Environmental and Experimental Botany 72 (2011) 351–357

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

The timing of leaf fall affects cold acclimation by interactions with air temperature through water and carbohydrate contents

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ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form
20 December 2010
Accepted 27 December 2010

Keywords:
Winter biology
Juglans regia
Water content
Carbohydrate
Photoperiod
Temperature

ABSTRACT

Three parameters (i.e. the water content, soluble sugar content and minimal air temperature) can be used to predict the cold acclimation process of walnut trees. In order to test this assumption, two-year-old walnuts were defoliated at two different dates, i.e. mechanical defoliation in early October (early leaf fall, EF) or natural defoliation in early November (natural leaf fall, NF) and conditioned in either outdoor freeze-deprived or cold-deprived (T_{min} > 13 °C) greenhouses over winter. Even if early defoliation date could have affected short day signal perception (SDSP), water balance and carbohydrate metabolism were more altered. EF treatment, by stopping transpiration, significantly increased tree's water content and at warm temperature high root activity stopped normal winter dehydration. Starch content decreased in all treatments, but there was only a significant increase in soluble sugar content when water content had sufficiently decreased. Thus, depending on date of defoliation, cold-deprived trees were or were not able to acclimate to frost (minimal frost hardiness = −21.8 °C *vs.* −22.1 °C in controls (freeze-deprived) for NF and −13.7 °C *vs.* −25.3 °C in controls for EF). Different treatments showed the relationship between minimal water content observed during winter and maximal soluble sugars synthesized. Thus, the cold acclimation process appeared dependent on these physiological parameters (water and soluble sugar contents) through the interaction between air temperature and timing of leaf fall.

1. Introduction

Winter biology of perennial plants is mainly driven by environmental factors, especially cold acclimation and dormancy release. Previous studies on cold acclimation showed that two successive and induced differently stages follow themselves to permit frost resistance. In a first stage, cold acclimation is induced only after budset, leaf senescence and buds dormancy by a photoperiod decrease (Leinonen, 1996; Welling and Palva, 2006). At this time, meristematic cells become dormant, leaves compounds are translocated in perennial parts and cold related proteins are synthesized. After this first stage, a relative resistance level is reached. For *Betula pendula* cv Roth, it has been demonstrated that phytochrome A in leaves is used for the short day perception (Olsen et al., 1997) and potentiates low temperature response in leaf tissue (Puhakainen et al., 2004).

Thereafter, during second stage, chilling temperatures are the main driving factor of acclimation (Sakai, 1966; Aronsson, 1975; Christersson, 1978). Different metabolic synthesis are observed with different chemical compounds maintaining membrane fluidity (polyunsaturated fatty acids, sphingolipids), cryoprotectant (anti freeze protein, dehydrins) or lowering freezing point (carbohydrates, amino acids, organic acids); (Sakai and Larcher, 1987). Impact of chilling temperatures has been extensively studied. Thus warm temperatures should slow down cold acclimation during autumn and, as a consequence, generate less frost resistance at first freezing event time (Sakai and Larcher, 1987; Repo et al., 1990).

In autumn after leaf fall, water uptake decreased with soil temperature, related with root cell membrane fluidity (Ewers et al., 2001), meaning trees become dehydrated (Sparks et al., 2001). Moreover, osmotic content increased, as temperatures drop and starch accumulated during the previous summer was converted into soluble sugars (Sauter, 1988; Witt and Sauter, 1994). Thus, for walnut trees, Poirier et al. (2010) were able to predict cold acclimation in trees using water and soluble sugar contents, combined with minimal temperatures as inputs.

So, defoliation treatment during growing season has a significant repercussion on cold acclimation potential through decrease in carbohydrate amount (Thomas and Blank, 1996), but could have also a potential effect on short day signal perception (SDSP). In this survey, two different leaf fall dates (i.e. early and natural leaf fall) and three conditioning temperatures (i.e. outdoor, freeze-deprived,

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doi:10.1016/j.envexpbot.2010.12.019

cold-deprived) on frost resistance pattern were used to test the SDSP-climate interaction on the physiology (i.e. water and carbohydrate contents) of cold acclimation process.

2. Materials and methods

2.1. Plant material and treatment

Two-years-old walnuts (*Juglans regia* cv Franquette) were housed in a greenhouse for an entire winter after the same growing season in a natural summer climate. Treatments were started either in early October (08/10/2008, daylength = 11h09mn) with mechanical defoliation (early leaf fall: EF) or early November (1/11/2009, daylength = 10h00mn) after natural leaf senescence (natural leaf fall: NF). For each treatment, 15 trees were placed in a warm greenhouse with a minimal temperature higher than +13 °C (cold-deprived trees) while 15 control trees with the same characteristics and history were housed in a freeze-proof greenhouse all wintertime. For NF treatments, 15 added potted trees were left in natural conditions (outdoor trees). For each treatment, one-year-old twigs were sampled every month over two winter periods (2007–2008 for EF and 2008–2009 for NF) on five different trees.

2.2. Frost hardiness tests

The electrolyte leakage test is based on the principle that damage to cell membranes results in an enhanced leakage of electrolytes from the cell. Recording the amount of leakage will thus provide an estimate of tissue damage. The test is fairly simple and rapid, yields quantitative data and requires only small amounts of plant material. However, certain concerns limit the validity of the technique to determine the real temperature at 50% lethality (LT_{50}). For this, several authors indicated that the precision of freezing stress resistance could be estimated by simultaneously using two or more viability tests and by combining the results of these tests (Zhang and Willison, 1987; Lindén, 2002). Nevertheless, temperature of frost hardiness obtain with this method represented a good approximation of this temperature at 50% lethality and permitted to assess frost hardiness dynamics on same trees during whole winter with the same and comparable way for different treatments.

For every sample date, each one-year old twigs ($n = 5$), they were cut into six 5-cm long segments without buds per dates to assess frost hardiness. All fresh harvested segments were washed in distilled-deionised water and placed in a moistened tissue and wrapped in aluminum foil. Shoot segments were cooled to one of four sub-zero temperatures. In addition, there was an unfrozen control in a cold room at +5 °C (control) and a lethal control in deep freezer at −80 °C. In each case, sample temperatures were monitored using copper-constantan thermocouples inserted into the foil pouch. For control and lethal control, samples were cooled at the rate of ≈7 °C h^{-1} in pre-chilled vacuum flask. For temperature-controlled boxes, cooling and warming cycle 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 °C h^{-1} and with freeze-thaw cycle between +5 °C to −5 °C, −10 °C, −20 °C and −30 °C was applied, respectively. Before thawing, the air temperature was maintained during 1 h to the freeze temperature. Temperature were recorded with data logger (Campbell, Logan, United States) as one-minute averages and averaged at 5 min intervals. After conditioning, segments were used to assess frost hardiness with electrolyte leakage method (Zhang and Willison, 1987; Sutinen et al., 1992).

After this freezing treatment, the segments were cut into 5 mm-long sections and placed in glass vials with 15 ml of distilled-deionized water. The vials were shaken for 24 h at +5 °C (limiting non-frozen induced lysis) on a horizontal gravity shaker (ST5, CAT, Germany). The electrolytic conductivity of the water in which the stem segments were immersed (C1) was measured at room temperature with a conductimeter (Held Meter LF340, TetraCon® 325, Germany).

Samples were then autoclaved at +120 °C for 30 min, cooled to room temperature, and a second conductivity measure (C_2) was done. Relative electrolytic leakage (REL) was calculated as $(C_1/C_2) \times 100$ as described in Zhang and Willison (1987). We assumed the following relationship between REL and percentage of cellular lysis (L) for each sample:

Frost hardiness of living cells in stems was estimated as the temperature where we observed inflection point (C) of the logistic sigmoid function (Repo and Lappi, 1989)

$$y = \frac{A}{1 + e^{B(C-x)} + D} \quad (1)$$

where y is relative electrolyte leakage (REL) and x is exposure temperature. The parameters A and D define the asymptotes of the function, and B is the slope at the inflection point C. The main disadvantage of the logistic sigmoid model is that the freeze-death probability curves are assumed to be symmetric. The results revealed that freeze-response may be inherently asymmetric and thus, fitting the data with a symmetric model may lead to biased lethality estimates (Lindén et al., 1996; Lindén, 2002). However, the bias was largest for point estimates near the asymptotes of the logistic function, while the inflection point estimates given by the symmetric and the asymmetric function were quite similar (Lindén, 2002).

The parameter estimation of Eq. (1) was performed by nonlinear regression using ExcelStat ver. 7.5.2. Mean frost hardiness was calculated for each treatment from the individual frost hardiness values.

2.3. Water content

Stem sample fresh weights (FW) were measured, then the stem samples were frozen with liquid nitrogen and lyophilized. After freeze-drying, dry weights (DW) were measured and water content was calculated as (FW − DW)/DW.

2.4. Extraction and quantification of carbohydrates

Lyophilized samples ($m > 2$ g) were ground into a powder that was used (50 mg) for extraction of soluble carbohydrates. It was melted with 1 ml of mannitol (5 g l^{-1}) in ethanol 80%, and shaken for 30 min at 80 °C, then centrifuged for 10 min at 15,775 × g (SR2000, Prolabo, France). The supernatant was filtered in a cartridge containing AGX-1 anion-exchange resin (150 μl), polyvinylpolypyrrolidone (100 μl) and activated carbon (200 μl). The solid was melted three more times with ethanol 80% (1 ml), ethanol 50% (0.5 ml) and ethanol 80% (0.5 ml), before the cartridge was rinsed with ethanol 80% (1 ml). The liquid fraction was SpeedVac-dried for carbohydrates analysis and the solid was SpeedVac-dried for starch analysis.

For carbohydrate analysis, dried samples were made soluble in 0.5 ml water and separated on an Aminex-HPX87C Column with a refractometer (R12000, Sopares).

To measure starch content, solid was melted with NaOH 0.02 N and autoclaved (2 h, 120 °C, 1 bar). Samples were then incubated with amyloglucosidase (1h30mn, 52 °C) in a microplate well, where each well contained 12 μl ATP (5×10^{-4} mol l^{-1}), 12 μl NADP (1.4×10^{-4} mol l^{-1}), 60 μl triethanolamine buffer (triethanolamine 0.48 mol l^{-1}, magnesium sulfate 1×10^{-2} mol l^{-1}, pH = 7.6), 96 μl water and 12 μl of sample supernatant. A spec-

trophotometric measurement was made at 340 nm (Power Wave 200, BioTek instruments) as a blank before incubation with 10 μl of hexokinase/glucose-6-phosphate dehydrogenase (EC 1.1.1.49) for 40 min under shaking after which another absorbance measurement was taken.

2.5. Statistical analysis

Statistical comparisons between treatments were performed with five replicates for each date and treatment by using an ANOVA test (i.e. Tukey's Honestly Significant Differences test) with an error $\alpha = 5\%$. Correlation tests were parametric (Pearson's test) with a 0.05 threshold.

3. Results

Fig. 1A and B shows minimal daily air temperatures for all treatments. Broad temperature differentials were recorded in the greenhouse treatments (average daily mean $\Delta T_{(\text{cold}-\text{outdoor})} = 2.51\,^{\circ}\text{C}$ in NF; daily mean $\Delta T_{(\text{warm}-\text{cold})} = 5.40\,^{\circ}\text{C}$ in NF and $7.40\,^{\circ}\text{C}$ in EF).

Date of defoliation had a significant impact on water content (WC). In the early leaf fall (EF) experiment, just after defoliation, WC increased significantly in control and cold-deprived trees from 1.2 to 1.6 and 1.8, respectively. Later similar tendency was observed for both temperature treatments but with a stronger WC in cold deprived trees. On the contrary, in natural leaf fall (NF) experiment, only WC of cold-deprived trees increased during winter (1.35 ± 0.2)

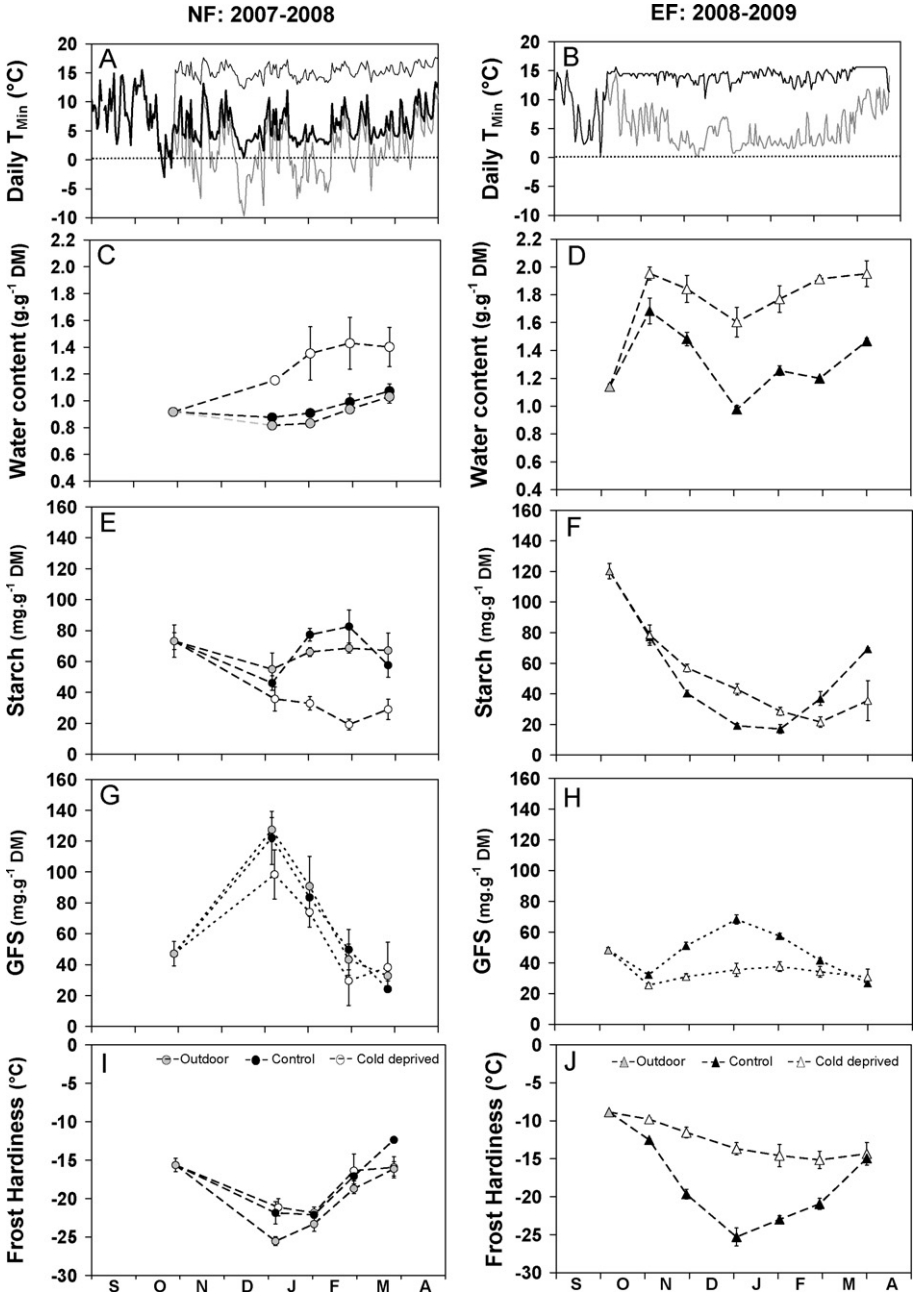

Fig. 1. Results in left panel for natural leaves fall (NF) experiments during 2007–2008 winter periods and in right panel for early leaves fall (EF) experiments during 2008–2009 winter periods. Time course of:(A) and (B) minimum daily air temperature for control cold greenhouse (bold line), cold deprived greenhouse (fine line) and outdoor treatments (grey line). (C) and (D) water content in NF (circle) or EF (square) treatments for Control (black), Outdoor (grey) and cold deprived (white) treatments. (E) and (F) starch content. (G) and (H) soluble sugar content. (I) and (J) frost hardiness levels as determined by mean temperature causing 50% of increase in electrolyte leakage. For figures (C–J), mean ± SE ($n = 5$) are given.

whereas no significant differences in WC appeared until March (WC around 1.0 ± 0.1) in control and outdoor trees (Fig. 1C).

Starch content pattern was not significantly different whatever the defoliation date. It decreased in all temperature treatments during autumn and remained stable in cold deprivation treatment (around 20 mg g^{-1} DM) while in control and outdoor trees a re-synthesis of starch before budburst was observed. For soluble sugar content (glucose + fructose + sucrose: GFS), in the NF treatment (Fig. 1G), cold-deprived trees and control trees showed similar patterns throughout the winter period, with an increase in autumn until deep winter (107 mg g^{-1} DM for cold-deprived trees and 134 mg g^{-1} DM for controls), decreasing thereafter.

Defoliation date obviously affected carbohydrate content in the EF treatment, GFS remained stable throughout the experiment and was significantly different of controls from December to February only (Fig. 1H). Even in control trees, carbohydrate content increased during winter but maximal level observed was significantly lower than in NF experiment.

Finally, for frost hardiness, in the NF experiment (Fig. 1I), all trees showed similar cold hardening (−21.8 °C for cold-deprived trees and −22.1 °C for controls, with $P = 0.011$ at 02/04/2008). In outdoor trees, minimal values were significantly lower and occurred earlier (Frost hardiness: −25.55 °C on 01/08/2008 at $P < 0.001$ for controls and $P = 0.004$ for cold-deprived trees) but were non-significantly different at 02/04/2008. Thereafter, dehardening started in all temperature treatments, with no between-group differences. Conversely, under the EF treatments (Fig. 1J), there were strong differences between control and cold-deprived trees. Control trees followed a regular pattern, with no significant difference with NF treatments in terms of maximal hardiness in February ($P = 0.69$) or dehardening in March. However, cold-deprived trees already demonstrated a significant decrease in cold hardening between October and December that was very different to controls, and frost hardiness remained stable until the end of the experiment.

Fig. 2 illustrates the relationships between frost hardiness and physiological parameters (water and soluble carbohydrate contents) or climatic parameter (minimal daily air temperature). Non-acclimated trees (frost hardiness higher than −20 °C) contained low amounts of GFS (below 60 mg g^{-1} DM; Fig. 2A) and maximal frost resistance levels were observed for a clearly higher soluble carbohydrate content (around 100 mg g^{-1} DM). This relationship between cold acclimation and GFS content is non-linear, fitting a polynomial equation: frost hardiness = $-4.64 - 0.35 \cdot \mathrm{GFS} + 1.6 \times 10^{-3} \cdot \mathrm{GFS}^2$ ($r = 0.81$), with weak changes in carbohydrate content for higher frost hardiness values and stronger changes when frost hardiness reached around −20 °C.

Less acclimated trees were also significantly more hydrated, whereas more cold-acclimated ones had less than 1.0 g g^{-1} DM in water content (Fig. 2B). The relationship is not as strong as between frost hardiness and GFS content ($r = 0.61$, $P < 0.001$). But less hydrated trees are obviously more frost-resistant.

In natural conditions (i.e. outdoor treatment), minimal air temperature seemed strongly correlated with frost hardiness level. However, a query arose regarding the best way to express minimal air temperature as an input factor to the frost resistance model. Given that the physiological processes directly altered such as carbohydrate metabolism, are unlikely to be instantaneous, the most relevant 'daily' temperature should not account for thermal profile on the individual day where frost hardiness was measured but should integrate temperatures over a several-day period. Consequently, we did a preliminary statistical analysis (data not shown) showing that frost hardiness was best correlated with the average minimal daily temperature over the 19 days before (and not including) the sampling day. Thus, Fig. 2C shows the relationship between frost hardiness and climate data. Frost hardiness and climate data were strongly correlated in the outdoor treatment

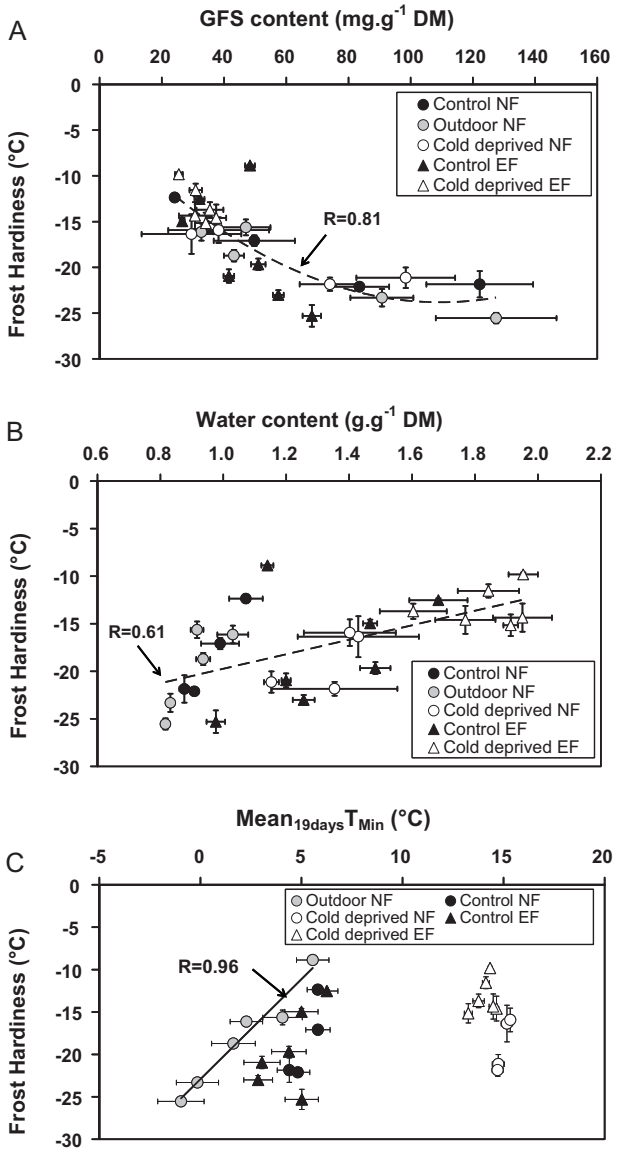

Fig. 2. Relationship between frost hardiness levels and (A) soluble sugar content (glucose + fructose + sucrose: GFS), (B) water content, (C) the average of minimal daily temperatures of the last 19 days before (and not including) the sampling day for all thermal and leaves fall treatments (mean ± SE; $n = 5$). Linear regression for outdoor treatment is given.

($r = 0.96$; $P = 0.010$), but with warmer temperatures no relationship were observed.

Finally, Fig. 3 showed that the two physiological parameters GFS and WC were related. Thus, on five different treatment (defoliation timing × temperature treatments), maximal GFS observed in trees during winter appeared strongly related to minimal WC observed with the same trees during winter ($R = -0.8481$).

4. Discussion

4.1. Effect of water content on cold hardiness

Our results show a significant correlation ($r = 0.608$; $P < 0.001$) between WC and frost hardiness which is not specific to walnut (Chen et al., 1976; Chen and Gusta, 1978; Tanino et al., 1990; Ogren, 1999; Gusta et al., 2004). Indeed, when liquid water turns into ice, there is a volume increase of about 9% that requires enough room free of water in the tissue structure to accommodate the mechan-

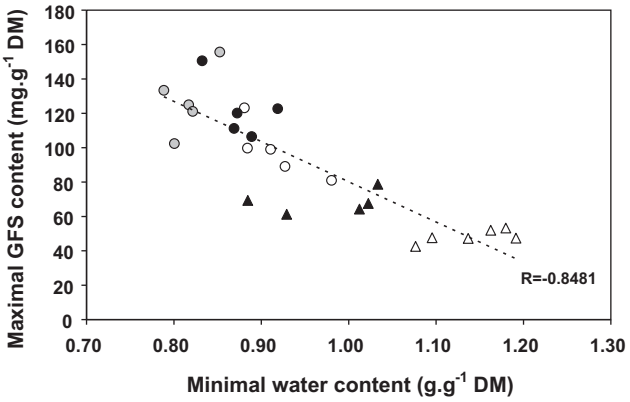

Fig. 3. Relationship between minimal water content and maximal soluble sugar content (glucose + fructose + sucrose: GFS) for all treatments (defoliation timing × temperature treatments). Mean ± SE ($n = 5$) are given.

ical effects of this volume change. A decrease in water also means that cell sap concentration increases, lowering the freezing point of cytosol.

In natural conditions, after leaf fall, WC decreases: more water was evaporated by bark than was absorbed by roots. It has long been established (Kramer, 1940) that decreasing soil temperatures results in decreasing water absorption as cold temperature reduces root cell membrane permeability (Levitt, 1980). Thus, when temperatures drop in autumn, stem water content decreases allowing efficient frost hardening. Later in spring, as soil temperatures increase, water absorption picks up again resulting in plant rehydration (Ewers et al., 2001; Turcotte et al., 2009). Améglio et al. (2002) demonstrated that this rehydration in walnut trees occurred when soil temperature is above +8 °C at 50 cm belowground.

In cold-deprived conditions, soil temperature remained high enough to maintain root activity. If the soil is moist enough, water supply is maintained, and we could observe rehydrating of trees, as it is usually occurring in spring (Turcotte et al., 2009). This was particularly evident when trees were defoliated in early October (Fig. 1D), when soil temperatures were still high. At this date, removing the leaves led to a loss in the balance between absorption and evaporation. Transpiration was stopped while water uptake was still strong resulting in a sharp rising in water content. Thus, control trees experienced high hydration before "normal" dehydration. Furthermore, for cold-deprived trees (both EF and NF treatments), this water uptake explains why water content increased and remained exceptionally high all winter long, whereas under natural defoliation and 'normal' autumn temperatures, soil temperature was already low and the trees did not have the possibility to recover water lost by transpiration.

4.2. Role of carbohydrates in cold hardiness

Cold hardiness was positively related to soluble sugar content (Figs. 1G and 2A) in all treatments, which is consistent with numerous studies (Siminovitch et al., 1953; Kramer and Kozlowski, 1979; Sauter and Ambrosius, 1986; Sauter and Van Cleve, 1994; Améglio et al., 2004; Thomas et al., 2004; Morin et al., 2007). In this study, GFS were pooled and represented more than 90% of total soluble sugars in walnut trees during winter. Literature reports state that the development of freezing tolerance is invariably accompanied by the conversion of starch to sucrose (Parker, 1962; Sakai and Yoshida, 1968; Essiamah and Eschrich, 1985; Ögren et al., 1997; Pearce, 2001; Klotke et al., 2004). A higher soluble sugar content lowers the freezing point of the intracellular solution, but the measured differences in concentration between hardened and de-hardened tissues can only explain 1 or 2 °C of the 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 sugars 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). At the same temperature, the ice will have a lower chemical potential than the super cooled water in the cell (Mazur, 1963; Rajashekar et al., 1982, 1983). This extracellular ice formation prevents concurrent intracellular ice (Burke et al., 1976). This is why water diffuses from the cells to the ice-growing zone, resulting in cell dehydration (Loris et al., 1999; Zweifel and 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 depress the effects associated with dehydration (osmotic effect and a volumetric effect: Wolfe and Bryant, 1999, 2001; Bryant et al., 2001; Lenne et al., 2007, 2009). Moreover, sucrose has the ability to stabilize biological membranes via direct interaction with lipids during dehydration when most of the free water is outside the cell (glassy state: Crowe, 2002). Thus, the in-tree amount of carbohydrate reserves appears to be one crucial parameter in cold acclimation and summer stresses which could hugely impact amount of reserves could have a non-negligible effect on sensitivity to frost damages. A severe defoliation during summer not only reduces the photosynthetic activity of leaves and total carbohydrate reserves but also limits cold hardiness during winter (Poirier et al., 2010; Poirier, 2008; Poirier and Améglio, 2006).

In addition, as shown in Sakai (1966), low temperature (<5 °C) or warm temperature (>15 °C) can generate starch hydrolysis, as was observed in our study. This was explained as an increasing of the catalytic activity of amylase at warm temperature or through the precipitation of endoamylase on starch granules with low catalytic activity but a closer relationship between enzymes and substrates at low temperatures (Elle and Sauter, 2000). Finally, according to soluble sugar content, whatever the temperature is below +5 °C or above +15 °C, cold hardiness seems possible.

4.3. Impact of climate on cold hardiness

Low temperatures are more often recognized as a key constraint in tree distribution (Shreve, 1914; Sakai and Weisner, 1973; George et al., 1974), especially at the higher latitudinal limit of a species (Gusta et al., 1983; Arris and Eagleson, 1989) or at treeline limit (Cavieres et al., 2000), and there are numerous reports of the effect of low temperatures on frost hardening (Mac Irving and Lanphear, 1967; Howell and Weiser, 1970; Levitt, 1980; Sakai and Larcher, 1987). In our study, cold acclimation is observed for trees submitted to low temperatures, but also cold-deprived trees and air temperature appeared not directly related to hardiness (*cf*. Fig. 2).

Schwarz (1970) in *Pinus cembra* or Taulavuori et al. (2000) in *P. sylvestris* showed also more complex relationship between cold hardiness and temperature. Thus, in this last species, reducing the day length appeared sufficient to obtain significant hardness in warm temperature (+20 °C). Moreover, some authors considered photoperiod as the key driver to cold acclimation.

In our study, a lower photoperiod for EF treatment (i.e. 10h00mn *vs*. 11h09mn for NF treatment) had a strong effect on maximal frost hardiness for cold-deprived treatment (Fig. 1I and J) whereas no significant difference was observed for control and outdoor temperature treatments. Welling et al. (1997) showed in *Betula pubescens*, that cold acclimation was initiated with a 3 weeks exposure to a photoperiod less than 12 h which was the case in our experiment as well for NF that for EF conditions. Differences observed in cold acclimation pattern between treatments were

probably triggered through physiological impact and not only on the inability of the EF trees to capture without leaves the short day signal perception (SDSP).

4.4. Interaction between timing of defoliation, temperature and physiological parameters

For WC, in the EF treatment, warm temperatures inhibited dehydration, while in the NF treatment, dehydration had already occurred. This difference could explain why in one case, at warmer temperatures, GFS content increased in one treatment (NF) but not in the other (EF). A previous study in poplar trees (Ruttink et al., 2007) reported that similar dehydration induced dormancy mediated by ABA content in buds. Li et al. (2003), studying birch, reported that ABA content increased in autumn simultaneously with cold acclimation and dormancy release. Thus, ABA content is often associated with abiotic stress (Popko et al., 2010). Applying exogenous ABA has an effect on cold tolerance in wheat (Veisz et al., 1996) and cultured cells (Galiba et al., 1993) via carbohydrate metabolism (Kerepesi et al., 2004). Also, ABA has an impact on cold signal transduction (Tahtiharju and Palva, 2001). Hence, in the EF treatment, it is possible that the higher WC altered carbohydrate metabolism due to low ABA levels. Furthermore, previous literature on the role of ABA suggests an interaction between WC and GFS content, as shown in Fig. 3.

In the EF treatment, in cold-deprived trees, starch content decreased at a similar rate of controls but without a significant increase in GFS content, whereas in NF treatments, GFS dynamics were similar in cold-deprived and control trees, with starch hydrolysis occurring at two temperature levels (below +5 °C or above +13 °C). Thereafter, soluble sugars were resynthesized into starch in controls but not cold-deprived trees. As a consequence, dehydration signal transduction via ABA modulation is probably not initiated in EF treatments. This could explain why soluble carbohydrate did not increase while trees stayed fully hydrated, whereas the decrease in starch content could be explained by high respiration levels.

To conclude, the timing of leaf fall could have a huge impact on the cold acclimation of trees depending on tree history and through their effect on water balance and soluble sugar content, and on the interaction of these two parameters. In the event of natural leaf fall, when dehydration has started, temperatures below +5 °C or above +15 °C enable cold hardening via starch hydrolysis and soluble sugar synthesis. However, if temperatures at leaf fall stay warm (e.g. early defoliation due to hard summer drought or phytophagous attack), trees stay highly hydrated if water is available in soil, and consequently disturbances in carbohydrate metabolism could lead to high frost sensitivity at the first winter freezing event. In all cases, the level of these two parameters (WC and GFS) appeared related with cold hardiness in walnut.

Acknowledgments

This work was supported in part by INRA–department of Agronomy and Environment and by a MRES PhD grant to G. Charrier. We are grateful to Christian Bodet and Christophe Serre for frost hardiness measurement contributions and Brigitte Girard, Brigitte Saint-Joanis and Marc Vandame for biochemical analysis contributions. We would like to thank Dr. Kari Taulavuori, editor-in-chief and the two anonymous reviewers for the long comments on the first draft that greatly improved this manuscript and Dr. D.G. Biron for the improvement of the English throughout the paper.

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