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Research paper

Frost hardiness in walnut trees (*Juglans regia* L.): How to link physiology and modelling?

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In the literature, frost hardiness (FH) studies in trees have often been restricted to one organ (buds, leaves, needles or twigs). To extend our knowledge and gain a unified view, FH differences between organs and tissues or throughout the life of the tree have to be characterized in relation to physiological changes. In this study, different organs and tissues of young potted and mature orchard walnut trees (*Juglans regia* L.) were compared for seasonal changes in FH during different years. FH was assessed using the electrolyte leakage method. Physiological parameters were concomitantly monitored focusing on two significant traits: water content (WC) and carbohydrate content (glucose + fructose + sucrose, GFS). No seasonal variation in FH was observed in the root system, but acclimation and deacclimation were observed aboveground. Among organs and tissues, cold sensitivity levels were different in deep winter, with buds most sensitive and bark most resistant, but acclimation/deacclimation dynamics followed similar patterns. Physiological variation was also similar among organs: FH increased when WC decreased and/or soluble carbohydrates increased. Based on these results, relations between soluble carbohydrate content, WC and FH were calculated independently or in interaction. The key results were that: (i) the relationship between FH and physiological parameters (GFS and WC), which had previously been shown for branches only, could be generalized to all aboveground organs; (ii) lower WC increased the cryoprotective effect of GFS, showing a synergic effect of the two factors; (iii) the best fit was a non-linear function of WC and GFS, yielding a predictive model with an root mean square error of 5.07 °C on an independent dataset and 2.59 °C for the most sensitive stages; and (iv) the same parameters used for all organs yielded a unified model of FH depending on physiology, although the variability of GFS or WC was wide. The model should be of value for predicting FH in walnut independently of previous growing conditions (i.e., after sublethal stress accumulation).

Keywords: carbohydrates, water content.

Introduction

To survive low winter temperatures, woody plants in temperate zones have to develop freezing resistance. Whereas extracellular freezing is generally well tolerated by frost-resistant plants, the critical factor is the ability to prevent intracellular freezing, which in natural conditions usually proves lethal (Levitt 1980). From autumn until spring, trees are able to transiently modulate their frost resistance (Sakai and Larcher 1987).

Frost resistance is triggered by a decrease in photoperiod and temperature as the growth season ends (Weiser 1970, Aronsson 1975). Parallel dynamics of decreasing temperature and increasing frost resistance are observed in autumn (Greer et al. 2000, Luoranen et al. 2004). This has allowed the development of models (such as in Greer and Warrington 1982,

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Leinonen 1996) where low temperatures (freezing or chilling) promote the resistance increase, whereas mild temperatures tend to promote deacclimation (reduction of acclimated frost resistance). However, various studies have shown that the relationship between frost resistance and temperature is not straightforward (Schwarz 1970, Charrier and Améglio 2011).

Walnut trees (*Juglans regia* L.) can exhibit different levels of frost resistance under the same thermal conditions (including constantly mild temperatures). It has been shown that water content (WC) and soluble carbohydrate (glucose + fructose + sucrose, GFS) content could explain most of the temporal variability in frost resistance (Poirier et al. 2010, Charrier and Améglio 2011).

During the process of frost acclimation, plant tissues exhibit a decrease in free WC (Pavel and Fereres 1998, Ewers et al. 2001). The decrease in soil temperature decreases membrane permeability, so water supply via the root system gradually diminishes, while the water continues to evaporate in aerial parts, leading to tissue dehydration (Turcotte et al. 2009).

Trees simultaneously synthesize soluble compounds from the reserves accumulated during the growing season (Sakai and Yoshida 1968). These compounds exert a protective role through several effects: osmosis (lowering the freezing point of cytosol), cryoprotection (stabilizing the solvation layer of macromolecules) and increasing viscosity. The major compounds are directly linked to specific metabolism: e.g., polyols (sorbitol) in mountain ash and Rosaceae, lipids and amino acids in conifers (Sakai 1962) and GFS in walnut (Améglio et al. 2004) and *Arabidopsis thaliana* (L.) Heynh. (Zuther et al. 2012).

Recent developments in climate simulations suggest that winter temperatures should get milder in the future (Christensen and Christensen 2007), which makes predicting the frost hardiness (FH) of trees a challenge for ecology, forestry and agriculture. In some cases, meteorological factors are not sufficient to explain hardening of trees: depending on treatment, differences in FH were observed across different branches from the same tree (Poirier et al. 2010); depending on the timing of leaf fall, cold-deprived trees were able or unable to harden (Charrier and Améglio 2011). These two studies emphasized the importance of physiological factors (WC, starch and soluble carbohydrates) at the onset of frost hardening. Furthermore, most studies have tended to focus on mature tree branches or seedlings, but economic consequences may be dramatically different depending on the injured part. Here, our aim was to develop a robust mechanistic model for FH, using physiological parameters monitored in various organs. This could clarify the determinism of FH in plants. To meet this objective, we (i) characterized the variability of these parameters according to year, organ, age and season, (ii) explored and unified the relationship between these parameters and FH, and (iii) investigated the percentage of variability explained by this unifying model.

Materials and methods

Frost hardiness tests, WC and soluble carbohydrate content measurements were performed on several organs from potted trees and branches from orchard trees (see below).

Potted trees

Different 2-year-old potted walnut trees (*J. regia* cv *Franquette*) were observed during 3 years (2004–05–06 and 2007–08). Samples ($n = 3$ trees per date in 2004–05 and 2007–08, $n = 5$ trees per date in 2005–06) were harvested in autumn (23 October 2004, 17 October 2005 and 30 October 2007), winter (25 January 2005, 27 January 2006 and 8 January 2008) and spring (1 April 2006 and 21 April 2008). At each sampling date, trees were collected, and different organs (fine roots, coarse roots, tap root, trunk and branches) were independently assessed for WC, carbohydrate and FH measurements.

Orchard trees

Branches were sampled on mature walnut trees (15 trees) grown in an orchard in central France (E 03°08′50″, N 45°46′20″, 340 m above sea level (a.s.l.)). Samples ($n = 5$) were collected from autumn to spring at dates similar to the potted trees experiment (22 October 2008, 19 January 2009, 27 April 2009), and during deacclimation (17 March 2009) and growing season (3 August 2009). Branches were separated into bark + cambium, wood and buds, and analysed independently. Whole branches (bark and wood mixed, without buds) from the same trees were analysed for comparison with existing literature data (15 September 2008, 13 October 2008, 12 January 2009, 17 March 2009, 28 April 2009).

Frost hardiness tests

Different segments from 1-year-old branches or parts from other organs/tissue (fine roots, coarse roots, tap root, trunk, bark + cambium, wood and buds) were taken at every sampling date to assess FH using the electrolyte leakage method (Zhang and Willison 1987, Sutinen et al. 1992). One-year-old branches were cut into six segments 5 cm long without buds at each date. Several pieces of thin (Ø <3 mm) and thick (Ø >3 mm) roots of length ~ 5 cm were also taken. For trunks and tap roots, several slices (4 cm thick) were used for each temperature treatment. Buds were kept intact on 1 mm branch segments. Different pieces were either cooled to one of the four sub-zero temperatures or used as one of the two controls (unfrozen and frozen at –75 °C). For temperature-controlled boxes, cooling and warming cycles were computer controlled by a circulator bath (Ministat Huber, Offenburg, Germany) with an external Pt100 probe in the chamber. Freezing was applied at a steady rate of –5 K h^{-1} down to –10 °C, –20 °C, –30 °C and either –5 °C in summer, autumn (until November) and

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spring (from April) or −40 °C in winter. Air temperature was then held at a minimal temperature for 1 h, and thawing rate was 5 K h^{-1} back to 5 °C. Temperatures were recorded with a datalogger (Campbell, Logan, USA) as 1-min averages. In addition, an unfrozen control was stored at +5 °C (control) and another control was stored in a −75 °C freezer with a freezing rate approximately −7 K h^{-1}.

After freezing treatment, branches, coarse roots and fine roots were cut into sections 5 mm long, and trunk and tap root were cut into ~2 mm^3 cubes. Five to 10 buds per temperature were used. Bark + cambium were separated from wood. Samples were transferred into glass vials with 15 ml of distilled-deionized water. Vials were shaken for 24 h at +5 °C (to limit bacterial growth) on a horizontal gravity shaker (ST5, CAT, Staufen, Germany). The electrolytic conductivity of the solution (C_1) was measured at room temperature with a conductimeter (Held Meter LF340, TetraCon® 325, Weiheim, Germany).

After autoclaving at +120 °C for 30 min and cooling down to room temperature, the conductivity was measured again (C_2). Relative electrolytic leakage (REL) was calculated as C_1/C_2 as described in Zhang and Willison (1987). We assumed the following relationship between REL and percentage of cellular lyses for each sample:

$$\mathrm{REL} = \frac{a}{1 + e^{b(c-\theta)}} + d \qquad (1)$$

where θ is the test temperature. Parameters a and d define asymptotes of the function, and b is the slope at the inflection point c. The FH level was estimated as the temperature of the inflection point (c) of the adjusted logistic sigmoid function (Eq. (1) above: Repo and Lappi 1989). Parameter estimation was performed by non-linear regression using ExcelStat ver. 7.5.2.

Water content

Sample fresh matter weights (FM) were measured, and the samples were then frozen with liquid nitrogen. After freeze-drying, dry matter weights (DM) were measured and WC was calculated as: (FM − DM)/DM.

Carbohydrate extraction and quantification

Lyophilized samples ($m > 2$ g) were ground into a powder, which was used (50 mg) to extract and measure the soluble carbohydrate content using high-performance liquid chromatography. Starch content was measured using an enzymatic method. Details are given in Charrier and Améglio (2011).

Statistical analysis

Comparison of means was performed by one-way analysis of variance (ANOVA) after testing for homogeneity of variance with the Brown–Forsythe test, followed when appropriate by the Tukey honestly significant difference post hoc multiple comparison test at the significance threshold of $P = 0.05$, using ExcelStat ver. 7.5.2 software.

Calibration

The relationship between FH, WC and GFS was fitted by minimization of the sum of squared deviations using the linear model function in R software (R Development Core Team 2005). Different equations were tested using non-transformed (WC and/or GFS, models (1–5) in Table 1) or transformed variable (1/*WC* and/or ln(GFS), models (6–10) in Table 1). Transformations of WC or GFS were suggested by graphical interpretation of FH depending on WC or GFS. Significantly better results were obtained with transformed than that with non-transformed variables ($R^2_{WC} = 0.268$ vs 1/WC = 0.399 and $R^2_{GFS} = 0.064$ vs $R^2_{\ln GFS} = 0.069$). The use of non-transformed variables in modelling could be justified, because they could not be considered as Gaussian after the Shapiro–Wilk test (WC: $W = 0.716$, $P < 0.001$; GFS: $W = 0.909$, $P < 0.001$). Conversely, the distribution of transformed variables was not significantly different from Gaussian: 1/WC ($W = 0.994$, $P = 0.30$) or at least closer to Gaussian: ln(GFS) ($W = 0.989$; $P = 0.02$) than non-transformed. Hence transformed variables were suitable for modelling in place of non-transformed variables. The architecture of the model was either strictly additive (models (1–3, 6–8) in Table 1), only through interaction (models (4, 9)) or additive with interaction (models (5 and 10)). The global goodness-of-fit of the model was evaluated by root of mean squared error (RMSE).

Validation

The validation dataset was taken from published data on branches of walnut trees (*J. regia*) cv *Franquette* or *Lara* from similar and colder environmental conditions (another year in the same orchard and in mountain conditions (800 m a.s.l.), from Charrier et al. 2011). Another dataset was taken from potted trees, either cold-exposed or cold-deprived (>15 °C; Charrier and Améglio 2011). Comparison of observed vs predicted FH level as yielded by the best model is shown with root mean square error of prediction (RMSEp) values.

Results

Frost hardiness

Adult orchard tree branch sample tissues were analysed separately (buds, bark + cambium and wood) over a year (Figure 1a). From autumn until spring, the time course of FH appeared to be split into acclimation and deacclimation stages. In October, FH values were similar among tissues (FH ≈ −10 °C, $P = 0.10$), but maximum FH values (in winter) were significantly different. In winter, buds were the most sensitive organs (FH = −18.5 ± 0.4 °C), and bark was significantly more resistant than wood (FH = −30.9 ± 1.4 °C vs −23.0 ± 0.3 °C,

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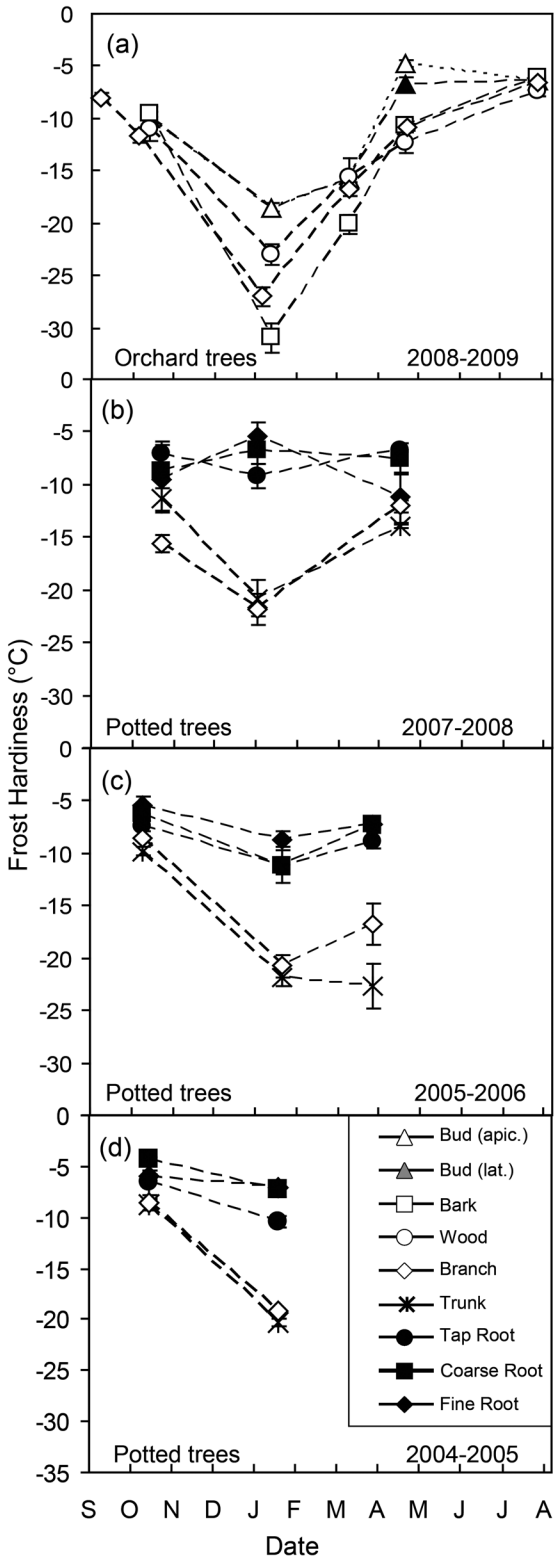

Figure 1. Frost hardiness (estimated by 50% electrolyte leakage) in different organs of young walnut trees (fine roots, coarse roots, tap root, trunk and 1-year-old branch (whole branch or different tissues (wood, bark, buds at budburst) sampled in three different years on adult trees in an orchard 2008–09 (a) and young potted trees in 2007–08 (b), 2005–06 (c) and 2004–05 (d)). The symbols and bars indicate means ± SE.

$P < 0.001$). At whole branch level, FH values were intermediate –27.0 ± 0.8°C).

In spring, buds and wood showed similar levels of hardiness (–15.8 vs –15.6 °C, respectively) while bark remained more resistant (–20.1 ± 1.0 °C). At bud break, the level of hardiness then dropped back to the autumn values (≈ –10 °C) except for the buds, which were extremely frost sensitive (≈ –5 °C). At the end of the growing season, FH values of different tissues were uniform, close to the previous autumn level.

On young trees, experiments were performed during three winters (autumn and winter in 2004–05, autumn, winter and spring in 2005–06 and 2007–08; Figure 1b–d). Despite slightly different sampling dates among years, a general trend emerged: (i) FH was greater in branches in autumn 2007–08 than in earlier years, but this was not observed in all the other organs (roots or trunk); and (ii) there was no significant difference in maximal hardiness during winter. However, dynamics of frost acclimation evolved differently depending on the position in the tree (mostly above- vs belowground). Thus a significant increase in FH between autumn and winter was observed in aboveground parts (branches and trunk), while belowground parts (tap root, coarse roots and fine roots) exhibited no difference, regardless of sampling period within a sampling year, the only exception being coarse roots in 2005–06.

Comparisons were performed on average values for several years and depending on the season and organ. In the aboveground parts, FH levels were comparable between branches and trunk, in autumn (trunk: –9.96 ± 1.6 vs branches: –11.28 ± 3.8 °C, $P = 0.29$) and winter (trunk: –21.1 ± 2.0 vs branches: –20.8 ± 2.6 °C, $P = 0.75$). In spring, the trunks were still hardened, whereas branches were more sensitive (trunk: –20.2 ± 2.2 vs branches: –14.2 ± 1.2 °C, $P = 0.02$). In belowground parts, FH was similar regardless of organ or period. One exception was observed in tap root during mid-winter (FH = –10.4 ± 0.6 °C) relative to fine roots (FH = –7.3 ± 0.6 °C, $P = 0.02$) or tap root during autumn ($P < 0.03$).

Physiology of frost acclimation

Water content

Water content decreased significantly in wood and buds between autumn and winter, and increased from early spring to budbreak, whatever the tissue (Figure 2a). This rehydration diverged strongly between apical and lateral buds, with a 300% increase in WC in the apical part (flushed), but stable WC in lateral buds (before flush). In summer, WC was similar in wood and new buds, while bark was more hydrated.

In potted trees (Figure 2b–d), WC in fine roots could not be measured without disturbing water balance in the tissues. Roots had to be extensively washed with water to remove soil particles. In other organs, almost all different sampling periods showed significant between-year differences (Figure 2). For example, coarse roots during winter were significantly more

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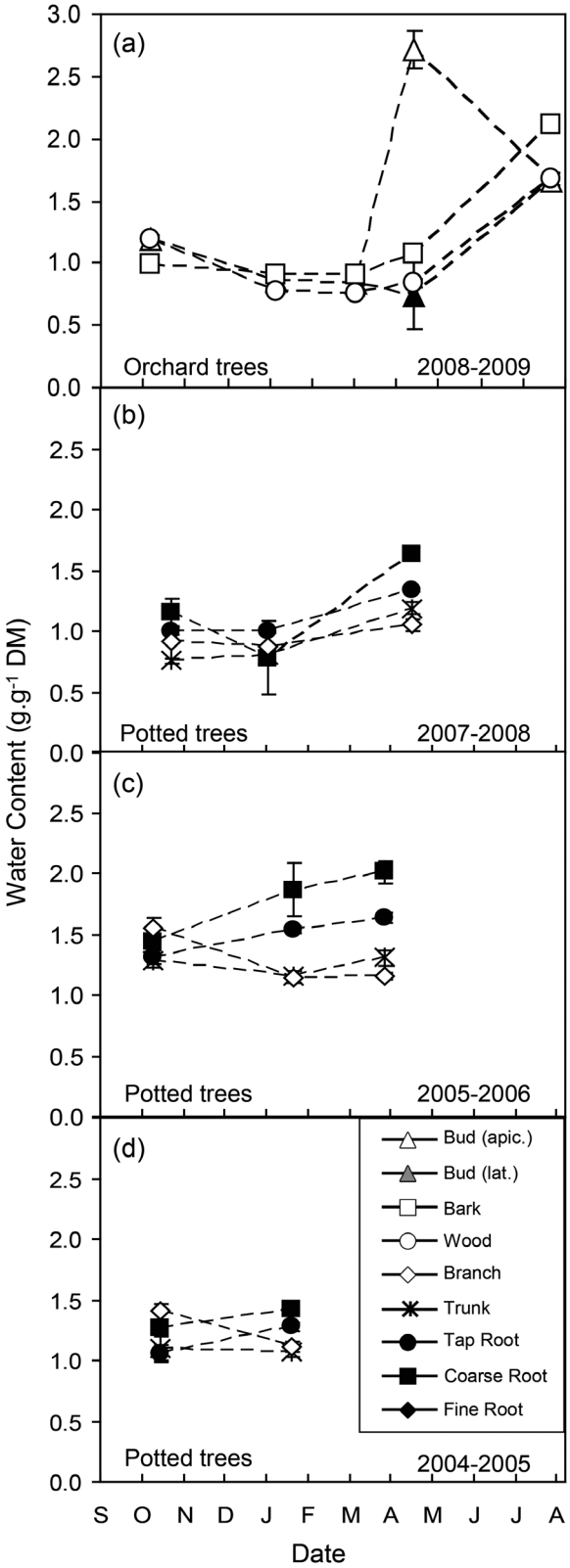

Figure 2. Water content in different organs of young walnut trees (fine roots, coarse roots, tap root, trunk and 1-year-old branch (whole branch or different tissues (wood, bark, apical and lateral buds at bud-burst) sampled in three different years on adult trees in an orchard in 2008–09 (a) and young potted trees in 2007–08 (b), 2005–06 (c) and 2004–05 (d)). The symbols and bars indicate means ± SE.

hydrated in 2006 than in 2008 ($P = 0.02$). Despite these significant between-year differences, trends were observed between organs. Thus a decrease in the WC from roots to shoots was observed.

In autumn, only coarse roots and trunk had a significant WC difference (1.31 vs 1.09; $P = 0.04$). During the winter, underground parts (large and tap root: 1.45 vs 1.32, $P = 0.37$) were significantly different ($P < 0.05$) from aboveground organs (trunk and branches: 1.034 vs 1.035, $P = 0.99$). Finally, in spring, a WC gradient was observed from roots to branches, with significant differences among organs.

WC changes during the leafless period were also organ-dependent. Thus no difference was observed between autumn and winter for coarse roots ($P = 0.45$) and tap root ($P = 0.08$), but a significant increase was observed in spring 2008 (coarse roots: $P < 0.04$; tap root: $P < 0.03$). A similar pattern was also observed for trunk in 2008 with similar WC between autumn and winter ($P = 0.53$) and rehydration in spring ($P = 0.02$). In branches, WC decreased between autumn and winter ($P = 0.01$).

Carbohydrate content

Within a branch, soluble carbohydrate (GFS) content time course (Figure 3a) was similar in bark and buds until bud-break. In wood, after autumn, GFS content was significantly lower for all sampling dates. Starch content decreased significantly in all shoot tissues between autumn and winter, and then rose until budbreak (Figure 3b). During the growing season, starch content decreased in all tissues and reached minimal values, close to the minimum observed in winter. Levels of starch and GFS contents were similar in young potted trees and mature orchard trees.

In underground parts, few inter-annual differences were observed, regardless of sampling period, for GFS content (Figure 3c, e and g) or starch content (Figure 3d, f and h). In aboveground parts, GFS was significantly different ($P = 0.01$) in 2007 samples compared with 2004 and 2005, but only in autumn and spring.

Starch content significantly decreased from underground parts, tap root, then coarse roots and fine roots, to aboveground parts, trunk and finally branches. This pattern was observed whatever the sampling date. Between autumn and spring, there was a ≥50% decrease in starch content in all underground parts. Trunk showed a similar-scale but still non-significant decrease between winter and spring ($P = 0.23$). In branches, after a sharp early decline between autumn and winter ($P < 0.001$), starch content tended to increase between winter and spring, but not significantly ($P = 0.17$).

In parallel with starch dynamics, GFS significantly increased between autumn and winter in all organs. Thereafter, GFS decreased significantly between winter and spring in all organs, reverting to autumn levels.

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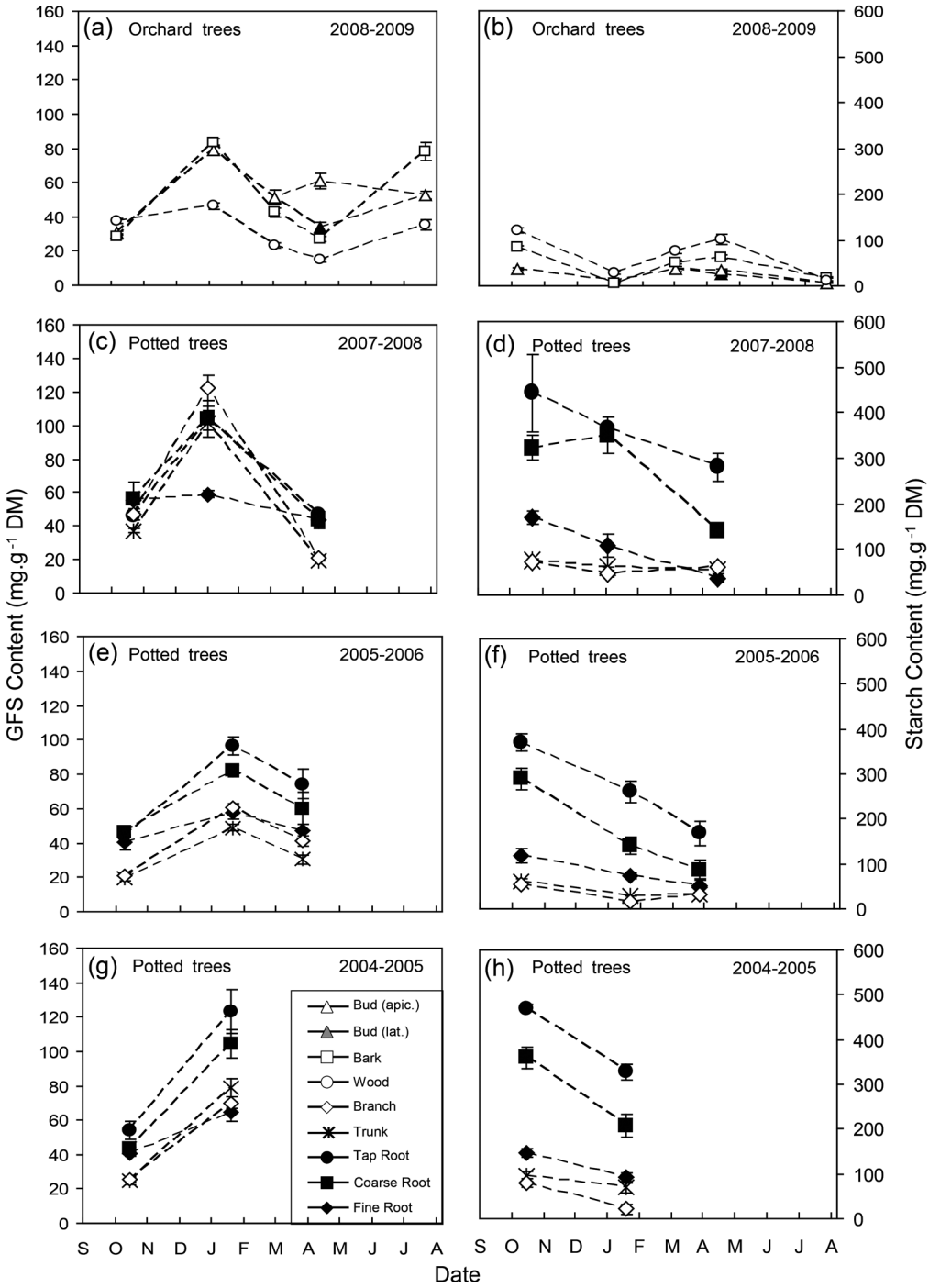

Figure 3. Soluble carbohydrates (GFS: a, c, e and g) and starch content (b, d, f and h) in different organs of young walnut trees (fine roots, coarse roots, tap root, trunk and 1-year-old branch (whole branch or different tissues (wood, bark, apical and lateral buds at budburst) sampled in three different years on adult trees in an orchard in 2008–09 (a and b) and young potted trees in 2007–08 (c and d), 2005–06 (e and f) and 2004–05 (g and h)). The symbols and bars indicate means ± SE.

Relationship between WC, carbohydrate content and FH

The relationship between FH and WC followed an inverse relationship bounded by two asymptotes (Figure 4). For high WC values, FH changed very slightly. This first asymptote (FH ≈ –3 °C) is defined by the minimum observed hardiness. In addition, when FH approached its highest values, WC no longer fell. Thus the second asymptote (WC ≈ 0.45) is in turn defined by the minimum WC values. Accordingly, we fitted the data using WC or transformed 1/*WC* variable (Table 1). Both relationships were significant, but using the transformed variable improved the result ($R^2_{(1)} = 0.266 < R^2_{(6)} = 0.397$).

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FH also depended on GFS (Figure 5a), but the relationship was not so obvious as with WC ($R^2_{(2)} = 0.061$). Even when removing points that potentially disrupted the relationship (sugars recently synthesized during summer), the relationships appeared to be strongly organ- or tissue-dependent. Slopes of these regressions can be grouped into three categories according to the slope value: gentle slopes in root organs (fine roots: −0.01; coarse roots: −0.03; tap root: −0.04), intermediate slopes in trunk (−0.12), buds (−0.13) and twigs (−0.15), and steep slopes in wood (−0.24) and bark (−0.34). All of these linear regressions were significant (except for fine roots, for which the slope was near-nil), and originate in the same area ($0 < GFS < 25$ mg g DM^{-1} and $-5 < FH < -10$ °C). Although regressions were significant (except for fine roots), the R values were in most cases (except for bark) weak (fine and coarse roots) or moderate (the other tissues). There was also a significant difference between belowground and aboveground organs ($P < 0.01$).

These different relationships can also be explained by WC (Figure 5b). When data were reorganized according to WC class ($WC < 0.75$, $0.75 < WC < 1.00$, $1.00 < WC < 1.25$ and $WC > 1.25$), different significant regressions appeared that even allowed the previously withdrawn summer points to be included. Four regressions intersected at a more clearly defined point (GFS = 0, FH ≈ −11 °C). Finally, lower WC induced higher absolute value of slope ($WC > 1.25$: 0.013, $1.00 < WC < 1.25$: −0.056, $0.75 < WC < 1.00$: −0.104, $WC < 0.75$: −0.157).

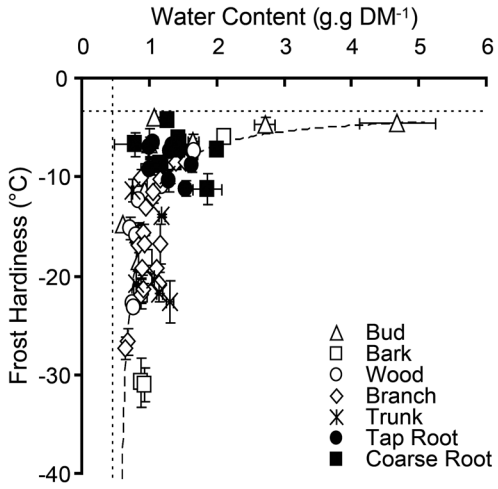

Figure 4. Frost hardiness vs WC in different organs and tissues of walnut trees. Symbols and bars represent mean ± SE. Dotted lines represent the two limiting asymptotes (WC = 0.45 and FH = −3.35).

Modelling FH

The relationship between FH and WC depends inversely on WC, and we observed that the potential effect of soluble carbohydrate content on FH was also WC dependent. Accordingly, we fitted the whole dataset with different models where variables WC and/or GFS were used either alone or combined, testing three combinations: additive with or without multiplicative interaction, and interaction alone. As explained in the Materials and methods section, variables were used either directly (WC and GFS) or after transformation (1/WC and ln(GFS); Table 1). Two models (9) $FH = (c \cdot \ln(GFS) / WC) + d$ and (10) $FH = (a / WC) + b \cdot \ln(GFS) + c \cdot \ln(GFS) / WC + d$ had good RMSE (<5.00 °C) and R^2 (>0.500) and both take into account interaction between transformed variables. In model (10), ln(GFS) / WC was highly significant ($P < 0.001$) and other ones were also significant ($P < 0.05$). In model (9), ln(GFS) / WC was highly significant ($P < 0.001$). Bayesian information criterion was lower for model (9), which means

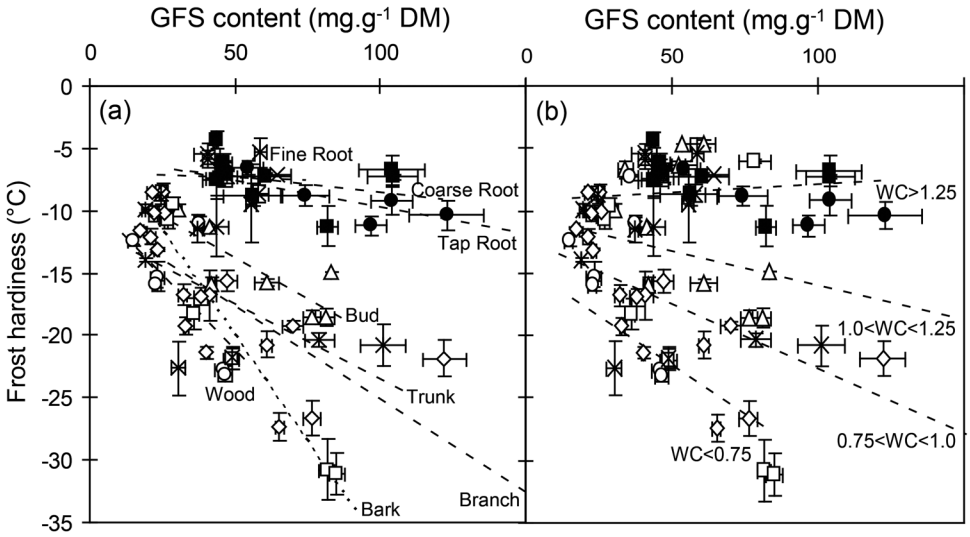

Figure 5. Frost hardiness vs GFS content in different organs and tissues of walnut trees. Regression lines are drawn (a) for different organ classes excluding summer samples or (b) for different WC classes including summer samples. Triangles, buds; squares bark; open circles, wood; open diamonds, branch; asterisks, trunk; closed circles, tap root; closed squares, coarse root; and closed diamonds, fine root.

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Table 1. Comparison between different models of FH depending on non-transformed or transformed WC and/or GFS with and without interaction.

Model	RMSE	Adjusted R^2	Df	*F*-statistic	*P*-value	BIC	Parameter estimate ± SE			
							a	*b*	*c*	*d*
Non-transformed variables										
(1) $FH = a \cdot WC + d$	6.00	0.266	291	106.6	$<2.2e^{-16}$	1897	**7.27 ± 0.70*****			**−22.86± 0.89*****
(2) $FH = b \cdot GFS + d$	6.79	0.061	291	19.99	$1.11e^{-05}$	1969		**−0.062 ± 0.014*****		**−11.27 ± 0.81*****
(3) $FH = a \cdot WC + b \cdot GFS + d$	5.66	0.348	290	79.07	$<2.2e^{-16}$	1866	**7.57 ± 0.67*****	**−0.071 ± 0.012*****		**−19.54 ± 0.99*****
(4) $FH = c \cdot WC \cdot GFS + d$	6.89	0.032	291	10.79	0.001	1978			0.030 ± 0.009**	**−16.240 ± 0.678*****
(5) $FH = a \cdot WC + b \cdot GFS + c \cdot WC \cdot GFS + d$	5.58	0.365	289	57.01	$<2.2e^{-16}$	1863	*1.33 ± 2.22*^{ns}	**−0.186 ± 0.041 *** **	0.107 ± 0.036**	**−12.88 ± 2.46*****
Transformed variables										
(6) $FH = \frac{a}{WC} + d$	5.44	0.397	291	193.5	$<2.2e^{-16}$	1839	**−16.19 ± 1.16*****			*1.17 ± 1.17*^{ns}
(7) $FH = b \cdot \ln(GFS) + d$	6.77	0.066	291	21.65	$4.98e^{-06}$	1967		**−3.36 ± 0.72*****		*−1.71 ± 2.77*^{ns}
(8) $FH = \frac{a}{WC} + b \cdot \ln(GFS) + d$	5.04	0.483	290	137.4	$<2.2e^{-16}$	1799	**−16.57 ± 1.08*****	**−3.77 ± 0.54*****		**15.85 ± 2.35*****
(9) $FH = \frac{c \cdot \ln(GFS)}{WC} + d$	4.94	0.504	291	297.5	$<2.2e^{-16}$	1782			**−4.185 ± 0.243*****	*0.845 ± 0.933*^{ns}
(10) $FH = \frac{a}{WC} + b \cdot \ln(GFS) + \frac{c \cdot \ln(GFS)}{WC} + d$	4.91	0.508	289	101.6	$<2.2e^{-16}$	17897	16.78 ± 8.44*	4.63 ± 2.17*	**−8.47 ± 2.13*****	−17.28 ± 8.63*

Results are RMSE, adjusted R^2, degree of freedom, *F*-statistic, *P*-values, Bayesian information criterion (BIC) and estimation of parameters ± standard errors and their degree of significance (*** < 0.001; ** < 0.01; * < 0.05; ^{ns} > 0.05). Numbers in italics indicate that the correlation is not significant and numbers in bold that it is highly significant.

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that the model has a strong likelihood with fewer parameters. We also tested whether 1/WC and ln(GFS) significantly improved the model (10) in comparison with (9) using ANOVA analyses, but they did not ($P = 0.10$). Consequently, we selected model (9) taking into account only interaction between GFS and WC for which the sum of squares was lowest with maximal degree of freedom. This equation respects the relationship observed between FH and WC, and adds the effect of WC on the nonlinear effect of GFS.

From a mechanistic perspective, the model allows a relatively good prediction of observed FH (RMSE = 4.94 °C). In wood (2.17 °C), branches (3.37 °C) and buds (4.08 °C), the prediction capacity seems good with similar slopes for regression between FH and ln(GFS) / WC. A greater deviation is observed in root system (coarse roots 5.43 °C and tap root 5.71 °C), and trunk (7.45 °C) with non-significant regressions ($R^2 < 0.06$; $P > 0.22$) and more gentle slopes (> –1.26). In bark, RMSE was also relatively high (7.10 °C), but FH was closely correlated to ln(GFS) / WC ($R^2 = 0.815$; $P < 0.01$) with a steeper slope (8.9 vs 4.2 on whole dataset). Overall, this model fitted data from various organs and years, and explained 50.5% of variance with 62% of data in the 95% confidence interval, which was significantly better than the other models tested (Table 1).

Validation

For external validation, an independent dataset was selected from independent samples: branches harvested in the same orchard and in another orchard exposed to different meteorological conditions (mountain: 850 m a.s.l) on two different genotypes '*Franquette*' or '*Lara*' (Figure 6b; from Charrier et al. 2011). Deviation was similar to the calibration dataset in *Franquette* (RMSEp = 5.11) and *Lara* (RMSEp = 4.59) in both environmental conditions, and 56.7% of variance is explained by the model. The source of deviation mainly occurred for lowest resistances. For instance, RMSEp is higher for FH < –20 °C (7.06 °C) than for FH > –20 °C (2.49 °C).

We also tested the predictive capacity of the model in conditions of cold deprivation in potted trees (>15 °C during the whole winter, cf. Charrier and Améglio 2011). Prediction of the model was weaker (RMSEp = 6.68); however, it explained 71.9% of the variance. By contrast, simulation was more accurate in control potted trees (RMSEp = 5.24) although it explained only 53.5% of total variance.

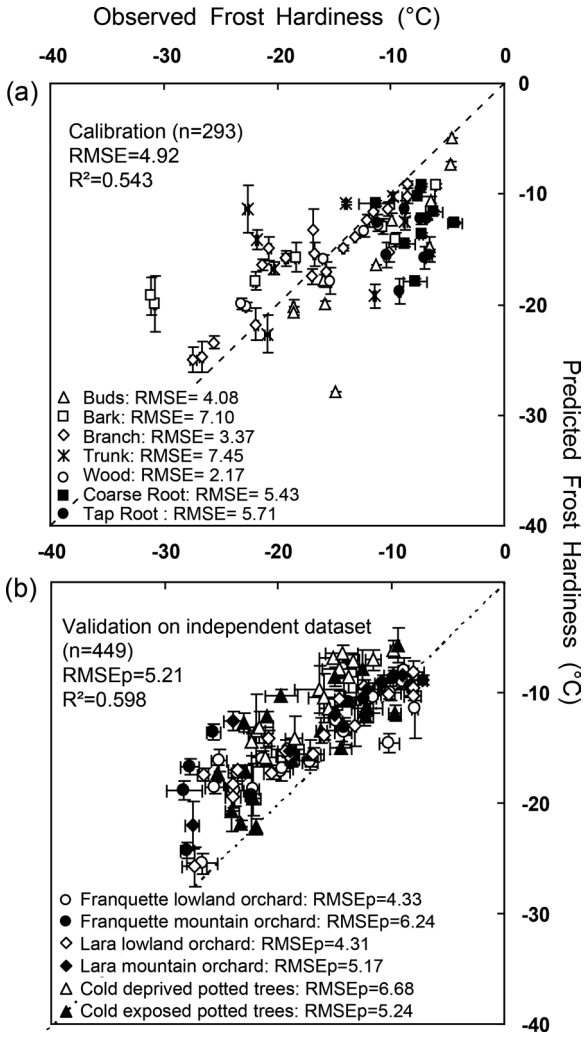

Figure 6. Simulated vs measured FH level. (a) Calibration on a dataset including all organ types; (b) validation on independent dataset gathering branches from adult trees of two genotypes (*Franquette* and *Lara*) from the same (lowland) or another (mountain) orchard, and from cold-deprived and cold-exposed potted trees. Simulation model equation: $FH = c \cdot \ln(GFS) / WC + d$ (parameter values: $c = -4.19$, $d = 0.85$).

Discussion

Frost hardiness variability within a tree

As an overall pattern, measurements of FH made during several years were not significantly different. Small differences can easily be explained by sampling dates, primarily autumn and spring when profound physiological alterations are occurring (acclimation or deacclimation). In autumn, trees were more hardened in 2007 (in relation to later date of experiment: 30 October and colder mean temperature of October: 11.2 °C) than in 2004 and 2005 (23 October with 14.0 °C and 17 October with 15.2 °C, respectively), while FH was moving quickly due to decreasing temperature. In spring, the date of experiment in 2006 (1 April) was 3 weeks earlier than in 2008 (21 April), which explains the differences in FH.

No significant difference was observed in winter during a relatively steady state of maximal FH (25, 27 and 8 January). On the one hand, it has been observed that maximal FH achieved in winter is not dependent on environmental conditions (Aitken et al. 1996, Morin et al. 2007). On the other hand, speed of hardening or dehardening is closely related to temperature changes during these periods (Pogosyan and Sakai 1969, Charrier et al. 2011).

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For the root system, this study did not show any ability to harden, except for tap root, which is histologically closer to aboveground tissues. Although the electrolyte leakage method is not common for roots, results have been shown to be similar to other methods (phenolic leakage, ninhydrin reactive compounds leakage; Bigras and Calmé 1994). We also had to use the same method for inter-organ comparison. It is relevant in roots even though it is not as good as the gene activity method (Stattin et al. 2012). Numerous studies on other species described hardening capacity in seedling roots, in *Pseudotsuga menziesii* (Mirb.) (Tinus et al. 2000), *Picea abies* (L.) Karst (Stattin et al. 2000), *Acer saccharum* L. (Bertrand et al. 1997) or *Quercus rubra* (L.) and *Betula alleghaniensis* Britton (Calme et al. 1994), but not always (in *Pinus radiata* D. Don and *Pinus halepensis* Mill.; Tinus et al. 2000). Under natural conditions, seedling roots are not frost-exposed, protected by the thermal inertia of the substrate. In general, FH of roots exhibited relatively low annual amplitude compared with aboveground organs (Sakai and Larcher 1987).

Aboveground parts had hardening (from autumn to winter) and dehardening abilities (from winter to spring). FH values observed between trunk and branches in young trees were similar under our experimental conditions. In 1-year-old branch, differences were observed among tissues after acclimation. Thus bark and cambium, which are relatively sensitive during the growing season, were able to harden more than xylem parenchyma in winter. A similar result was observed in *Hedera helix* L. (Andergassen and Bauer 2002). In contrast, buds hardened but remained more sensitive than other organs in winter. It became extremely sensitive during budbreak with –2 °C frost sensitivity limit, as observed in *P. abies* (Neuner and Beikircher 2010). Andergassen and Bauer (2002) state that leaf primordia remained the most sensitive parts of bud throughout the year, while procambium and parenchyma hardened significantly (similar to differentiated tissues). Although this study did not characterize FH in as much detail, interesting results nevertheless emerged. Values measured on whole branches were the mean of wood and bark. These histological differences will have to be taken into account in further studies. Freezing events can have low impact on bark cells, yet a dramatic effect on wood or meristematic cells. To achieve such accuracy, damage should be estimated using techniques such as exotherm detection. In our case, FH measured via the electrolyte leakage method is an indicator of hardening/dehardening, and the average value could be related to physiological parameters without bias.

Physiological modulations and impact on FH

Water status of plants during the leafless period is not easy to characterize (Cottignies 1990, Améglio et al. 2000). This is especially true for the walnut tree, where the xylem sap can be alternatively under pressure or tension (Améglio et al. 1995). Failure to measure water potential induced numerous authors to use WC (Tanino et al. 1990, Améglio et al. 2002, Gusta et al. 2004).

After leaf fall, WC decreases: bark evaporates more water than roots absorb. Decrease in soil temperature induces root absorption decrease due to decreased membrane permeability of root cells (Wan et al. 2001, Lee et al. 2012). Thus WC of branches decreased in autumn, producing hardening, while later in spring, when soil temperatures rose, root absorption restarted and rehydrated aboveground organs (Ewers et al. 2001, Turcotte et al. 2009). Améglio et al. (2002) had shown that hydration in walnut trees occurred when soil temperatures (50 cm deep) rose above 8 °C. Thus the highly hydrated buds flush, as full turgor is necessary for the new leaves to unfold.

Starch reserves decreased between autumn and spring in roots, accompanied by a much less visible decrease in aboveground parts only between autumn and winter. Moreover, in all organs, depletion of starch was associated with soluble carbohydrates increase. This starch/soluble carbohydrates interconversion is very classical and observed in many studies (Witt and Sauter 1994, El Zein et al. 2011). Here, we observed that levels of sugars and starch were similar in young potted and mature orchard trees. This suggests that carbohydrates were not limiting in young trees and that metabolism is similar.

This increase in soluble carbohydrates is usually related to hardening (Améglio et al. 2004, Morin et al. 2007, Zuther et al. 2012). The literature reports that FH is invariably accompanied by starch-to-sucrose hydrolysis (Ögren 1997, Klotke et al. 2004). High GFS concentration decreases freezing point, but only by 1–2 °C:1.86 °C per mole of solute dissolved in 1 kg of water (Hansen and Beck 1988, Cavender-Barès 2005). Soluble carbohydrates also play an indirect role in increasing FH, as they induce ice nucleation in the extracellular space because of lower osmolarity (Levitt 1980). The low water potential of ice induces cellular dehydration (Zweifel and Hasler 2000, Améglio et al. 2001). However, soluble carbohydrates also show a protective effect on cellular dehydration (Bryant et al. 2001, Lenné et al. 2007, 2009) and stabilization of biological membranes (Crowe 2002). Ability to produce soluble carbohydrates is essential for hardening (Poirier et al. 2010).

Two monitored physiological parameters (WC and GFS) drove, in interaction, FH of all aboveground organs and tissues. They are two major factors underpinning acclimation to frost (Pogosyan and Sakai 1969, Junttila et al. 1983). In the walnut tree, both predict the state of frost resistance (Poirier et al. 2010).

The relationship between FH and WC is inverse, limited by two physiological asymptotes: the freezing temperature of highly diluted solution (between 0 and –5 °C; Levitt 1980) and the fraction of freezable (unbound) water in plant tissue (Wolfe et al. 2002). Thus a higher proportion of freezable water triggers higher freezing temperature, which will generate cell damage (Gusta et al. 1975).

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Differences observed between tissues in the relationship between FH and GFS content are well explained by WC. As WC increased, solute concentrations decreased and became ineffective for freezing point decrease. This explains why sugarcane, even with the highest sugar contents observed in plants, could not survive freezing events (Levitt 1980). However, metabolically active plants have high WC, which inhibits the cryoprotective effect of sugars. It was also observed in this study on summer samples (with high carbohydrate but also WC), which were accurately predicted by the model.

Frost damage is reduced when the amount of freezable water is limited by reducing the amount of water and binding residual water with solutes. The best model for FH is based on these two parameters in interaction, and has yielded strongly significant results in various tree organs and ages. More variance is observed in root systems, trunk and bark than that in wood, buds or whole branches. Roots showed no significant acclimation due to high hydration, and appear to act as a source for carbohydrates moving up to aboveground parts. Trunk comprises dead tissues containing other compounds such as tannins that could influence the relationship between GFS, WC and FH. This could also be the case in bark tissues, where predicted FH is highly underestimated for deep resistance (<20 °C). Furthermore, bark WC could be impacted during sampling due to separation from wood. Another effect could be due to the presence of other cryoprotectant compounds not detected during this study. For example, nitrogen compounds, such as proline, are known to have a potential effect and could accumulate in these tissues. Nevertheless, GFS and WC have a major impact on the FH of trees in all organs and ages of trees. Different models demonstrated that frost hardening is not due to WC alone (models (1, 6)), GFS alone (models (2, 7)) or independently added (models (3, 8)). Moreover, the interactive effect was clearly demonstrated as being more significant than the individual effects ($RMSE_{(9)} < RMSE_{(8)}$) using transformed variables. This fact demonstrates that interaction is stronger than individual effects: decreasing WC or increasing soluble carbohydrate content trigger higher solute concentration in intracellular sap, enhancing FH. Thus this model could make it possible to predict FH in walnut in all organs and, probably, throughout the whole year.

The model explained more than 50% of variance, even in cold deprivation conditions, which would have been impossible with a classical temperature-driven model. The RMSE is ~5 °C across different environments and genotypes. This might appear too inaccurate, but in terms of agricultural management, most freezing damage occurs when trees are not fully resistant (during spring frosts and after transient heat waves in autumn), and during these periods, the model proved far more accurate (≈2 °C). One promising way forward would be to uncouple these two physiological parameters by varying the carbon balance during the growing season (defoliation or girdling; Poirier 2008) or by manipulating the WC in autumn through drought treatments.

This wide-range predictive model should be able to take into account the impact of physiological changes on the ability to acclimate after defoliation (Thomas et al. 2004), drought (Kreyling et al. 2012) or warming (Charrier and Améglio 2011). As mechanisms of hardening are similar in most species (differences from the chemical nature of osmotic compounds), the model could be expected to find wider use for a broad spectrum of tree species. This is a first step for a process-based model that simulates intermediate WC and carbohydrate concentration from meteorological data. This ultimate model should prove very valuable in predicting changes in frost acclimation of trees after several accumulated stresses over the coming century.

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Conflict of interest

None declared.

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