finally similar for each defined phenological stage (from leaf fall through budburst).

Thus, the signals identified as driving dormancy and cold acclimation are the same. While endodormancy occurs during winter, trees are acclimating; then, during the ecodormancy stage, they are deacclimating. Some authors have studied those two phenomena and have shown that they occur in parallel (Druart et al. 2007; Welling and Palva 2006) leading, in some cases, to parallel modelling of both processes (Fuchigami et al. 1982; Leinonen 1996).

Deviation in budburst dates were observed between different genotypes under the same environmental conditions, which could be assigned to differences in temperature sensitivity (Vitasse et al. 2009). For example, in different genotypes of walnut trees (*Juglans regia* L.; Mauget and Germain 1980), budburst dates were staggered over 2 months. As it is now well established that environmental conditions will be different in the future, it appears critical to weigh the environmental and genotypic effects on the developmental cycle of trees. This genotypic difference could be developed during endodormancy, ecodormancy or both. Furthermore, another frost resistance process, frost tolerance, could also be influenced by genotypic differences. Hence, this study examined different genotypes of walnut trees under two environmental conditions, and measured dormancy (chilling and heat requirements) and frost acclimation (estimated by relative electrolyte leakage method) from leaf fall until budburst.

## Material and methods

### Orchards

Trees were between 15 and 20 years old and planted in two different locations in the middle of France: Crouël (Lowland: 03°08'50"E, 45°46'20"N, alt: 340 m) and Theix (Mountain: 03°01'00"E, 45°43'10"N, alt: 880 m). The two locations are 20 km apart and exhibit a mean difference in temperature of 3°C (Poirier et al. 2010). In the lowland orchard, fruit-oriented trees, *Juglans regia* L. cv Chandler ( $n=4$ ), Franquette ( $n=5$ ), Lara ( $n=5$ ), Serr ( $n=4$ ) were sampled. Some wood-oriented trees, *Juglans regia* x

*nigra* cv NG38 from the same breeding were split into early hybrids (E-Hybrid;  $n=5$ ) and late hybrids (L-Hybrid;  $n=5$ ) and also sampled. In the mountain orchard, only Franquette ( $n=5$ ), Lara ( $n=5$ ), early hybrids ( $n=5$ ) and late hybrids ( $n=5$ ) could be sampled because low temperatures did not allow Serr and Chandler survival.

#### Phenological data

Phenological data were measured in the spring of 2008 and 2009 on individual trees. For each tree, the average proportion of apical and lateral buds that were broken was estimated every 2 days. Day of budburst was estimated for each tree as the day when 50% of lateral buds reached the threshold of unfolding new leaves (stage 15 of BBCH scale; Meier, 2001, last picture on Fig. 1).

#### Temperature requirements

Chilling and heat requirements were evaluated in climatic chambers on single-bud cuttings. On each tree, stems were sampled on 29 November when endodormancy was usually close to its maximal value [as determined on walnuts (Mauget and Germain 1980; M. B., unpublished results), and cut in 7-cm-long pieces with only one bud left on each piece of stem. Then, for non apical buds, the top of the stem segments were covered with wax and the bottom was dipped in water. For chilling requirement assessments, buds were placed at 4°C with light for 8 h/day for a between 0 and 1600 hours. After chilling exposure, buds were placed in climatic chambers with long-day treatment (16 h) and warm temperature (25°C). Every 2 days, each bud was observed for bud bursting status: bud closed, becoming green, or leaf unfolding. For each chilling exposure duration, the mean individual time spent at 25°C was calculated and referred to as mean time until budburst (MTB). Endodormancy was considered released and chilling exposure satisfied when no further significant decrease in MTB was observed with increased chilling exposure.



**Fig. 1** Different phenological stages of buds in walnut trees (*Juglans regia* L.) from winter dormancy until budburst (from Germain et al. 1999)

#### Bud growth during ecodormancy

Bud growth rates were evaluated after endodormancy was broken under natural conditions (29 January). Single-bud cuttings (see above) were placed under long-day conditions at different temperatures (5, 10, 15, 20 and 25°C) and checked for burst every 2 days. MTB was calculated for each genotype and temperature.

#### Chilling model

Since the last century, studies on chilling requirements have generated lots of different models for chilling accumulation. In order to compare between both locations, thermal models were selected from the literature (Bidabe 1967; Fishman et al. 1987a, 1987b for the Dynamic model; Hanninen 1990; Landsberg 1974; Richardson et al. 1974 for the Utah model; Sarvas 1974; Weinberger 1950). For details on chilling effect calculations, refer to the original papers. Sums of chilling units were computed on an hourly basis starting on 15 September during the frost acclimation stage.

#### Forcing Models

During the ecodormancy stage, warm temperatures were used to compare between locations and genotypes. A preliminary statistical analysis was performed using different usual models (counts of hours above a given threshold; growth-compatible degree days, parameterized with different thresholds; Pouget 1964; Bidabe 1967; Landsberg 1974; Sarvas 1974; Anderson et al. 1986; Hanninen 1990).

#### Frost hardiness tests

Different segments from 1-year-old twigs were used at each sampling date to assess frost hardiness by the electrolyte leakage method (Zhang and Willison 1987; Sutinen et al. 1992). In 2007–2008, stems were sampled every month from 5 December through budburst in lowland location. On 2008–2009, sampling started 15 September through budburst on mountain and lowland locations. From February through budburst, sample dates were closer (between 15 and 20 days). Each 1-year-old twig was cut into six 5-cm long segments without buds per date. All segments were washed quickly in distilled-deionised water, wrapped in a moistened tissue that was then wrapped in aluminum foil. Shoot segments were cooled down to one of four sub-zero temperatures. For temperature-controlled boxes, cooling and warming cycles were computer-controlled by a circulator bath (Ministat Huber, Offenburg, Germany) with external

Pt100 into the chamber. A steady rate of cooling and thawing of  $5^{\circ}\text{C h}^{-1}$  and with freeze-thaw cycle between  $+5^{\circ}\text{C}$  to  $-5^{\circ}\text{C}$ ,  $-10^{\circ}\text{C}$ ;  $-20^{\circ}\text{C}$  and  $-30^{\circ}\text{C}$ , respectively, was applied. Before thawing, the air temperature was maintained for 1 h at the freeze temperature. Temperatures were recorded with a data logger (Campbell, Logan, UT) as 1-min averages. In addition, there was an unfrozen control in a cold room at  $+5^{\circ}\text{C}$  (control) and a lethal control in a deep freezer at  $-80^{\circ}\text{C}$ . In this case, sample temperatures were monitored using copper-constantan thermocouples inserted into the foil pouch, and the rate of cooling was  $\approx -7^{\circ}\text{C h}^{-1}$  in pre-chilled vacuum flasks.

After this freezing treatment, the segments were cut into 5 mm-long sections and placed in glass vials with 15 ml distilled-deionized water. The vials were shaken for 24 h at  $+5^{\circ}\text{C}$  (to limit non-frost-induced lysis) on a horizontal gravity shaker (ST5, CAT, Germany). The electrolytic conductivity of the bathing solution ( $C_1$ ) was measured at room temperature with a conductimeter (Held Meter LF340, TetraCon<sup>®</sup> 325, Germany).

Samples were then autoclaved at  $+120^{\circ}\text{C}$  for 30 min, cooled down to room temperature, and a second conductivity measurement ( $C_2$ ) was taken. Relative electrolytic leakage ( $REL$ ) was calculated as  $(C_1/C_2)*100$  as described in Zhang and Willison (1987). We assumed the following relationship between  $REL$  and percentage of cellular lyses ( $L$ ) for each sample:

$$REL = A / \left( 1 + e^{B(C-x)} \right) + D \quad (1)$$

where  $x$  is the test temperature. The parameters  $A$  and  $D$  define the asymptotes of the function, and  $B$  is the slope at the inflection point  $C$ . The frost hardness level ( $LT_{50}$ ) was estimated as the temperature abscises of the inflection point ( $C$ ) of the adjusted logistic sigmoid function in Eq. 1 (Repo and Lappi 1989). Parameter estimation was performed by nonlinear regression using ExcelStat ver. 7.5.2. Mean  $LT_{50}$  was calculated for each treatment from the individual  $LT_{50}$  values.

#### Statistical analysis

Mean comparison was performed by variance analysis (ANOVA) with subsequent post-hoc multiple comparison test of Tukey-HSD (honestly significant difference) at the significance threshold of  $P=0.05$  using ExcelStat ver. 7.5.2. The respective significance of environmental vs genotypic effects was evaluated by covariance analyses (ANCOVA). The quantitative co-variable was Julian day or chilling units, and qualitative factors were genotype or location. In the results, the proportion of total variance explained by each factor was calculated from sums of squares.

## Results

### Phenology and thermal requirements of buds

#### *Phenological difference between genotypes*

As shown in Table 1, differences in date of budburst reached up to 50 days, among genotypes for Lowland in 2009: from April through June. For each genotype, the different replicates are bud bursting within a few days in the same location: from a 3–4 day spread for wood walnuts to less than 2 or even 1 day for fruit walnuts. We observed significant differences between apical and lateral buds (between 4 and 8 days delay;  $P=0.007$ ) observed in both years.

In the mountain location, Serr and Chandler were not able to survive winter conditions. All other genotypes showed delayed budburst in comparison with Lowland (around 20 days) except L-Hybrid which showed a similar day of budbreak in both climatic conditions.

Day of budburst was similar for most genotypes in both years. Earliest genotypes, however, exhibited a difference between the two years, especially Serr in which buds burst 2 weeks later in 2008 than in 2009. A severe freezing event that occurred just before budburst may explain this between-year difference. Also, after this late frost in 2008, Chandler, Lara and Serr were not significantly different ( $P>0.791$ ) whereas in the freeze safe year (2009), differences were significant between Lara and Serr ( $P=0.036$ ).

#### *Temperature requirements*

Chilling requirements were measured as the chill exposure duration after which no significant difference was observed in the time spent at  $25^{\circ}\text{C}$  until budbreak. Between genotypes, no differences in chilling requirement, calculated as a count of hours cooler than  $7.2^{\circ}\text{C}$  (Weinberger 1950), were observed (less than 36 h: Table 1); between 900 and 950 hours of chilling are required for endodormancy release. Similar results are observed using other models.

Nevertheless, after the chilling requirement was satisfied, the MTB at  $25^{\circ}\text{C}$  was clearly different between genotypes (Table 1); earlier genotypes required only 470 h whereas later ones required 728 h. This suggests that most of the difference in the observed day of budburst was generated during the ecodormancy stage. The temperature response on those genotypes was monitored during this stage at between 5 and  $25^{\circ}\text{C}$  (Fig. 2).

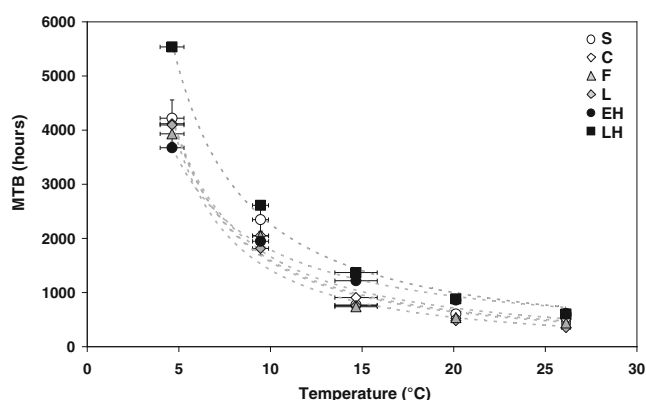
The relationship between temperature and delay to bud break is non linear, with a clearly higher efficiency of

**Table 1** Day of budbreak in walnut tree (*Juglans* sp.) genotypes in two locations in central France for lateral buds, in Julian day $\pm$ standard deviation; different lower case letters indicate significant groups ( $\alpha=0.05$ ) within year/location by Tukey test. MTB Mean time until budbreak

Genotype	Day of Budbreak (average $\pm$ Standard Deviation)			Chilling hours until endodormancy release	MTB after chilling requirements satisfied (hours at 25°C)
	Lowland		Mountain		
	2008	2009	2009		
Serr	117 $\pm$ 1.43 c	102 $\pm$ 0.58 d		924	470 c
Chandler	115 $\pm$ 0.70 c	111 $\pm$ 0.39 cd		924	477 c
Lara	116 $\pm$ 0.16 c	117 $\pm$ 1.72 bc	139 $\pm$ 3.97 b	905	559 c
Franquette	126 $\pm$ 1.40 b	125 $\pm$ 1.31 b	142 $\pm$ 0.44 b	905	543 c
E-Hybrid	129 $\pm$ 2.41 b	128 $\pm$ 4.36 b	141 $\pm$ 2.26 b	941	662 b
L-Hybrid	163 $\pm$ 4.54 a	155 $\pm$ 3.80 a	154 $\pm$ 3.64 a	940	791 a

warmer temperature. These results fitted a power function (Rate=a. $\theta^b$ ), with  $R^2$  ranging from 0.903 (Chandler) to 0.982 (L-Hybrid) (Table 2).

Depending on genotype, the relationship between temperature and growth rate of buds is not as obvious as suggested by the observed phenological differences. Highly significant differences are observed between wood-oriented and fruit-oriented genotypes, with higher growth rates in fruit-oriented genotypes at warm temperatures (15°C and more). Within fruit-oriented genotypes, no significant differences were observed at all temperatures tested (except Lara vs Serr, at most temperatures). On the other hand, wood walnuts appeared to grow at a similar rate at warm temperatures and differences between them occurred only at cold temperature (5 and 10°C).

**Fig. 2** Mean time until budbreak (MTB) after endodormancy release in six different walnut tree genotypes depending on temperature. Dashed lines Fitted power function: Late-Hybrid (LH):  $R^2=0.982$ ; E-Hybrid (EH):  $R^2=0.928$ ; Chandler (C):  $R^2=0.903$ ; Franquette (F):  $R^2=0.914$ ; Lara (L):  $R^2=0.940$ ; Serr (S):  $R^2=0.916$ . Error bars Standard error

### Cold acclimation/deacclimation

**From autumn to full acclimation** Frost acclimation changes were evaluated in parallel on twigs from the same trees. Throughout the acclimation process (between September and January), no significant difference was observed between either Lara and Franquette ( $P=0.994$ ) or E-Hybrid and L-Hybrid ( $P=0.512$ ) (Fig. 3a), so Lara and Franquette, and E-Hybrid and L-Hybrid, were bulked as fruit-oriented and wood-oriented genotypes, respectively, in all subsequent statistical analysis. Fruit- and wood- walnuts were significantly different ( $P<0.0001$ ).

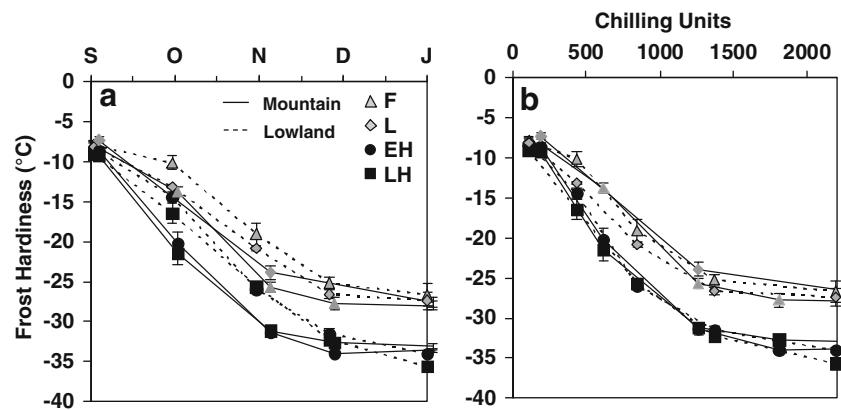
The level of frost hardiness between genotypes and locations prior to acclimation (15 September) was not significantly different ( $P=0.144$ ). During hardening, large differences were observed ( $P<0.0001$ ), explained by both genotype (55.2% of variance) and location (34.8%). Moreover, at maximum hardiness level (beginning of January), differences were still observed between wood and fruit walnuts ( $-35^\circ\text{C}$  vs  $-28^\circ\text{C}$ ) but much less between locations ( $LT_{50}=-34.9^\circ\text{C}$  in lowland and  $-34.3^\circ\text{C}$  in

**Table 2** Relation between temperature ( $\theta$ ) and MTB after endodormancy release in six different walnut tree genotypes (two wood walnuts *J. regia* x *nigra* (E-Hybrid: Early Hybrid and L-Hybrid: Late Hybrid) and four fruit walnuts *J. regia* (Chandler; Franquette; Lara and Serr))

Genotypes		Relation	$R^2$
Wood walnuts	L-Hybrid	$34,263.0 \cdot \theta^{-1.180}$	0.982
	E-Hybrid	$15,622.0 \cdot \theta^{-0.941}$	0.928
Fruit walnuts	Chandler	$29,248.0 \cdot \theta^{-1.264}$	0.903
	Franquette	$30,748.0 \cdot \theta^{-1.293}$	0.914
	Lara	$34,636.0 \cdot \theta^{-1.388}$	0.940
	Serr	$27,431.0 \cdot \theta^{-1.217}$	0.916



**Fig. 3** Frost resistance variation of different walnut genotypes (Franquette: F, Lara: L, Early hybrid: EH and Late hybrid: LH) in two locations in central France: Crouël (alt: 340 m a.s.l., dashed line) and Theix (alt: 870 m a.s.l., solid line) as plotted vs **a** calendar date and **b** chilling units (from Landsberg). Symbols and bars represents respectively mean and standard error calculated on  $n=5$  replicates



mountain for wood and  $-27.0^{\circ}\text{C}$  in lowland and  $-27.8^{\circ}\text{C}$  in mountain for fruit;  $P<0.0001$ : genotypes 78.6% of variance, location 5.7%).

In order to compare and explain the source of differences between locations during acclimation, chilling accumulation was calculated from 15 September as evaluated according to different chilling models. Thereafter, ANCOVA was performed using cumulative chilling units as a quantitative co-variable (chilling time), and compared to the simpler ANCOVA using date as a quantitative co-variable (Tables 3 and 4). Compared to Julian days, use of chilling time explained a little bit less variance for most models, but was still highly significant ( $P<0.0001$ ). Between genotypes (wood- vs fruit-), the difference was similar (between 9.1 and 9.6% of total variance), but most of the difference between locations disappeared using

chilling time for all chilling models except Sarvas and Weinberger. Thus,  $LT_{50}$  evolutions became synchronised, especially with the Landsberg model (0.05% of variance explained by variance), as shown in Fig. 3b.

*From full acclimation until budburst* During deacclimation, three different factors interacted differently: genotype, year and location (Fig. 4). Analysis of covariance depending on calendar date was performed. Differences between years were significant, according to Tukey's test, for Lara ( $P=0.001$ ) and Franquette ( $P=0.016$ ) but neither for E-Hybrid ( $P=0.269$ ) nor L-Hybrid (0.378). Significant differences were observed among locations for Franquette ( $P=0.0002$ ) and L-Hybrid ( $P=0.021$ ), but not for Lara (0.998) and E-Hybrid ( $P=0.787$ ). The same procedure as shown for cold acclimation evolution was applied to cold deacclimation dynamics. Analysis of

**Table 3** Analysis of covariance between frost hardiness of different walnut genotypes ( $LT_{50}$  calculated by electrolyte leakage method) during endodormancy period, with genotypes and locations as

qualitative variable and date or chill units as quantitative variable calculated from 15 September until full acclimation. SCR Signal to clutter ratio

		Julian day	Bidabe	Dynamic	Hanninnen	Landsberg	Utah	Sarvas	Weinberger
$R^2$		0.950	0.931	0.948	0.929	0.937	0.933	0.909	0.903
SCR		390.8	531.0	406.5	550.0	490.9	515.8	702.2	751.6
$P<F$		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Variation Source	Quantitative variable	79.83% ****	82.67% ****	82.68% ****	82.19% ****	83.12% ****	81.64% ****	77.90% ****	76.18% ****
	Genotype	9.55% ****	9.17% ****	9.50% ****	9.25% ****	9.38% ****	9.11% ****	9.26% ****	9.23% ****
	Location	4.13% ****	0.20% ns	1.23% ****	0.35%*	0.05% ns	1.31% ****	3.08% ****	4.22% ****
	Varieties* location	0.03% ns	0.02% ns	0.02% ns	0.01% ns	0.01% ns	0.01% ns	0.00% ns	0.00% ns
	Quantitative variable* genotype	0.91% ****	0.83% ***	0.87% ****	0.79%***	0.83%***	0.79% ***	0.68% ns	0.66% ns
	Quantitative variable* location	0.50%**	0.26%*	0.46%**	0.31%*	0.27%*	0.49%**	0.02% ns	0.00% ns

\*\*\*\*  $P<0.0001$ ; \*\*\*  $P<0.001$ ; \*\*  $P<0.01$ ; \*  $P<0.05$ ; ns  $P>0.05$

**Table 4** Analysis of covariance between frost hardness of different walnut genotypes ( $LT_{50}$  calculated by electrolyte leakage method) during the ecodormancy period, with genotypes and locations as

qualitative variable and date or forcing units as quantitative variable calculated from 15 September until full acclimation. Experimental data refers to fitted equation shown in Table 2

		Julian Day	Bidabé	Hanninen	Landsberg	Anderson	GDD5	Sarvas	Exp. data
$R^2$		0.937	0.877	0.880	0.833	0.799	0.822	0.886	0.873
SCR		1,190.186	2,312.751	2,268.092	3,149.412	3,792.378	3,354.863	2,142.319	2,389.205
$P < F$		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Variation Source	Quantitative variable	78.16% ****	68.44% ****	68.71% ****	61.88% ****	57.16% ****	59.95% ****	70.07% ****	75.82% ****
	Genotype	12.03% ****	15.47% ****	15.22% ****	14.71% ****	15.27% ****	15.19% ****	14.95% ****	5.69% ****
	Location	1.46% ****	1.27% ****	1.71% ****	4.46% ****	4.28% ****	4.19% ****	1.60% ****	2.30% ****
	Genotype* location	0.74% ****	1.61% ****	1.55% ****	1.47%***	1.75% ***	1.70% ****	1.40% ****	1.17% ****
	Quantitative variable* genotype	1.27%****	0.33% ns	0.27% ns	0.23% ns	0.62% ns	0.46% ns	0.22% ns	2.10% ****
	Quantitative variable* location	0.02% <sup>ns</sup>	0.62%***	0.51%***	0.55%**	0.82%**	0.72%**	0.41%**	0.25%*

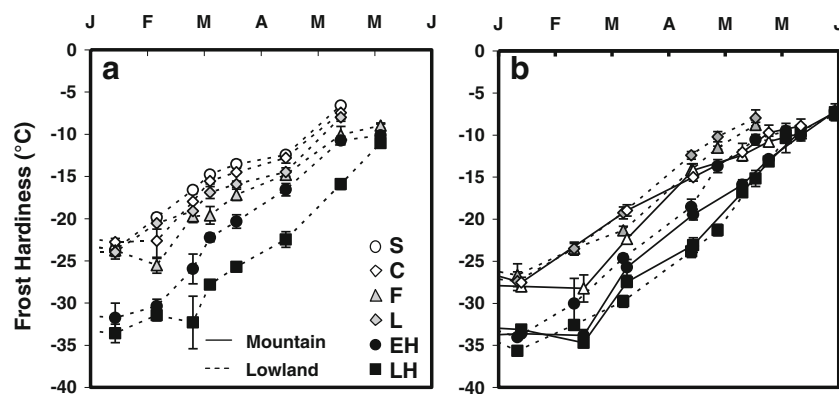
\*\*\*\*  $P < 0.0001$ ; \*\*\*  $P < 0.001$ ; \*\*  $P < 0.01$ ; \*  $P < 0.05$ ; ns  $P > 0.05$ 

covariance using different thermal times classically used for growing stages or depending on the response to temperature observed for buds (experimental data, Fig. 2) was performed in order to decrease difference among locations. However, none of these analyses (Table 4) were more significant than the simple calendar date (Table 3). Correlation between date and frost hardness was linear for all genotypes and highly significant ( $P < 0.0001$ ;  $R \geq 0.924$ ). Analysis of covariance indicates that the slope is similar among four different fruit walnuts, whereas wood walnuts deacclimated significantly faster ( $P < 0.0001$ ). At the last sampling date, in June, all genotypes had similar frost resistance of around  $-10^\circ\text{C}$  at the day of budbreak.

## Discussion

### Endodormancy and cold acclimation

Dormancy induction is considered as a prerequisite to cold acclimation (Weiser 1970). Moreover, it has been shown in many species that photoperiod decrease is the main driving factor in both endodormancy (Moshkov 1935; Bogdanov 1931; Welling et al. 1997) and cold acclimation inductions (Irving and Lanphear 1968; Weiser 1970; Aronsson 1975; Christersson 1978). In our study, in both locations, the photoperiod is similar (same latitude, 20 km distant) but the climate is really different (around  $3^\circ\text{C}$  less at the higher elevation). Cold resistance was similar in mid-September



**Fig. 4** Frost resistance variation of different walnut genotypes (Franquette: F, Lara: L, Early hybrid: EH and Late hybrid: LH) in two locations in central France: Crouël (alt: 340 m a.s.l., dashed line) and Theix (alt: 870 m a.s.l., solid line) during ecodormancy stage:

$LT_{50}$  calculated by electrolyte leakage method (y-axis) and calendar date (x-axis) in 2008 (a) and 2009 (b) in both locations (lowland and mountain: dashed lines and solid lines, respectively). Symbols and bars represent mean and standard error from  $n=5$  replicates

(Fig. 3). Thus, endodormancy release and cold acclimation processes likely started at the same time in both locations and genotypes.

Short day signal alone is only sufficient to provide a first level of hardiness (Howell and Weiser 1970; Schwarz 1970; Greer and Warrington 1982), e.g., to around  $-20^{\circ}\text{C}$  in *Picea sitchensis* (Cannell et al. 1985). When endodormancy and cold acclimation processes are enabled, chilling temperature becomes the main driving factor (Sakai and Larcher 1987). Proteins synthesized during the first stage (short-days-induced) will allow, during the second stage of hardening, synthesis of cryoprotectants such as sugars (Guy et al. 1992; Taji et al. 2002), solutes or proteins (Xing and Rajashekar 2001).

Freezing temperatures trigger deeper frost resistance than chilling temperatures (Glerum 1973). In this study, a faster rate of cold acclimation was observed in the colder climate: Mountain vs Lowland but maximal hardiness in deep winter was equal, as reported in Dantuma and Andrews (1960) and Pogosyan and Sakai (1969).

Maximal cold hardiness (in January) was similar within fruit walnuts or within wood hybrid walnuts but not between. Wood walnut, a hybrid between *J. regia* and *J. nigra* (more frost resistant), is more frost resistant than *J. regia* (Poirier et al. 2010). However, even if potential maximal hardiness is different, the time course for acclimation is similar. In other species, several studies reported inter-population variation in cold hardiness during autumn (acclimation) or spring (deacclimation). However, no significant differences were observed either in summer (minimal hardiness period) or winter (maximal hardiness period) in *Pinus sylvestris* (Nilsson and Walfridsson 1995) or *Pinus albicaulis* (Bower and Aitken 2006).

In order to compare both locations, we calculated accumulated chilling units according to Landsberg's model (Landsberg 1974), from September until maximal hardiness was reached (Table 3, Fig. 3b). In this case, differences between locations were no longer observed. Thus, differences in the cold acclimation process between both sets of environmental conditions were of strict thermal origin in all different genotypes. Moreover, small differences were observed in chilling requirement (around 900–950 hours of chilling; Table 1), which is in agreement with simulated data from Luedeling et al. (2009). Thus, it appeared that neither endodormancy release nor cold acclimation evolution were genotypically different despite the large differences in budburst dates. Some intraspecific differences in chilling requirements have however been observed in peach trees (Linsley Noakes and Allan 1994), and also in walnut trees (Aslamarz et al. 2010). Conversely, in our study, we observed similar chilling requirements among genotypes. Chilling temperatures should be considered as the main

driving factor for these processes under strict environmental, even thermal, control.

In the context of global warming, some studies have reported that the date of endodormancy release could be delayed significantly (Legave et al. 2008). It is also likely that the onset of endodormancy should be delayed mainly for species that are poorly sensitive to photoperiod. But very few reports are available in the literature. Regarding cold acclimation, hardening could be affected significantly (Pogosyan and Sakai 1969) but would still be possible (Charrier and Améglio 2011). We can hypothesize that the differences among walnut genotypes should not be impacted significantly during cold acclimation and endodormancy release.

#### Ecodormancy and cold deacclimation

After endodormancy was released, huge differences among genotypes in sensitivity to forcing temperatures appeared (Table 1). The earlier the genotype in natura, the lower the MTB at  $25^{\circ}\text{C}$  after endodormancy release [470 h in earlier (Serr and Chandler) and 550 h in later fruit walnut trees (Lara and Franquette), which is consistent with earlier studies on different walnut trees (Mauget and Germain 1980; Mauget 1981)]. This was also observed within wood walnuts (660 vs 730 h).

This relationship between growth rate and temperature is non linear (Fig. 1) consistent with earlier reports (Myking 1999; Sarvas 1974), although some authors use linear response curve (Karlsson et al. 2003; Linkosalo et al. 2009; Clark and Thompson 2010). This linear approach has been contested for years (Arnold 1959; Wang 1960; Cannell and Smith 1983). This is indeed likely to be due to the relationship with the underlying metabolism driving bud growth, and differences in temperature sensitivity between genotypes, which are not linear processes. Lots of models have been published predicting phenology of trees (e.g., Fuchigami et al. 1982; Kellomaki et al. 1992; Linkosalo et al. 2000; Rea and Eccel 2006) and have used a linear relationship between time to budbreak and temperature (Cannell and Smith 1983; Linkosalo et al. 2009; Clark and Thompson 2010). As indicated by Cannell and Smith (1983), a non-linear response “would not invalidate the use of thermal time if there were temperatures without any marked tendency to increasing temperatures”. But, with global change, this approximation could lead to large discrepancies between predicted and actual future phenology.

Among fruit walnuts, for most temperatures, only a few significant differences were observed in growth rate. They involved mainly the Serr genotype, which seemed to grow slower than Lara whereas its budburst occurred earlier. Thus, growth rate in ecodormancy cannot explain pheno-



logical deviation among fruit walnut cultivars. But, among wood walnuts, under warm temperatures, growth rate is similar; in colder temperatures, the growth rate of the earliest hybrids is higher than that of later hybrids. Thus, below 15°C, the growth rate of early hybrids was similar to that of fruit walnuts. After endodormancy release, while temperatures were still cold, earlier hybrids are able to grow faster than later ones. Thereafter, in milder temperatures, the growth rate was similar but phenological deviation between wood walnuts already existed.

Trends in cold deacclimation highlighted the difference between fruit and wood walnuts (Fig. 4). Some studies observed that the evolution of cold deacclimation is correlated with budbreak time in natural populations (Hannerz 1999). Indeed, between locations, deacclimation started later in mountain climate, limited by cold temperatures. Two differences were observed between fruit and wood walnuts: the rate of deacclimation was significantly different, with wood walnuts deacclimating faster but starting at a lower level. Then, among fruit walnut, or also among wood or fruit walnut, earlier genotypes were deacclimating with the same rate but a little bit earlier. In some studies on *Pseudotsuga menziesii*, it was observed that earlier genotypes deacclimate faster (Jermstad et al. 2001, O'Neill et al. 2000). Nevertheless, the heritability of spring frost resistance is higher than for autumn resistance (Anekonda et al. 2000; Aitken and Adams 1997). We can hypothesize that sensitivity to temperature is different and probably linked to inherent metabolic activities. During cold deacclimation, starch reserves, used initially as substrate for acclimation, are re-synthesized (Witt and Sauter 1994a, 1994b; Essiamah and Eschrich 1985). Alpha amylase involved in carbohydrate metabolism could be the critical factor in this process (Schrader et al. 2004). Indeed, genetic analysis identified this enzyme as a strong quantitative trait locus (QTL) for budburst date in different *Quercus petrae* altitudinal populations (Derory et al. 2006, 2009). Thus, among fruit or among wood walnuts, differences in the activation energy of isoenzymes could trigger metabolic activities in colder conditions for earlier genotypes, and thus, in fruit walnuts, enzymatic activity could be higher than that in wood walnuts. These hypotheses could better explain why we observed budburst when an LT<sub>50</sub> value reached around -10°C, because of the strong link between frost hardiness and carbohydrate content (Sakai and Larcher 1987).

The environmental response of cold deacclimation is not totally clear among genotypes. Some interactions were indeed observed between genotype and location: for L-Hybrid or Franquette but not for Lara and E-Hybrid; or between genotype and year: for Lara and Franquette but

not E-Hybrid and L-Hybrid. These differences among location and years could not be explained by temperature alone, but maybe through a photoperiod effect, acting as a forcing factor, whereas temperature was limiting. Recently, Korner and Basler (2010a) argued that “phenological events (...) are not primarily controlled by temperature”, which initiated a controversy (Chuine et al. 2010, Korner and Basler 2010b). These results serve to show that temperature is not the only factor involved in differences between locations. But, if there is a photoperiod effect, this could better explain why late hybrids are bud bursting at similar dates at different locations (Table 1).

Our results appear to contradict several earlier studies on fruit trees. Couvillon and Erez (1985) proposed that, in fruit tree cultivars, there is no genetic difference in heat requirement during ecodormancy, but Citadin et al. (2001) showed the reverse. A negative correlation between chilling requirement and heat requirement has been reported in apricot (Ruiz et al. 2007). Our findings are supported by experiments in which endodormancy release dates were similar between walnut cultivars whereas budburst dates were highly different (Mauget and Germain 1980; Mauget 1981). Several studies concluded that frost acclimation and endodormancy release were strongly related (Fuchigami et al. 1982; Leinonen 1996; Druart et al. 2007). Moreover, strong genetic control of spring cold hardiness was observed in natural populations, while for autumn the cold hardiness relationship is weak (Aitken and Adams 1997).

According to our results, we could expect that, in the future, the phenological response of walnut would differ significantly among genotypes, exacerbating the range of deviation. This could lead to increasing freezing damage in the earliest genotypes, mostly through earlier exposure of buds and new leaves. But, this impact could be mitigated if endodormancy release is delayed enough to result in delayed budburst. More likely there will be extended budbreak and flowering periods, and maybe some problems for pollination if male and female flowers do not respond exactly in the same way to increasing temperatures. This point should be investigated as it is not certain that we can continue to consider the whole population of buds as homogeneous regarding their response to temperature. Interaction between genotype and environment (temperature but also photoperiod) should also lower these risks.

## Conclusion

In this study, two main frost resistance mechanisms were monitored: frost avoidance and frost tolerance. Both

processes appeared to be similarly driven. First, during endodormancy and cold acclimation, they were strictly environmentally driven. Thereafter, during ecodormancy and cold deacclimation, beside environmental effects, a strong genotypic effect was identified. During this stage, these two driving factors interact within the same species, *J. regia* L. This explains why we observed a deviation in budbreak date in walnut trees. These results are of great importance in modelling approaches to predict dates of budburst in the coming decades. Thus, during the endodormancy/acclimation stage, climatic data only would be sufficient as a parameter, but in ecodormancy/deacclimation, genotypic differences have to be taken into account, probably through metabolism and expression of some related protein. This will need further experiments.

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