ADAPTATION TO COLD TEMPERATURE AND RESPONSE TO FREEZING IN WALNUT TREE

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Keywords: cold hardiness, frost hardiness, freezing avoidance, freezing tolerance, cell dehydration, diameter variation, carbohydrate reserves, electrolyte leakage conductivity.

Abstract

In south-central France, Walnut exhibited freezing tolerance by acclimation in the fall and deacclimation in the spring. This involved a sequence of active processes that result in well-defined changes in cellular composition (starch level in particular). At the same time, the response to freezing by dehydration of cells showed that Walnut trees present also freezing avoidance. We developed a new simple tool for testing cold hardiness and compared it with the classical LT_{50} test (electrical leakage conductivity). The new test gave similar results. We observed also similar results for both tests when applied to cold-deprived trees (in greenhouse >15°C). Frost hardiness was correlated with carbohydrate reserve status in the both cases (cold-deprived trees and orchard trees).

1. Introduction

It is commonly recognised that low temperatures are a major limiting factor that may explain species distribution in cold climates (George *et al*. 1974). For this reason, cold hardiness is often a selection criterion in breeding programs, and several research programs have placed a primary emphasis on elucidating mechanisms of freezing injury and temperature acclimation (Ashworth, 1986). Strategies that allow plants to survive freezing temperatures have been placed into two major categories: freezing tolerance for plants that exhibit deep supercooling characteristics (Ashworth *et al*., 1993) and freezing avoidance, characterising plants that exhibit extra-cellular freezing (Burke *et al*., 1976). Due to this formation of extra-cellular ice, frost leads to pronounced shrinking of the living bark (Loris *et al*., 1999). Although this mechanism was shown for the first time by Friedrich in 1897, it has received little attention to this days.

Perennial plants of temperate zones undergo cyclic change in hardiness each year. Classically, acclimation with the cessation of growth in the autumn is initiated by certain

environmental stimuli (e.g. shortening days, changes in light quality, decreasing temperature and drought stress; see Bowers, 1994) and during mid-winter, plants begin to deacclimate (Bowers, 1994). This deacclimatation becomes irreversible when spring growth temperature become favorable. Various methods exist for estimating the acclimation and the frost hardiness, but the most widely used one is the electrolyte leakage conductivity method based on the method of Zhang and Willison (1987).

In our study, frost hardiness was compared on walnut trees grown in two conditions (natural conditions *vs*. cold-deprived potted trees in greenhouse >15°C). For these two conditions, carbohydrate reserve and acclimation evolution were investigated. For the last point, two methods were applied for acclimation estimate, the electrolyte leakage conductivity method (LT_{50}) and a new test based on stem diameter changes (extra-cellular freezing).

2. Materials and methods

Measurements were made on excised 1-year old twigs Walnut (*Juglans regia* L. *cv*. Franquette scions) at the INRA PIAF station near Clermont-Ferrand, in south central France. Samples were taken from twelve-year-old trees in orchard and three-year-old trees in container. Each segment was used for one of three different measurements.

First, segment of twigs were frozen in liquid nitrogen, lyophylised, and their dry weight measured. Soluble sugars were then extracted from the stems 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 & Gaudillière (1992). Using a spectrophotometer at 340 nm, sucrose, glucose and fructose contents were determined after enzymatic assays (Boehringer 1984), and starch content was determined with a hexokinase, glucose-6-phosphate linked assay (Kunst, Draeger & Ziegenhorn 1984) after hydrolysis with amyloglucosidase (Boehringer, 1984).

Second, twig segments were used for estimating frost hardiness with an electrolyte leakage conductivity method (LT_{50}: ie, subzero temperature causing 50% mortality: based on the methods of Wisniewski and Ashworth, 1985; Zhang and Willison, 1986). Fresh segments harvested were washed in distilled-deionized water. Stem sections of uniform size (10 cm in length) and a moistened tissue were wrapped in aluminium foil and placed in pre-chilled Dewar flasks. Flasks were transferred to a deep freezer (-80°C). Sample temperatures were monitored using copper-constantan thermocouples inserted into the foil pouch. Samples cooled at the rate of 5-7°C/h. At any desired subzero temperature (ex. control+5°C, –5°C, -10°C, -15°C, -20°C, -30°C), Dewar flask were placed at +5°C in a refrigerator for 12-15 h to facilitate slow thawing of the samples. After thawing, internodal sections were quartered and sliced in several segments while immersed in 20ml of precooled (+5°C) distilled-deionized water into 2.5x20 cm test tubes. The capped tubes were placed on a gyratory shaker for a night. Initial conductivity was taken at the end of the night (12h) with an electrical conductivity meter (Conductivity hand-Held Meter LF340 with standard conductivity cell, TetraCon® 325). To obtain maximum conductivity, tubes were autoclaved at 120°C for 15 min. Relative conductivity was calculated by dividing the initial conductivity value by the maximum conductivity value

(Zhang and Willison, 1987; Boorse *et al*., 1998). After plotting the percentage of injury as a function of treatment temperatures, the temperature at which 50% injury occurs was defined as LT_{50}.

Third, segment of twigs were submitted to several freeze-thaw cycles. For this, a temperature-controlled chamber was designed to hold ten excised stem segments, of 5 cm in length and 1 cm in diameter. Cooling and warming cycles were computer-controlled by a circulator bath (Ministat Huber –25°C to +120°C) with external Pt100 into the chamber, with a steady rate of cooling and warming of 5°C per hour, and with freeze-thaw cycles repeated up to 10 times. Copper-constantan thermocouples were used to measure stem and air temperatures. Segment diameter variations were monitored with LVDT devices (models DF 2.5 and DF 5, Solartron Metrology, Massy, France) allowing sensitive measurements (± 1 µm) of diameter variation throughout the freeze-thaw cycles in controlled chamber. Temperatures and stem diameter fluctuations were recorded with data loggers as one-minute averages and averaged at five-minute intervals.

As a reference in natural condition, diameter variation (Huguet, 1985; Améglio and Cruiziat, 1992) was also measured on the trunk of 12-year old trees grown in orchard. In parallel, air and trunk temperature were measured.

3. Results

In winter, trunk shrank down during frost and expanded back during thaw (Figure 1). This shrinkage was very significant: 1400 µm when air temperature was near to –10°C, which is more than the diameter fluctuations that are related to water status during the vegetative period.

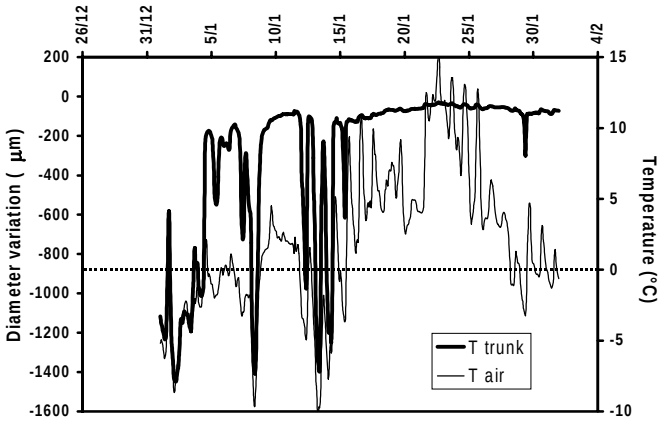

Figure 1: Time course of diameter variation of a trunk and air temperature in January. When air temperature decreases below zero, trunk diameter shrinks. When temperature increases, trunk diameter expands back to initial position.

During winter, total carbohydrate stored in the stem decreased slowly (Figure 2) but composition in sugars changed. We observed a classical interconversion between starch and soluble sugars (Figure 2), which could lower the freezing point by osmotic effect (-1.86°C for 1 osmole).

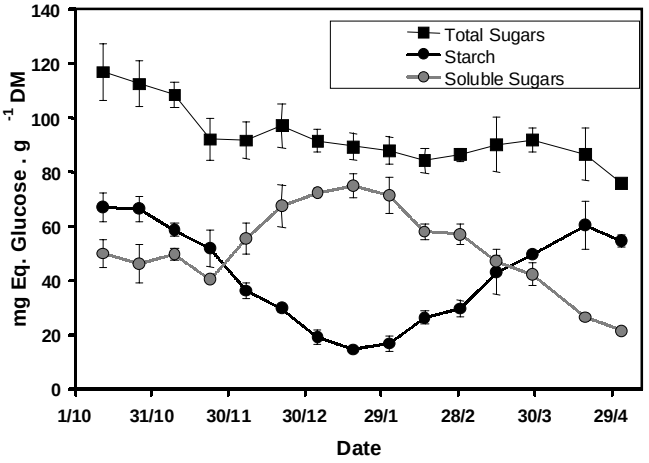

Figure 2: Evolution of sugars content: Total sugars = starch +soluble sugars. We observe an interconversion between starch and soluble sugars (sucrose essentially, glucose and fructose) during winter. Vertical bars represent standard errors.

Figure 3 shows acclimation during Autumn and deacclimation during mid-winter and spring. We observed no difference in pattern acclimation for the different conditions: orchard, potted trees and cold-deprived potted trees in greenhouse (Air temperature >15°C). Frost hardiness in winter was more developed for potted trees in natural condition (LT_{50} = -30°C) than for orchard trees (LT_{50} = -25°C) and potted trees in greenhouse for a temperature upper +15°C (LT_{50} = -20°C)

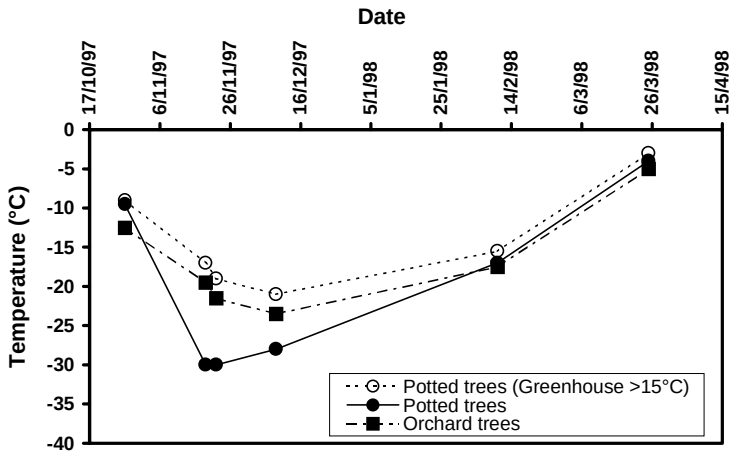

Figure 3: Evolution of the temperature at which 50% injury occurred (LT50) during winter for 3 treatments: - twelve-year-old Walnut in orchard. - three-year-old Walnut in container in natural conditions. - three-year-old Walnut in container in warmed greenhouse (Air temperature > +15°C)

Figure 4 shows diameter variation during two successive freeze-thaw cycles. We can observe an exotherm when stem freezes (arrow). This exotherm appeared for a temperature below –6°C (supercooling). At the exact time of the exotherm, stem shrinkage was observed for control stem. In contrast, stem swelling was observed for boiled stem.

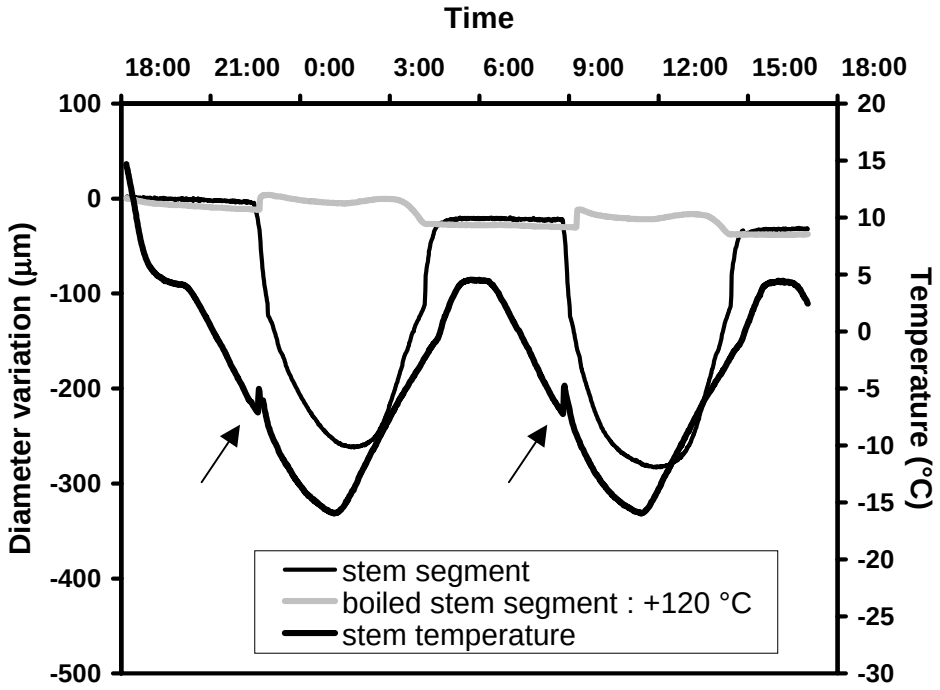

Figure 4: Diameter variation during 2 freeze-thaw cycles for a control stem segment and for a boiled stem segment (Arrow: exotherm).

The same measurements in control stem segment (not boiled) in October (Figure 5,a) and December (Figure 5,b) showed contrasting results. In October, we observed similar diameter variation in the first cycle. Stem diameter shrunk but after the first cycle, the diameter did not recover its initial dimension and after two and three cycles, we observed an increase of the diameter (Figure 5,a).

In December, stem diameter shrunk during freeze and swelled during thaw for all cycles and for the two treatments (control orchard tree and cool deprived potted tree in greenhouse : Figure 5,b).

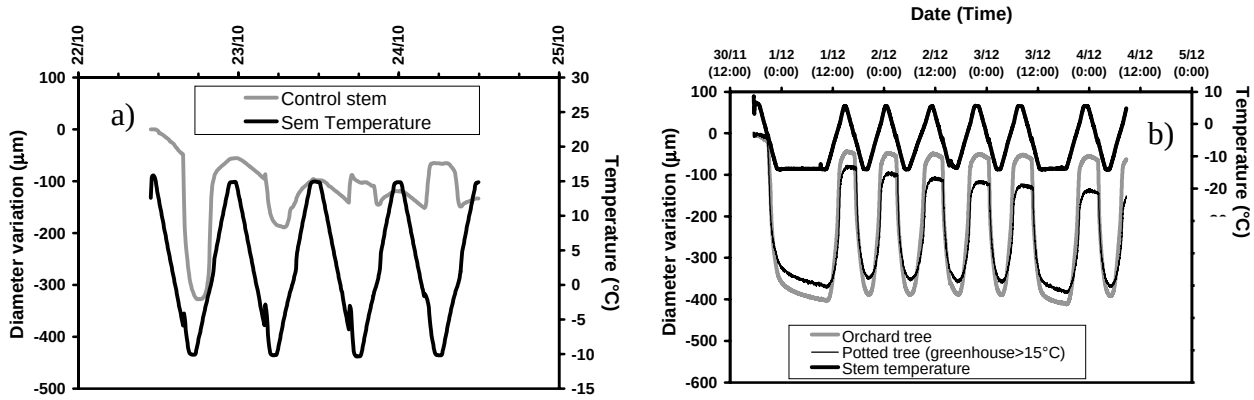

Figure 5: Diameter variation during several freeze-thaw cycles for a stem segment –a) Diameter variation for a control stem segment (orchard tree) in October. –b) Diameter variation for a control stem segment (Orchard tree) and for treated stem segment (potted tree in warmed greenhouse: air temperature>15°C) in December.

In the both conditions, in December, starch content was low and soluble sugar contents (GFS: glucose, fructose and sucrose) was high (Figure 6), which could be ascribed to the carbohydrate interconversion (*cf*. Figure 2).

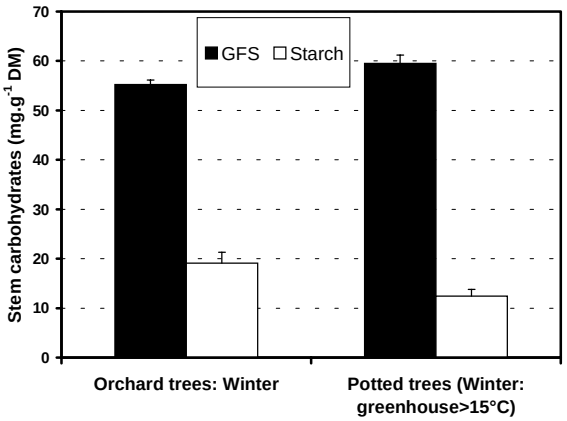

Figure 6: Evolution of stem carbohydrate: starch and GFS (glucose, fructose and sucrose). The bars represent standard errors

3. Discussion and conclusions

In our studies, we observed freezing tolerance by acclimation in the fall and deacclimation in the spring. This acclimation was concomitant to the interconversion between starch and soluble sugars. However, it can be easily calculated that one mole of sugar can only account a freezing point lowering of –1.86°C. Lowering freezing point by osmotic effects is not an essential factor; the increase in intracellular sugar content acts possibly much more by influencing the hydration degree of biomembranes and proteins (Sauter *et al*., 1996) or by protecting these structures from freezing events. Stem freezing was observed for a temperature of –6 °C, which show that some supercooling did occur

although its was not a deep supercooling (Ashworth *et al*., 1993). At the same time, the response to freezing by dehydration of cells show that Walnut trees present also freezing avoidance. In December, diameter variation decreased rapidly at the exotherm. This can be related to what is currently known of extra-cellular freezing in freezing tolerant plant (Burke *et al*., 1976). In this process, water in the cell walls freezes first in the course of cooling (Lewitt, 1972; Pearce an Beckett, 1985), because this water has a lower solute concentration than intracellular vacuolar and cytoplasmic water. Intracellular concentration is particularly high in cold condition due to starch breakdown. Once the temperature drops below the freezing point, the vapour deficit will be higher than that of the extracellular ice at the same temperature (Mazur P., 1969; Loris et al., 1999). Consequently water diffuses from the cells through the plasma membrane to the ice nucleation point in the extracellular space and, as a consequence, cell water is lost. The same phenomenon explained why bark shrunk. We showed that this mechanism was obtained for a living tissue, but not for a killed tissue (boiled stem: 120°C). In this case, swelling was observed. Similar swelling was observed during October. At this date, LT_{50} test (electrical leakage conductivity) indicated that 50% of cells were killed at the temperature of –9°C and explained why after two cycles at –10°C, swelling was observed at freezing time. With these results, we propose a new simple tool for testing cold hardiness based on diameter variation. In comparison with classical LT_{50} test, we observed similar acclimation response between the two tests. In example, cold deprived trees in greenhouse (>15°C) showed acclimation response in the both cases. This surprising result was consistent with carbohydrate reserve status. In the two cases (cold-deprived trees and orchard trees), we observed interconversion between starch and soluble sugars in December. In the both cases, difference in solute concentration of extracellular vs. intracellular compartment was sufficient to explain the cell dehydration and questions of the general accepted requirement for cold temperature in the acclimation process (Bowers, 1994).

Acknowledgments.

This work was supported by the “Ministère des Affaires Etrangères” under Project “Procope” (Procope N°97072: France-Germany) and by Institut National de la Recherche Agronomique (department Environment and Agronomy). Additional technical assistance was provided by Mrs P. Chaleil, M. Crocombette, and S. Ploquin.

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