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A semi-physiological model of cold hardening and dehardening in walnut stem

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Summary It has been hypothesized that the increase in temperature in this century could lead to an increase in frost damage to plant tissues. Several models have been proposed to describe the development of cold hardiness, but never taking into account extreme climatic and/or physiological events. Our results on walnut tree (*Juglans regia* L.) show that cold hardiness was best correlated with average daily temperatures minimal temperatures over the last 15 days before sampling ($\bar{T}_{\text{min 15 days}}$), indicating that the freezing tolerance depended on the tree's climatic history. Moreover, this study also shows that the accumulation of sucrose and the water content (WC) decrease are an essential step towards cold hardiness. Thus, a simple linear model based on climatic ($\bar{T}_{\text{min 15 days}}$) and physiological (soluble sugars, WC) explanatory variables was developed to predict the cold hardiness level in walnut stem at any time during the leafless period. Each of the three input variables can be assigned a specific role contributing to the simulated function, cold hardiness. The extent and robustness of this relation was assessed on extreme physiological events on walnut trees bearing three main branches. On each tree, one branch was defoliated to limit the local carbohydrate and transpiration, one was girdled to increase local carbohydrate and prevent carbohydrate export and the third one was kept untreated as control. As expected, these treatments impacted both local carbon reserves and WC in the stems born by each main branch in comparison with the control on the same tree. The impact of these treatments on stem's freezing tolerance, as evaluated by an electrolyte leakage method (LT_{50}), confirmed the direct impact of soluble sugar and WC on cold hardiness over a wide range of carbohydrate and WC. This is discussed in relation to the branch autonomy theory for carbon but also for water during summer growth and winter periods. The present study demonstrates the importance of physiological parameters in the prediction of cold hardiness and proposes a way to model cold hardiness with extreme climatic and/or physiological events.

Keywords: carbohydrate, climate, cold acclimation, defoliation, girdling, Juglans regia, temperature, water content

Introduction

Low temperature represents, together with drought and salt stress, one of the most important environmental constraints limiting the productivity and affecting the distribution of plants on Earth (Sakai and Larcher 1987). Over the course of the year, plants from temperate regions show dramatic changes in their ability to survive freezing temperatures. Cold acclimation, also known as cold hardening, is triggered late in the growing season by decreasing photoperiod as day length shortens, as temperatures decline (Huner et al. 1998, Li et al. 2003*a*, 2003*b*), and is under strong genetic control (Xin and Browse 2000). These environmental cues induce physiological and biochemical changes in the plant, which result in greater tolerance (Levitt 1980). In particular, the dynamics of the stem carbohydrate reserves have been largely investigated in relation to frost resistance (Siminovitch et al. 1953, Sakai 1966, Sakai and Larcher 1987, Sauter and Kloth 1987; Tinus et al. 2000, Morin et al. 2007) especially regarding the interconversion between starch and soluble sugars. An additional relevant factor is the water status, with many studies showing a decline in the water content (WC) of tissues as plants acclimate to low temperatures (Chen et al. 1976, Chen and Gusta 1978, Tanino et al. 1990, Ögren 1999, Améglio et al. 2001, Gusta et al. 2004).

During the course of the 21st century, the global-average surface temperatures will likely increase by +2 to +5 °C. Extreme climatic events such as hot spells and drought episodes like those experienced during summer 2003 are expected to occur with increased frequency in western Europe (Bréda et al. 2006). It has been hypothesized that the increase in temperature during this century could lead to an increase in frost damage to plant tissues (Hanninen 1991). Higher temperatures hasten phenological development and spring dehardening (Walther et al. 2002, Parmesan and Yole 2003). General circulation models (IPCC 2001, 2007) predict that the frequency of random frost events will remain quite stable as mean surface temperature increases. Therefore,

advanced leaf development could be vulnerable to late frosts (spring frost; Hanninen, 1991).

In the cold hardiness models initially developed by Repo et al. (1990) for Scots pine, air temperatures were used to describe the development of cold hardiness. These authors introduced a model with a linear relationship between the prevailing air temperature and the stationary level of cold hardiness, i.e., the target level that determines the direction of the change in cold hardiness. The rate of change of cold hardiness was assumed to be proportional to the difference between the prevailing frost hardiness and the stationary level. Kellomäki et al. (1992) modified the model of Repo et al. (1990) by dividing the annual cycle of trees into three periods (growth, lignification and dormancy), which included different cold hardiness responses to the environment. Leinonen et al. (1995) and Leinonen (1996) further developed the model of Repo et al. (1990) by introducing an additive effect of temperature and photoperiod on cold hardiness to the stationary level. These models were accurate in predicting changes in hardiness during cold hardening in autumn, but overestimated the hardiness during dehardening in spring. Moreover, they did not take into account the current physiological state of trees following extreme climatic events.

To better predict the impact of climate change on tree survival and geographical distribution of woody plants, we need a simple model that allows predict cold hardiness from the actual course of climatic conditions all the time, taking into account the tree development history. This should particularly include the impact of summer conditions of growth on tree freezing tolerance as shown by Poirier (2008). The first objective of the present study was to build such a predictive model yielding the stem hardiness (LT_{50}) of walnut at any time during the resting phase, based on air temperature, soluble carbohydrate reserves and WC as input variables. These variables, all recognized as important factors in the cold-hardening process as stated above, were measured on walnut to acquire a data set large enough to allow parameter fitting and validation on independent data. The second objective of the present study was to test the model in extreme experimental conditions drastically impacting on the carbon and/or WC, to evaluate its robustness and potential validity in the context of current or expected global climate changes.

Materials and methods

Plant material

This study involved two walnut varieties: one fruit-oriented cultivar (*Juglans regia* cv. 'Franquette') and one wood-oriented, seed-grown, hybrid *J. regia* × *J. nigra* 'NG38'. To investigate the impact of climate on freezing tolerance, two locations with contrasted climate were chosen near Clermont-Ferrand in south-central France: (i) the INRA site of Crouelle, altitude 330 m above sea level, 45° 46′30″N; 3°08′34″E and (ii) the INRA site of Theix, altitude 887 m, 45°43′09″N; 3°01′02″E. The model was built and parameterized from two sampling sets. The first set involved twenty 5-year-old trees, i.e., five trees of each cultivar in each location; each of them was sampled for one 1-year-old twig every month from 8 November 2004 through 24 May 2005. The other sampling set involved ten 8-year-old hybrids 'NG38' at Crouelle exhibiting heterogeneity in their timing of bud-break, with individual bud-breaking dates ranging from late April or early May (early bud-break) to late May or early June (late bud-break). Each tree was sampled for one 1-year-old twig monthly from 8 November 2004 through 6 June 2005.

Model validation was performed on three additional sampling data sets involving both fruit- and wood-oriented walnuts, planted in the Crouelle site under various growth conditions: (i) during winter 2002–03, using five 6-year-old hybrid 'NG38'; (ii) during winter 2004–05, using five 5-year-old hybrid 'NG38' trees from a mixed, close plantation, including several other trees species; and (iii) during winter 2005–06, using five 6-year-old *J. regia* cv. 'Franquette'. For each of those three data sets, a 1-year-old twig was collected from each tree monthly from October/November through June.

Extreme experimental conditions were applied to one additional sampling data set involving fruit-oriented walnuts, planted in the Crouelle site. Five 6-year-old *J. regia* cv. 'Franquette' were used. Two years before the experiment, these trees were pruned severely to three main branches. During summer 2005, each of the three main branches of each single tree was treated independently. The highest branch was nearly completely defoliated on 28 June, keeping all growing leaves but only the youngest two full-grown ones on each current-year shoot. Another main branch was girdled, i.e., the bark, including phloem, was removed all around the stem, preventing photoassimilate export out of the stem. The last main branch of the tree was left untreated as a control.

Freezing treatment and electrolyte leakage test

We estimated the cold hardiness of each individual tree using the electrolyte leakage test (Zhang and Willison 1987, Sutinen et al. 1992). As a consequence of frost damage to the plasma membrane, electrolytes leak from the symplast to the apoplast as the primary symptom of cellular damage. This leakage can be detected after 'washing' the electrolytes from the apoplast into distilled water and by measuring the electrical conductivity of the water solution. The conductivity of the heat-killed samples gives a measure of the total amount of tissue electrolytes. Frost injuries in cells may be determined by comparing the relative conductivities between the non-frozen and frozen samples (Flint et al. 1967, Burr et al. 1990, Sutinen et al. 1992).

The samples, 10 cm in length, were washed in distilled-deionized water. Stem sections of uniform size and accompanied by a moistened tissue were wrapped in aluminium foil and placed in pre-chilled Dewar flasks. The flasks were transferred into a deep freezer (−80 °C). Sample temperatures were monitored using copper–constantan thermocouples inserted into the foil pouch. Samples cooled down at the rate of 5–7 °C h^{-1}. At each desired temperature (e.g., +5 °C for control, −5, −10, −15, −20, −70 °C), the flasks were sampled and transferred into a refrigerator at +5 °C for 12–15 h to facilitate slow thawing of the samples at the rate of 5–7 °C h^{-1}.

After freeze–thaw treatment, internodal sections were quartered and sliced into several segments while immersed in 15 ml of distilled-deionized water in 2.5 × 20 cm test tubes. The capped tubes were placed on a gyratory shaker for 24 h at 5 °C. At the end of 24 h and after warming the samples up to room temperature, initial conductivity (C_1) was measured with an electrical conductivity meter (hand-held conductivity meter LF340 with standard conductivity cell, TetraCon® 325). The tubes were autoclaved at 120 °C for 25 min to kill any possible surviving cells, and after cooling down to room temperature electrical conductivity was measured again to yield the maximum conductivity (C_2).

The per cent electrolyte leakage was calculated, according to Zhang and Willison (1987), as:

$$R = 100 \times \frac{C_1}{C_2},$$

The temperature representing the freezing stress resistance level was estimated from the plot of % electrolyte leakage rate vs. test temperature as the 'average lethal temperature' (LT_{50}), for which (Sutinen et al. 1992):

$$\%\ \text{electrolyte leakage rate} = \frac{R_1 + R_2}{2},$$

where R_1 is % electrolyte leakage from the non-injured tissue (control temperature) and R_2 is % electrolyte leakage at the highest level of injury.

Soluble carbohydrates

When samples were prepared for the freezing tests, a 1-cm long segment of each twig was sampled to measure the carbohydrate content. These segments were immediately frozen in liquid nitrogen and subsequently lyophilized. Soluble carbohydrates (i.e., glucose, fructose and sucrose noted as GFS) were then extracted from the twigs with hot ethanol/water (80/20, v/v), and purified on ion-exchange resins (Bio-Rad AG 1-X8 in the carbonate form, Dowex 50W in the H+ form), as described by Moing and Gaudillère (1992).

Soluble sugars were assayed by high performance liquid chromatography (HPLC) (Gilson 715 HPLC) using an Aminex column (Bio-Rad, Fast Carbohydrate, 9 µm, 100 × 7.8 mm). The mobile phase (ultrapure water) was pumped through the column at a flow rate of 1 ml min^{-1}. The oven temperature was adjusted to 85 °C. A refractometer (RI 2000) was used. Sucrose, glucose and fructose were identified by their retention times and were quantified using mannitol as the standard.

The starch content was extracted from the pellet after hydrolysis with beta-amyloglucosidase (Boehringer 1984) and was quantified as glucose with a hexokinase, glucose-6-phosphate linked assay (Curt et al. 2005). All concentrations were expressed as mg glucose equivalent. g^{-1} dry matter (DM).

Water content

The twig samples were weighed before and after freeze-drying, yielding the WC (dimensionless) as:

$$\text{WC} = \frac{\text{FW} - \text{DW}}{\text{DW}},$$

where WC is the water content, FW the fresh weight (g) and DW the dry weight (g).

Meteorological data

Daily minimum and maximum air temperatures were recorded from 1 November 2004 through 30 June 2005, on the exact experimental site at Theix, and at a distance of 800 m away from the experimental site of Crouelle.

Statistical analysis

Mean LT_{50}, carbohydrate content (GFS and starch) and WC were calculated for each population from the individual values. Results are expressed as average ± standard error. The significance of differences between means was evaluated by the non-parametric test of Mann and Whitney (Sprent and Smeeton 1993).

The model yielding LT_{50} as a function of environmental and biological variables was designed as linear. It was built and parameterized with both quantitative (GFS, WC, temperatures) and qualitative (cultivar, location) input variables, using the general linear model (GLM) procedure (Statistica software v.5.5, StatSoft, USA). The general approach was stepwise, starting with simple linear regression and adding more input variables or interactions if that significantly improved the model, i.e., (i) the adjusted coefficient of determination (R^2_{adj}, see below) was increased and (ii) all input factors had significant Type III sums of squares (Hill and Lewicki 2007).

The global goodness-of-fit of the model was evaluated by three statistics:

(1) the coefficient of determination, adjusted for estimated parameters (Hill and Lewicki 2007) to yield an unbiased value, R^2_{adj},

(2) the index of agreement d as defined by Willmott (1982) and
(3) the quadratic mean deviation estimated from measured values, or root of mean squared error, RMSE (Janssen and Heuberger 1995)

The predictive capacity was evaluated by validation on data sets that were independent of the ones used to parameterize the model (see above), using the same three indices: R^2_{adj}, d and RMSE called here RMSE of prediction (RMSEP).

Results

Comparison between Theix climate and Crouelle climate

Of the two studied sites, Crouelle was the warmer and drier (Table 1). Variations of daily minimal and maximal temperatures between 1 November 2004 and 30 June 2005 are shown in Figure 1a for the Theix site and in Figure 1b for the Crouelle site. At Crouelle, the average minimum and maximum air temperatures during the experimental period were 4.1 ± 0.4 and 13.3 ± 0.6 °C, respectively (Table 2), both significantly ($P < 0.0001$) milder than at Theix (1.4 ± 0.4 and 9.8 ± 0.6 °C, respectively). Lowest temperatures recorded were −18.6 °C at Theix and −12.5 °C at Crouelle and occurred at the end of February. In both experimental sites, temperatures started to warm up in early March. Except for a few days (temperature inversion) during the experimental period, daily minimal temperatures in Crouelle were higher than those in Theix (Figure 1c), with an average difference of 2.8 ± 0.1 °C between the two locations. The mean thermal amplitudes did not significantly differ among the three studied years or between both locations. Among the three studied periods, 2002–2003 was the hottest period (significance level: $P < 0.0001$).

Changes in cold hardiness, WC and carbohydrate concentration over time

Times courses of frost resistance level, WC and carbohydrate content of both walnut cultivars on both experimental sites (Theix and Crouelle) are shown in Figure 2 as well as the dates of bud-break for each group (Franquette/Crouelle, Franquette/Theix, Hybrid/Crouelle and Hybrid/Theix). The frost resistance level, as determined by LT_{50}, ranged between −5 and −10 °C for cv. Franquette and −10 and −15 °C for hybrids at the end of the summer growth period. It increased (i.e., LT_{50} decreased) strongly in fall, then slightly further in early winter, reaching peak values of ca. −22 °C on average (cv. 'Franquette') and −24 °C (hybrids) in late February. Dehardening started soon in spring (from early March on Figure 2a and d) with air temperatures warming up (Figure 1). Before and during the hardening phase, in November and December, trees of cv. 'Franquette' were significantly hardier in Crouelle than in Theix (Figure 2a) That difference vanished at maximum hardiness, in February, then turned opposite as cold dehardening was faster in Crouelle, the warmer site.

In general, cold hardiness of hybrids was not significantly different between the two locations during the experimental period (Figure 2b), except for 5-year-old hybrids in December during temperature inversion (Figure 1). In Crouelle, 8-year-old hybrids tended to deharden slightly earlier than the 5-year-old, but the difference in LT_{50} was significant only in mid-March.

Regarding WC (Figure 2c and d), trees of cv. 'Franquette' exhibited a decrease in autumn down to a minimum of ∼0.8 g g^{-1}, whereas hybrids had already reached this minimal level in December. In spring, WC increased in all groups, likely in relation to temperature increase as suggested by the difference between Crouelle and Theix in June.

More precisely, before spring, WC was higher throughout the winter for hybrids in Theix than in Crouelle, consistent with higher rainfall and lower reference evapotranspiration (ET_o) (sum of precipitation from January to April: 205 vs. 179 mm and sum of ET_o for the same period: 144 vs. 205 mm, at Theix and Crouelle, respectively). No difference in WC was detected between 5- and 8-year-old hybrids (Figure 2d).

Regarding carbohydrate levels (starch and GFS), no difference was observed between either cultivars or locations in fall. The subsequent variations in winter showed significant interconversion between starch and GFS in relation to air temperatures. Starch hydrolysis occurred with decreasing temperatures in early winter, slightly more pronounced and earlier ($P = 0.047$) in Theix than in Crouelle in accordance with the respective temperature patterns in the two locations. Minimum starch and maximum GFS levels were concomitant with air temperatures and maximum cold hardiness levels (Franquette, Theix: GFS = 80.2 ± 3.7 mg g^{-1} DM, $LT_{50} = -21.2 \pm 0.3$ °C; Franquette, Crouelle: GFS = 79 ± 3.6 mg g^{-1} DM, $LT_{50} = -21.1 \pm 0.3$ °C; Hybrids, Theix: GFS = 84.2 ± 5.6 mg g^{-1} DM, $LT_{50} = -23.5 \pm 0.6$ °C; Hybrids, Crouelle: GFS = 75 ± 3.7 mg g^{-1} DM, $LT_{50} = -23.2 \pm 1.1$ °C) (Figure 2a, b and e, f). From late February on, as air temperatures increased, starch was synthesized back from soluble sugars, with different timing among combinations of cultivar × location. Those different timings appeared closely related to bud-break dates, i.e., depending both on cultivar-specific phenological characters and on location-specific thermal

Table 1. The mean annual temperature and the mean annual rainfall for the two locations from 1996 to 2005 and from 1990 to 2005 in Crouelle and Theix, respectively

Year	Location	Mean annual temperature (°C)	Mean annual rainfall (mm)
1986–2005	Aulnat (Crouelle site)	11.9 ± 0.1	556 ± 31
1990–2005	St-Genès-Champanelle (Theix site)	8.7 ± 0.1	770 ± 32

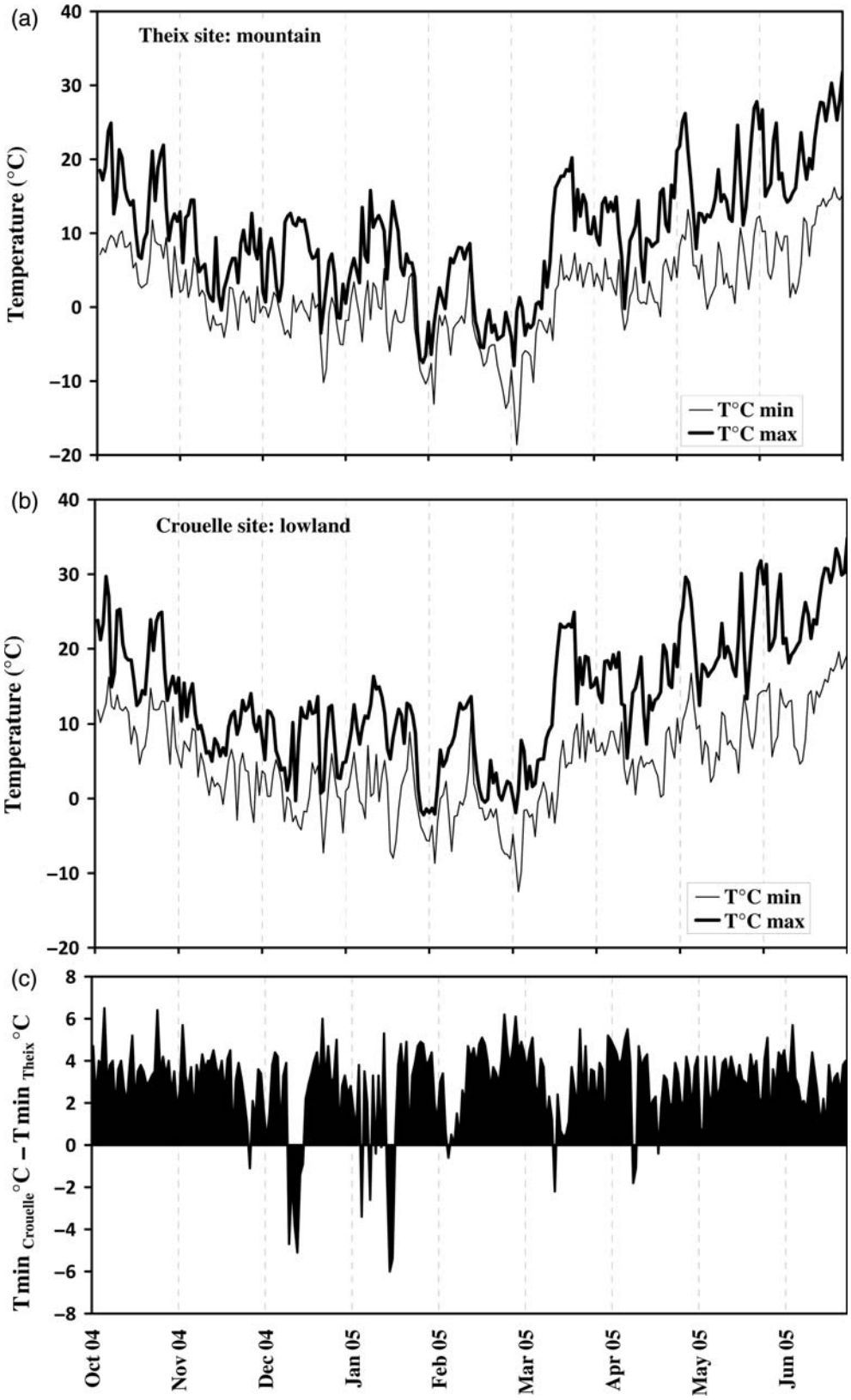

Figure 1. Summary of data for the 2004–05 winter period. (a) Time courses of minimum and maximum daily air temperatures in mountain Theix site: altitude 887 m, 45°43′09″N; 3°01′02″E. (b) Time courses of minimum and maximum daily air temperatures in plain Crouelle site: altitude 330 m above sea level, 45°46′30″N; 3°08′34″E. (c) Time course of mean thermal amplitude of both locations.

Table 2. The location and temperature regime (daily minimal or maximal air temperature, thermal amplitude) for each sample year studied.

Year	Location	Daily temperature (°C)						Thermal amplitude (T_{max} – T_{min} °C)		
		Minimal			Maximal			Lower	Higher	Mean on the period
		Lower	Higher	Mean on the period	Lower	Higher	Mean on the period			
2002–03	Aulnat (Crouelle site)	−14.1	20.7	5.4 ± 0.4	−5.9	37.1	15.5 ± 0.6	0.5	23.5	10.1 ± 0.3
2004–05	Aulnat (Crouelle site)	−12.5	20.7	4.1 ± 0.4	−2.2	34.8	13.3 ± 0.6	0.9	20.3	9.2 ± 0.3
	St-Genès-Champanelle (Theix site)	−18.6	17.3	1.4 ± 0.4	−7.9	31.8	9.8 ± 0.6	0.7	18.6	8.4 ± 0.3
2005–06	Aulnat (Crouelle site)	−9.9	17.5	3.6 ± 0.4	−2.6	32.2	12.9 ± 0.5	1	21.3	9.3 ± 0.3

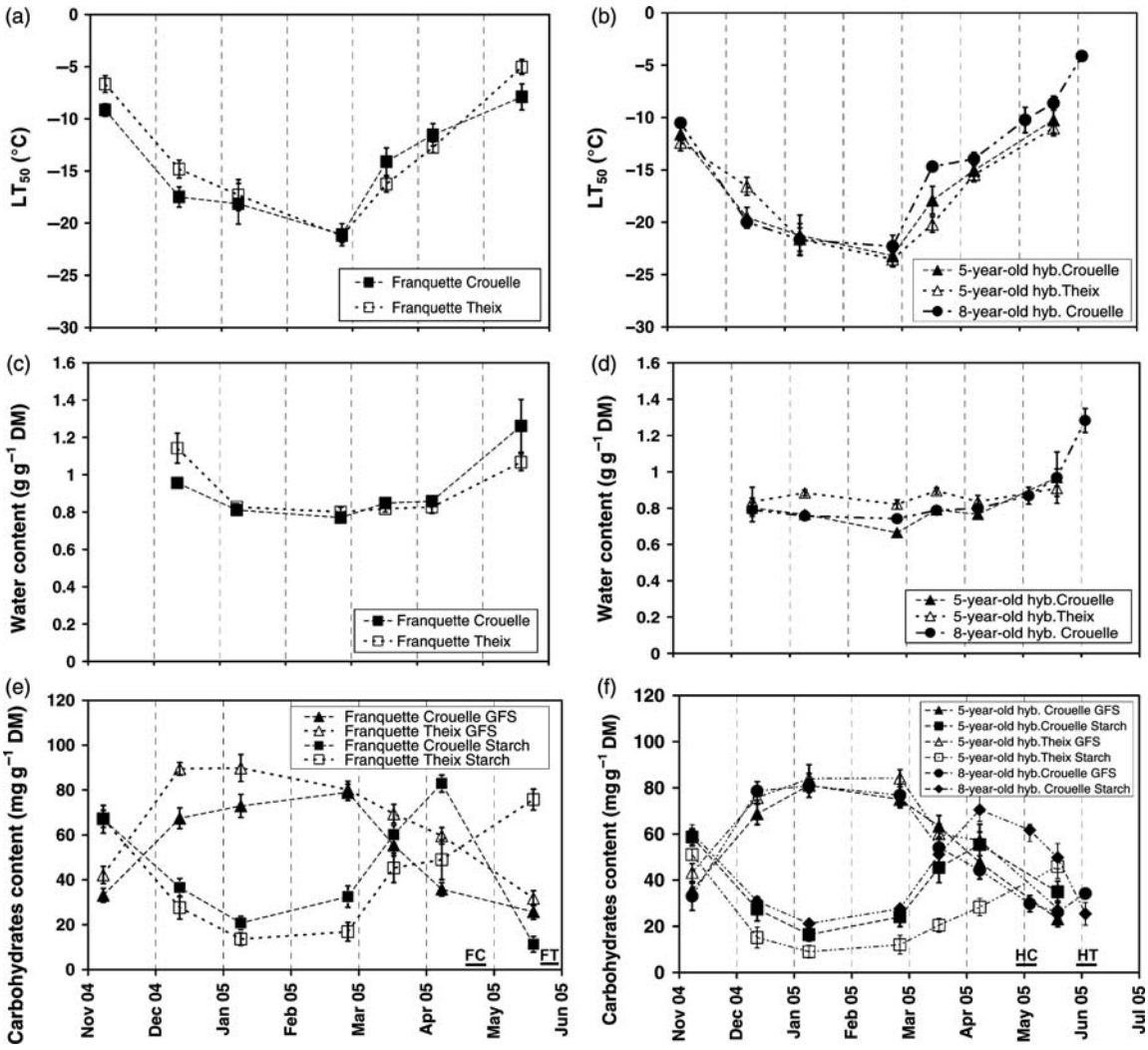

Figure 2. Summary of data for the 2004–05 winter period. Time course of (a, b) frost résistance level as determined by mean temperature causing 50% cell lysis (LT_{50}) for (a) Franquette cultivar planted in Theix or Crouelle and (b) hybrid 'NG38' planted in Theix or Crouelle. For the same trees, (c, d) presented the time course of WC and (e, f) showed the time course of carbohydrate content (starch and GFS). In (e) and (f), the thick line represents the time of bud burst. FC, Franquette Crouelle; FT, Franquette Theix; HC, Hybrid 'NG38' Crouelle; HT, Hybrid 'NG38' Theix. Values in panels are means ± SE ($n = 5$).

conditions. This pre-bud-break starch resynthesis phase was followed by massive reserve mobilization and consumption at and after bud-break.

No effect of age (8- vs. 5-year-old hybrids in Crouelle) was detected throughout the winter on the starch–GFS interconversion.

Correlation between LT_{50}, carbohydrate content, temperature and WC

According to the physiological results here above, the three variables GFS, WC and air temperature appeared to be strongly correlated with frost resistance level (LT_{50}). Consequently, the three of them were selected as possible input factors to the predictive GLM yielding the stem LT_{50} at any time during the leafless phase. Location and cultivar were entered as possible additional, qualitative factors. However, a question arose regarding the best way to express air temperature as an input factor to the model. As the physiological processes that it may directly impact, e.g., carbohydrate metabolism, are unlikely to be instantaneous, the most relevant 'daily' temperature should account not for the thermal dynamics of the single current day when LT_{50} was measured, but for its integration over a period covering several days. Consequently, a preliminary statistical analysis was performed (data not shown), which showed that LT_{50} was best correlated with $\bar{T}_{\text{min 15 days}}$, the average of daily minimal temperatures of the last 15 days before (and not including) the sampling day. The stepwise model selection procedure was then launched as explained in the Materials and methods section, starting from each single quantitative variable: GFS, WC and $\bar{T}_{\text{min 15 days}}$. This yielded (Table 3) nine models meeting the selection criteria, i.e., with all input factors significant ($P < 0.05$ as the criterion; actually $P < 0.0005$ for the least significant factor).

No interaction between input variables was found significant in any of the nine models, so that the GLM reduced to a classical linear regression model if only quantitative variables were involved, or to a classical covariance analysis (ANCOVA) model if qualitative variables were also involved.

Of the three quantitative variables, GFS, $\bar{T}_{\text{min 15 days}}$ and WC, the most explanatory was $\bar{T}_{\text{min 15 days}}$, as shown by simple linear regression results (Figure 3, Table 3). The goodness-of-fit was roughly uniform over the whole range, except for extreme values: very low resistance levels were overestimated (LT_{50} measured = −5 vs. −10 °C for simulated) whereas very high resistance levels were underestimated (LT_{50} measured = −25 vs. −20 °C for simulated). GFS was almost as explanatory as $\bar{T}_{\text{min 15 days}}$ (Table 3), with the same shortcomings at the extreme values (Figure 3b), but not completely redundant, as the double regression on both variables (Table 3) was statistically better than either of the simple ones. WC was the least explanatory quantitative variable; nevertheless, it was not redundant with the previous two, as the triple regression was significantly better than the double $f(\text{GFS}, \bar{T}_{\text{min 15 days}})$, regarding both goodness-of-fit and predictive quality (Table 3), notably for the simulation of the low-resistance levels (Figure 4). Adding the qualitative variable, 'cultivar' as an input factor slightly improved the goodness-of-fit, but not the predictive quality; 'location' did slightly improve both criteria, but the gain looked very modest (RMSEP was decreased by 0.2 °C) compared with the loss of generality that it would induce.

Thus, the best model yielding the frost resistance level (LT_{50}, °C) of 1-year-old walnut stems was the triple regression on all three quantitative variables (GFS, $\bar{T}_{\text{min 15 days}}$ and WC), without interaction:

$$LT_{50} = -0.1 \times \text{GFS} + 0.46 \times \bar{T}_{\text{min 15 days}} + 9.77 \times \text{WC} - 9.5$$

where GFS is the soluble carbohydrate content (mg g^{-1} DM), WC the water content (g g^{-1}) and $\bar{T}_{\text{min 15 days}}$ the average of daily minimal temperatures of the previous 15 days (°C).

The model explained a good percentage of the variance ($R^2_{\text{adj}} = 0.74$) with a highly significant ($P < 0.00000001$) effect of each variable (GFS, $\bar{T}_{\text{min 15 days}}$, WC). The mean residual error of fitting (RMSE) was ±2.9 °C. The mean

Table 3. Estimation and validation on the GLM built and parameterized with both quantitative (soluble carbohydrate, i.e. *GFS*, *WC*, the average daily minimal temperatures of the last 15 days before (and not including) the sampling day: $\bar{T}_{\text{min 15 days}}$) and qualitative (cultivar: cv., location: loc.) input variables.

Variables included in the model	GFS	$\bar{T}_{\text{min 15 days}}$	WC	GFS, $\bar{T}_{\text{min 15 days}}$	GFS, WC	$\bar{T}_{\text{min 15 days}}$ WC	GFS, $\bar{T}_{\text{min 15 days}}$ WC	GFS, $\bar{T}_{\text{min 15 days}}$, WC, cv.	GFS, $\bar{T}_{\text{min 15 days}}$ WC, loc.
Fitting ($n = 197$)									
RMSE	3.746	3.602	4.668	3.298	3.204	3.238	2.931	2.746	2.779
$R^2_{\text{adj.}}$	0.582	0.614	0.351	0.675	0.693	0.686	0.742	0.772	0.766
Willmott *d*	0.852	0.867	0.712	0.895	0.903	0.900	0.922	0.934	0.932
Validation ($n = 138$)									
RMSEP	4.499	4.216	5.254	3.960	4.105	4.015	3.785	4.025	3.581
$R^2_{\text{adj.}}$	0.504	0.565	0.324	0.613	0.584	0.602	0.644	0.594	0.679
Willmott *d*	0.811	0.836	0.651	0.858	0.854	0.860	0.878	0.877	0.895

The global goodness-of-fit of the model was evaluated by three statistics: the coefficient of determination adjusted, R^2_{adj}; root mean squared error, RMSE; and the Willmott index of agreement, *d*. The predictive capacity was evaluated with an independent data sets using the same three indices: R^2_{adj}, *d*, RMSEP (root of mean squared error of prediction).

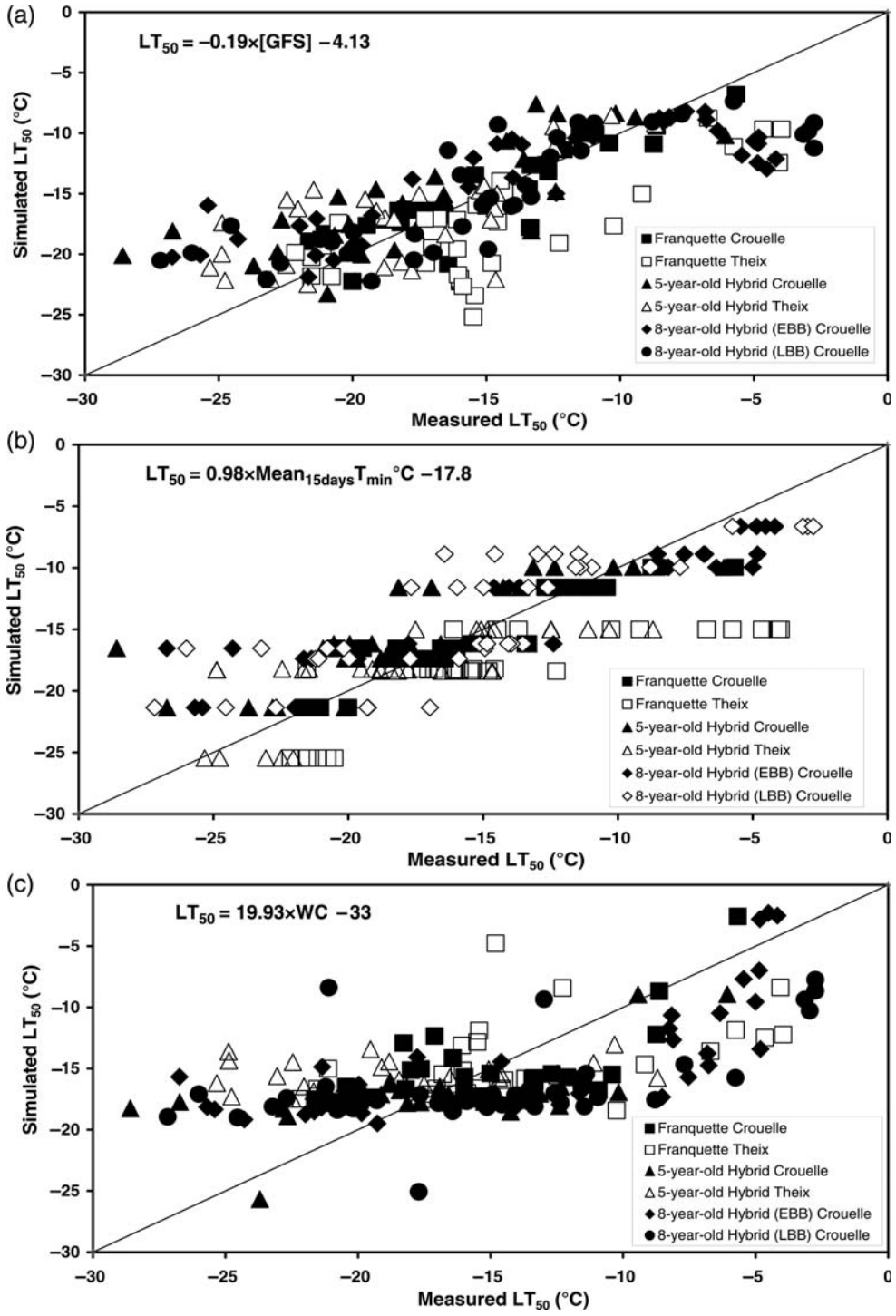

Figure 3. Relationship between frost resistance levels as determined by mean temperature causing 50% cell lysis (LT_{50}) measured in the field and simulated by a simple linear model for each cultivar and each location. Results are presented (a) for a model with only soluble carbohydrate (i.e., GFS; (b) for a model with only the average of daily minimal temperatures of the last 15 days before (and not including) the sampling day $\bar{T}_{\text{min 15 days}}$ (c) for a model with only WC.

prediction error (RMSEP) was still quite acceptable: ±3.8 °C on values ranging from −5 to −25 °C, showing the conservation of the model equation over the years and its usability on data independent of those to build it (Figure 4b, Table 3).

Summer experimental treatments, main branch leaf area and consequences on carbohydrate, WC and cold hardiness

Figure 5 shows the time course of the leaf area for control main branch (Figure 5a), girdled main branch (Figure 5b)

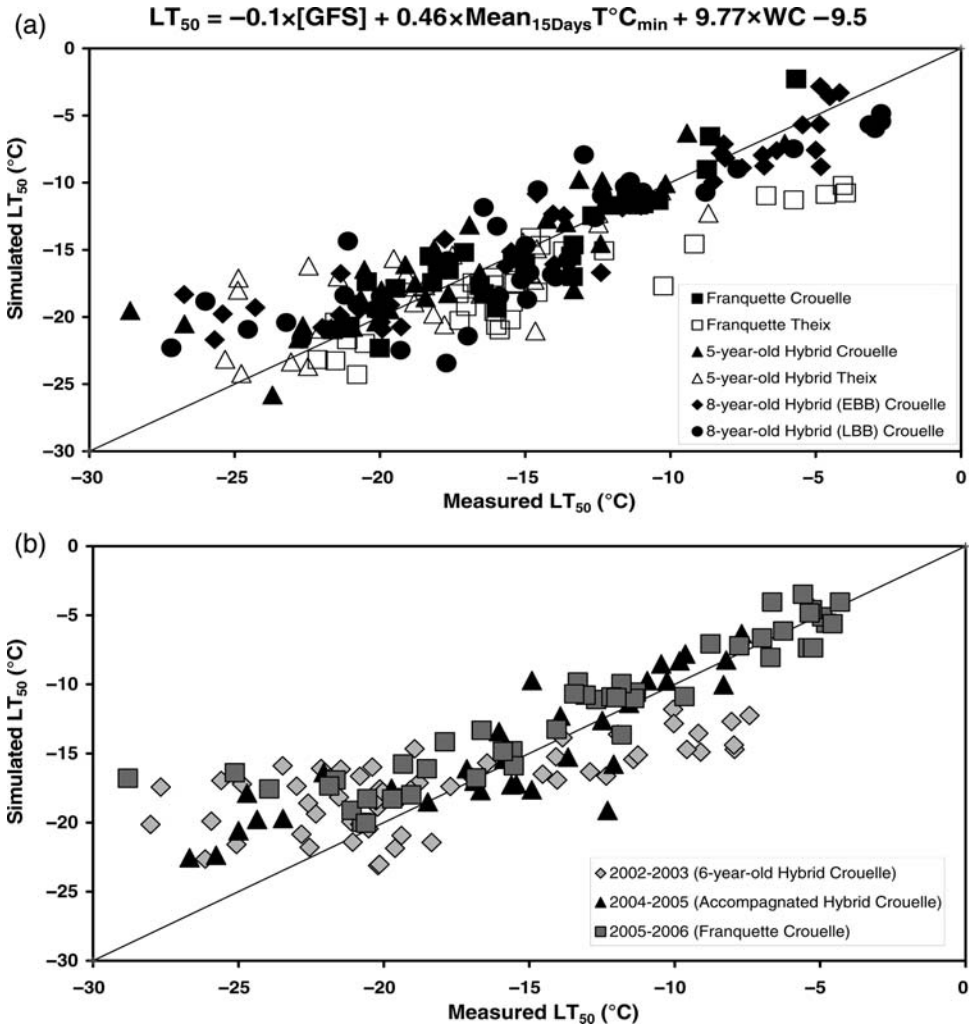

Figure 4. Relationship between frost resistance levels as determined by mean temperature causing 50% cell lysis (LT_{50}) measured in the field and simulated by a global linear model included three quantitative variables (GFS, WC and $\bar{T}_{\text{min 15 days}}$) for each cultivar and each location, (a) with the data for fitting, (b) with independent data to evaluate the predictive capacity of the model.

and defoliated main branch (Figure 5c), averaged for the five trees. On the control branch, leaf area doubled between July (4.6 ± 0.6 m^2) and October (10.5 ± 2.1), whereas in the same time, the girdled branch exhibited a low increment (5.5 ± 1.1 vs. 6.6 ± 1.4 m^2). On the defoliated main branch, leaf area remained constantly <3 m^2, although the total leaf area produced was equivalent to the control main branch (10.5 ± 1.5 m^2).

Figure 6 shows the consequences of these summer treatments on carbohydrate content (Figure 6a), WC (Figure 6b) and cold hardiness (Figure 6c) in autumn, winter and spring. The defoliated main branch had lower carbohydrate content in all seasons, whereas WC was significantly higher. In contrast, the girdled main branch had the highest carbohydrate content in all seasons, whereas WC was significantly lower. Regarding cold hardiness, the girdling increased LT_{50} in autumn and spring when compared with control, whereas defoliation significantly lowered LT_{50} during winter.

Finally, we tested the previous model on this new data set with extreme summer treatments (defoliation and girdling). Figure 7 shows the predictability of the model for all treatments: in spite of a slight overestimate for the girdling treatment and a slight underestimate for the defoliated treatment, the overall RMSEP is still quite acceptable: ±3.5 °C on values ranging from −5 to −25 °C, showing the conservation of the model equation for extreme physiological treatments.

Discussion

Impact of climate on cold hardiness

Low temperatures are recognized to be a key constraint in tree distribution (Shreve 1914, Sakai and Weisser 1973, George et al. 1974), especially at the species northern range limit (Gusta et al. 1983, Arris and Eagleson 1989) or at the

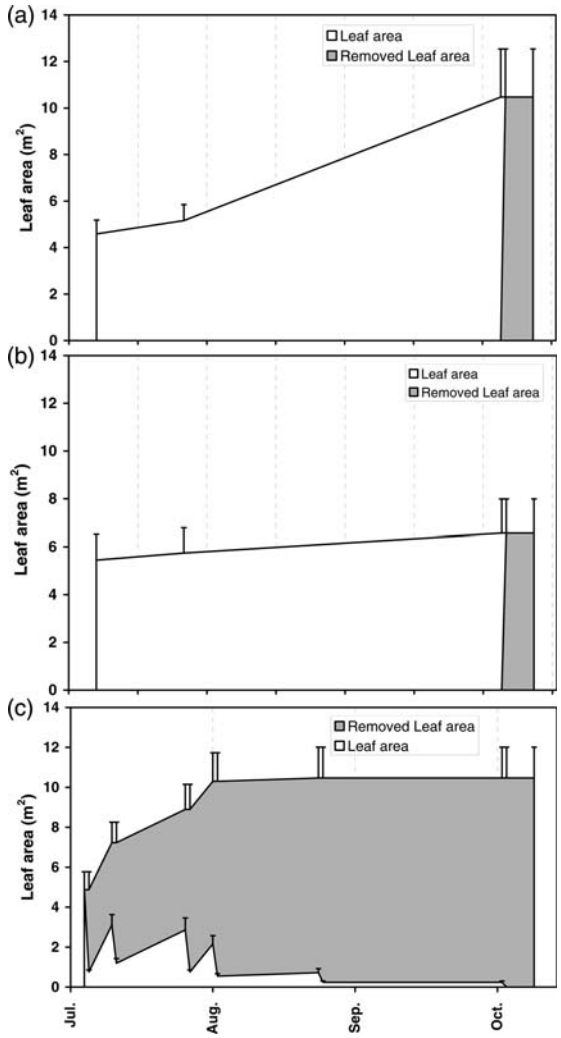

Figure 5. Summary of data for the 2005 summer treatments. Solid white area represents the time course of leaf carried by: (a) control main branch; (b) girdled main branch; (c) defoliated main branch. Solid grey colour represents the leaf area removed for the same branches. Thus, for control (a) and girdled (b) branches, leaves are totally removed at the beginning of October whereas defoliated branch (c) leaves are partially removed at the beginning of July and repeated six times until the total defoliation at the beginning of October. Means ± SE ($n = 5$) are presented.

treeline (Cavieres et al. 2000). Thus, the effect of low temperatures on frost hardening is well observed (Irving and Lanphear 1967, Howell and Weiser 1970, Levitt 1980, Sakai and Larcher 1987). Our results also showed a good correlation between cold hardiness and low temperature. LT_{50} was best correlated with $\bar{T}_{\text{min 15 days}}$, the average of the daily minimal temperatures over the last 15 days before sampling (Pearson correlation coefficient: $r = 0.830$, vs. 0.696 for the correlation with current day minimal temperature). This is an indication that the freezing tolerance depended on the tree climatic history, likely in relation with some structural and/or metabolic changes.

Moreover, for a given climate (e.g., Theix site or Crouelle site), differences in cold hardiness between cultivars were observed principally in autumn and spring, less in winter. These differences could be explained by differences in phenology, which is a highly plastic trait (Larcher 1980). In the literature, phenology and cold hardiness are intimately linked (Kruger and Trappe, 1967, Sakai and Larcher 1987, Fitter and Hay 1987, Dereuddre and Gazeau 1992). In the present experiment, the bud-break timing difference between both cultivars was the same (~2 weeks) as between their respective cold dehardening kinetics; this was found in both Crouelle and Theix, although the absolute dates differed by 1 month between the two locations. As dormancy release is linked with several metabolic changes (Marquat et al. 1999, Alves et al. 2007), this is further evidence for the involvement of metabolism in tree cold hardiness.

The role of carbohydrates in cold hardiness

Seasonal changes in carbohydrate content in woody plants have been investigated for a long time (Hartig 1860 in Sauter 1988) and have often been indicated in relation to cold hardiness (Siminovitch et al. 1953, Ziegler 1964, Kramer and Kozlowski 1979, Sauter and Ambrosius 1986, Frossard and Lacointe 1988, Sauter and Van Cleve 1994). These studies have shown that the accumulation of sucrose due to winter starch mobilization is an essential step towards freezing tolerance (Sakai 1966, Sauter et al. 1996). Our results in *J. regia* showed that LT_{50} was negatively, i.e., cold hardiness was positively, related to soluble sugar content (Figure 3b). These results were consistent with many other studies (Palonen et al. 2000, Améglio et al. 2004, Morin et al. 2007) where the maximum cold hardiness coincides with the maximum soluble sugar content, both occurring at the time of maximum risk of frost injury. The most commonly accepted hypothesis states that greater concentration of soluble carbohydrates lowers the freezing point of the intracellular solution. But the processes that are underpinning this relationship are still being debated. In fact, the measured difference in concentration between hardened and dehardened tissues can only explain 1 or 2 °C of difference in frost resistance (Hansen and Beck 1988): 1.86 °C per mole of solute dissolved per kg of water (Dereuddre and Gazeau 1992, Cavender-Bares 2005). Thus, soluble carbohydrates may also play an indirect role in cold hardiness development. Frost triggers ice nucleation within the extracellular space (Levitt 1980), which has a lower solute concentration and therefore a slightly higher freezing point than intracellular vacuolar and cytoplasmic water (where soluble sugar content has increased). This extracellular ice formation prevents concurrent intracellular ice (Burke et al. 1976), since at the same temperature, the ice will have a lower water potential than the supercooled water in the cell (Mazur 1963, 1968, Rajashekar et al. 1982, 1983). This is why water diffuses from cells to sites of ice formation, resulting in cell dehydration (Loris et al. 1999, Zweifel and

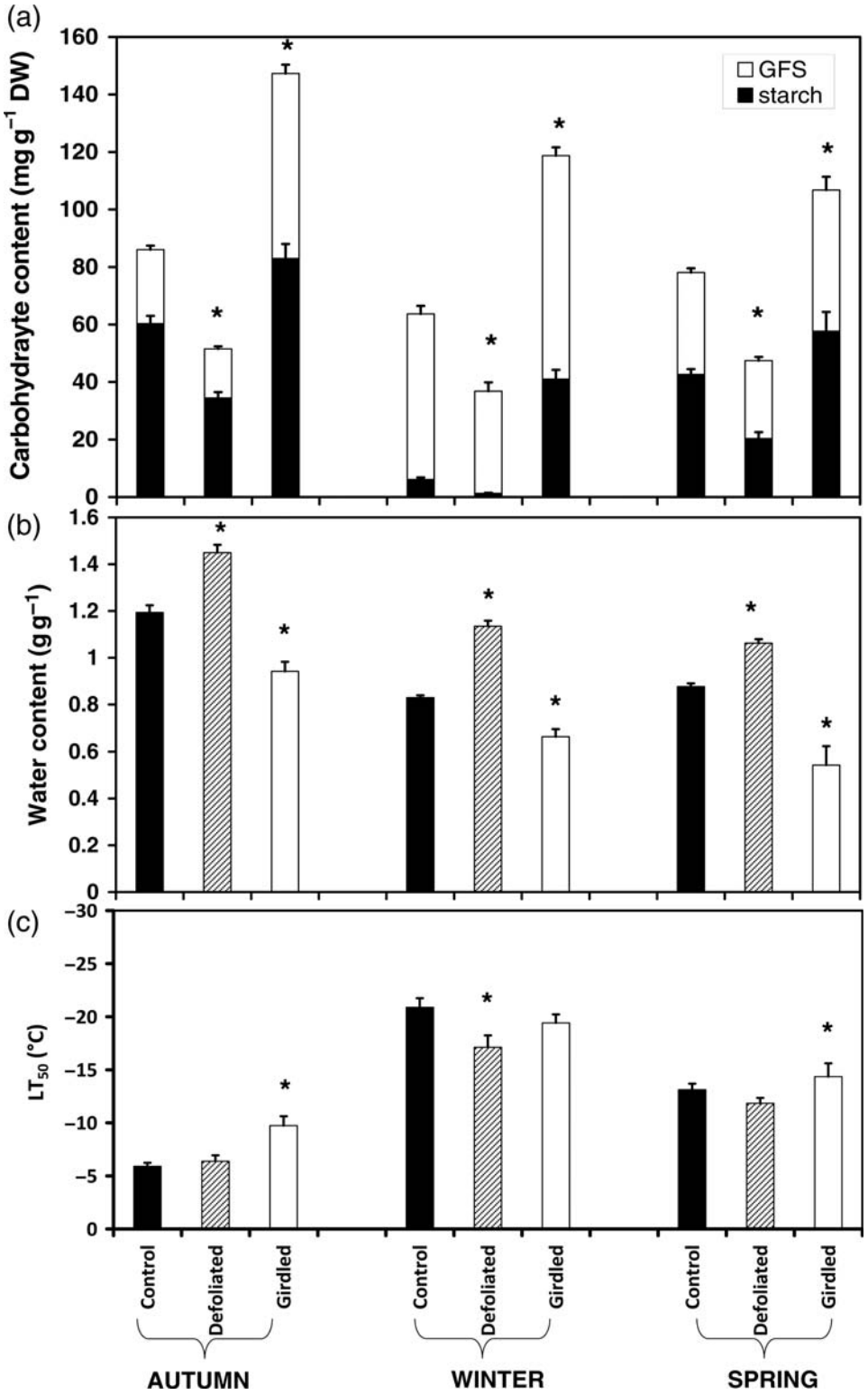

Figure 6. Summary of data for the 2005–06 winter period. Seasonal change in carbohydrate content (starch and GFS) (a), WC (b) and cold resistance as determined by mean temperature causing 50% cell lysis (LT_{50}) (c) in branches that were either defoliated, girdled or left untreated (controls) during summer prior the autumn cold hardening period. Each treatment was applied separately from one of the three main branches for the same tree (Franquette cultivar planted in Crouelle site). Values in panels are means ± SE ($n = 5$). Asterisks indicate significant differences ($P < 0.05$) between treatment (defoliated or girdled) and control for the measured period (autumn, winter or spring).

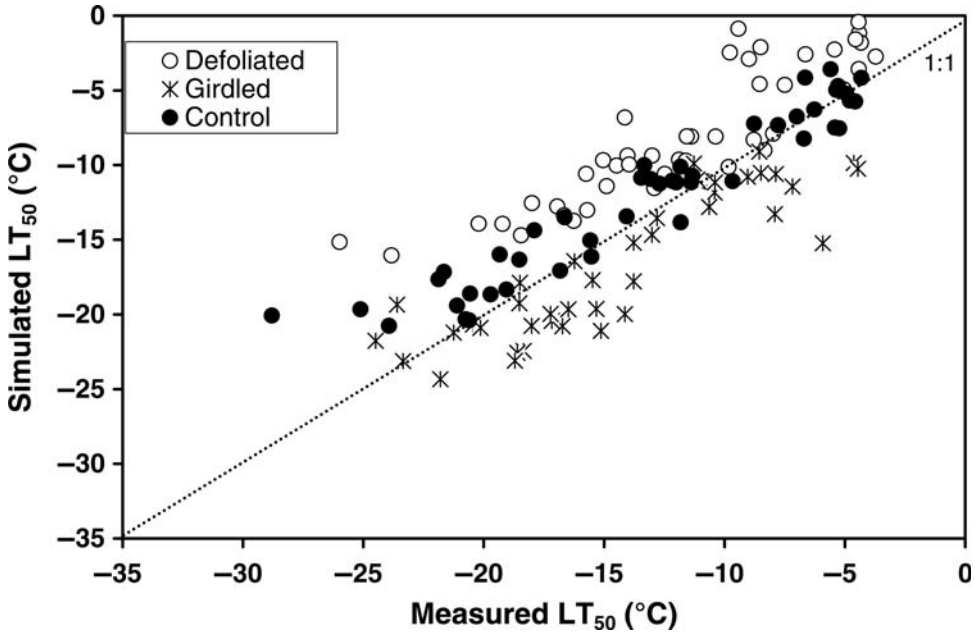

Figure 7. Model simulated LT_{50} (mean temperature causing 50% cell lysis) as a function of measured LT_{50} in branches that were either defoliated, girdled or left untreated (controls) during summer prior to the autumn cold hardening period.

Hasler 2000, Améglio et al. 2001), which is the primary cause of freezing injury in woody plants. However, it is now understood that sugars not only have a freezing-point depression effect but also stabilize membranes through osmotic and volumetric effects (Wolfe and Bryant 1999, 2001, Bryant et al. 2001, Lenné et al. 2007, 2009).

Thus, in our experience with extreme physiological treatments such as girdling or defoliation, the in-tree amount of sugar reserves appears a crucial parameter in cold acclimation (Figure 6). In the context of global climate change, where summer water deficits are predicted to happen more often, trees will therefore become more susceptible to cold. A severe summer drought not only reduces the photosynthetic activity of leaves and total carbohydrate reserves but also limits cold hardiness during winter (Poirier 2008).

The effect of WC on cold hardiness

Our results show a significant correlation between WC and frost resistance level (LT_{50}), which is not specific to walnut (Chen et al. 1976, Chen and Gusta 1978, Tanino et al. 1990, Ögren 1999, Gusta et al. 2004). Indeed, when liquid water turns into ice, there is a volume increase of ~9% that requires enough room free of water in the tissue structure to accommodate the mechanical effects of this volume change.

In normal conditions, after leaf fall, the WC decreases: more water is lost by bark or buds than supplied by roots. It has long been established (Kramer 1940) that decreasing soil temperatures result in decreasing water absorption. Thus, when temperatures dropped in autumn, stem WC decreased, allowing efficient frost hardening. Later in spring, as soil temperatures increased, water absorption peaked up again, resulting in plant rehydration (Ewers et al. 2001, Turcotte et al. 2009). Améglio et al. (2002) showed that in walnut trees, this rehydration occurred when soil temperature rose above +8 °C at 50 cm belowground.

In contrast, in the summer and/or in the warmer autumn conditions, soil temperature remained high enough to maintain root activity. If water was present in the soil and if the transpiration conditions were low, water supply was maintained, and we observed that trees rehydrated, as is usual in spring (Turcotte et al. 2009). This was particularly evident when a main branch was defoliated in summer (Figures 5c and 6b). At this date, removal of leaves led to an abrupt drop in transpiration, while water uptake was still strong. Thus, defoliated trees experienced high hydration in autumn before dehydration. This water uptake explains why WC increased and remained exceptionally high all winter, whereas for the girdling treatment, with bark removed the branch stem evaporation was higher and presented a decreased WC all winter.

Physiological model of cold hardening and dehardening in stem

The main purpose of this study was to develop a physiological model to predict cold hardiness level in walnut stem at any time during the leafless period. Why propose yet another model for cold hardening, when several were already available (Repo et al. 1990, Leinonen et al. 1995, Leinonen 1996) that allowed satisfactory simulation of cold hardening?

The main objective was to take into account the development history of trees to build a physiological model that

would be robust to extreme climatic events, which are expected to occur more frequently in the near future (IPCC 2001, 2007). In this context, a previous study (Poirier 2008) indicated that stressful summer conditions strongly impacted both the winter freezing tolerance and the carbohydrate levels of aerial organs, with a clear correlation between the two effects. Moreover, in the extreme physiological treatments (Figure 6), cold hardiness showed contrasting levels among branches in the same trees, consistent with differential levels in our two physiological parameters (water and soluble sugar content). Thus, girdling decreased WC and increased soluble sugars, whereas defoliation increased WC and decreased soluble sugars.

This result confirmed the direct impact of carbohydrate reserve and water status on cold resistance over a wide range of carbohydrate and water content. These results also confirmed the branch autonomy theory (Sprugel et al. 1991, Brisson 2001), which has been invoked as a mechanism for differential branch growth and/or survival according to local light conditions, which in turn can explain specific tree shapes in relation to stand density or age. The 'branch autonomy' concept has been included in several tree growth models (Ford et al. 1990, Takenaka 1994, Kellomäki and Strandman 1995), but Lacointe et al. (2004) showed for young walnut tree that during winter, branch autonomy was more questionable. In this study, for adult tree, we confirmed 'branch autonomy' for carbon and extended this concept also for water during winter.

The model that we propose in this study is a simple linear model based on climatic and physiological explanatory variables. Each of the three input variables (GFS, WC and $\bar{T}_{\text{min 15 days}}$) can be assigned a specific role contributing to the simulated function, frost hardiness. Sugars and WC account for the physiological status, dependent on the current and past environmental history, which can impact major functions such as the whole season carbon or water balance. The average of daily minimal temperatures over the last 15 days before sampling renders the impact of current or near-past environmental history on sugars and WC.

Currently, this model is limited to walnut, but recent results on oak (Morin et al. 2007) suggest it could be extended to other species. A more serious shortcoming is the amount of work needed to measure the physiological input variables GFS and WC. One way to solve this issue, and to provide additional insight into the physiological processes involved, would be to have these variables not measured, but simulated themselves from climatic data. This would involve investigating, then modelling, the action law of the air temperature on starch hydrolysis for GFS, or of ET_o and soil temperature for WC.

To conclude, the present study has demonstrated the importance of environmental history of the tree in the prediction of cold hardiness and proposed a way to model this history with few physiological parameters (carbohydrate and WC) and climatic data. Only if these are taken into account can we hope to better predict the impact of global climate change on tree survival and on the geographical distribution of woody plants.

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