Gelista™: A New Tool for Testing Frost Hardiness by Stem Diameter Variations on Walnut

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Abstract

Extracellular freeze-induced desiccation tolerance was observed by diameter variations on walnut in the field. To evaluate frost acclimation, stem segments were submitted to various freezing experiments in a climatic chamber. Reversible stem shrinkage was obtained for living tissue, but not for autoclaved tissue an injured tissue. In this case, swelling was observed with the freeze due to water expansion with the liquid solid phase change. Similar swelling was observed during early November for stem segments. Given that the diameter fluctuation patterns were dramatically different for acclimated versus non-acclimated plants, and for living versus autoclaved tissues LVDT sensors could represent a novel, non-invasive approach to testing frost hardiness. Practical conditions to apply the Gelista™ method to test the temperature of freezing injury was defined.

INTRODUCTION

Low temperatures are a major limiting factor that may explain species distribution in cold climates (Parker, 1963; George et al. 1974). For this reason, freezing tolerance is often a selection criterion in breeding programs (Stushnoff, 1972; Ashworth and Wisniewski, 1991; Palonen and Buszard, 1997) and several research programs have placed primary emphasis on elucidating mechanisms of freezing injury and cold acclimation (Ashworth, 1986, Rodrigo, 2000). Following a frost, the fate of the cell depends on where the formation of ice crystals takes place (Mazur, 1969). Thus, ice formation can be either intra–cellular, which is fatal and causes cell death if cooling is rapid, or extra-cellular, which protects the cells themselves at least temporarily (Levitt, 1980; Rodrigo, 2000). Strategies that allow plants to survive freezing temperatures have been placed into two major categories: those plants that exhibit deep supercooling characteristics (Ashworth et al., 1993) and those that exhibit extra-cellular freezing (Burke et al., 1976). Due to this formation of extra-cellular ice, on the surface of the cell-wall, in lumens of non-living fibres and vessels, or in the extra-cellular spaces (Guy, 1990), cell dehydration due to water redistribution occurs. Liquid water moves out of the cell (Mazur, 1969) and the osmotic concentration inside cells increases and thus prevents intra-cellular-freezing.

The same phenomenon has been used to explained why the living bark shrinks at freezing temperatures (Wiegand, 1906; Winget and Kozlowski, 1964). Although this mechanism was reported in the 19th century (Hoffmann, 1857; Sachs, 1860; Friedrich, 1897), it has received little attention until recently (Loris et al., 1999; Zweifel and Häsler, 2000, Améglio et al. 2001a and b). Zweifel and Häsler (2000) of mature subalpine conifers showed reversible shrinkage with LVDT sensors (Linear variable differential transformer) and concluded there was transport of water between bark and wood and that, when ice crystals melted, water returned to the living cells of the bark.

Perennial plants of temperate zones undergo cyclic change in hardiness each year. Classically, cold acclimation with the cessation of growth in the autumn is initiated by certain environmental stimuli (e.g. decreased daylength, changes in light quality, decreasing temperature and drought stress) and during the spring, plants begin to deacclimate (Bowers, 1994). In contrast, non-acclimated plants have cell death at freezing

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temperatures, caused by disruption of cell membranes and other cellular components (Burke et al., 1976, Steponkus, 1984). This disruption is usually manifested as a flaccidity and/or discoloration of the affected tissues (Burke et al, 1976; Rodrigo, 2000).

In the present study, to evaluate frost acclimation, stem segments were submitted to various freezing experiments in a climatic chamber.

MATERIALS AND METHODS

Temperature and diameter variation measurements were made on excised stems of orchard walnut trees (*Juglans regia* L. *cv*. Franquette scions on wild walnut root stocks) in the winters of 1999-2000 and 2000-2001. The trees were grown outdoors at the INRA PIAF station near Clermont-Ferrand, in south-central France when trees were 18 years old in 1999. Stem segment of twigs were submitted to several freeze-thaw cycles. For this, a temperature-controlled chamber with LVDT sensors, "Gelista™" (INRA, France: Améglio et al., 2001a) was designed to hold ten excised stem segments of 5 cm length and 1 cm diameter and to measure diameter variations. 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 linear rate of cooling and warming of 5°C per hour, and with freeze-thaw cycles (0/-10°C/0°C) repeated up to 10 times or with progressive freezing temperature (-2.5, -5, -7.5, -10°C) were imposed. 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 the chamber. Temperatures and stem diameter fluctuations were recorded with data loggers (DL2e, Delta T devices, UK) as five-minute averages and averaged at one-minute intervals.

In some cases the stem segment was autoclaved to kill all living cells. A minimum of ten replicates for each case were compared with regard to diameter fluctuations during the freeze/thaw cycles. We also calculated the percentage injury at a given temperature (Gelista™ method). For this, the loss of diameter after one cycle of freeze-thaw at a given temperature was compared to the maximal diameter loss. To obtain the maximal diameter loss, stem segments were submitted to one heat shock cycle (T°C>55°C).

$$\% \text{ injury} = 100 * a/b$$

with
a = initial diameter - diameter after 1 cycle at temperature tested
b = initial diameter – minimal diameter after heat shock.

RESULTS

With the Gelista™ chamber, progressive freezing temperatures (-2.5, -5, -7.5, -10°C) were imposed on isolated stem segments from orchard trees in early (figure 1a) and late (figure 1b) November, 1999. The cooling cycles with minimum temperatures from –2 to -4°C resulted in no diameter changes. We observed shrinkage only when exotherms appeared, which indicated a freezing event had occurred within the stem. In isolated stem segments these would typically occur at temperatures around -5°C to -8°C.

The LVDT results were quite different in early (Figure 1a) versus late (Figure 1b) November, suggesting an acclimation response. For instance, during early November, the diameter fluctuations were not entirely reversible, and repeated freeze/thaw cycles resulted in progressive loss of diameter. Furthermore, after repeated freeze-thaw cycles, there were spikes (transient increases) in stem diameter associated with exotherms and endotherms (Figure 1a). In contrast in late November, there were large, reversible declines in diameter that were initiated with the freeze events. Even with many freeze-thaw cycles, there were no positive spikes in diameter associated with the exotherm or endotherm events (Figure 1b).

Typical responses with LVDT sensors are summarised by the schematic Figure 2.

For acclimated stems, diameter fluctuations were entirely reversible. For non acclimated stems, diameter fluctuations was not entirely reversible and repeated freeze-thaw cycle resulted in progressive loss of diameter with positive spikes associated with an exotherm and endotherm.

In Figure 3, we observed diameter fluctuations for three freeze-thaw cycles for an acclimated stem segment, and a similar autoclaved stem segment. We can observe an exotherm when the stem freezes (arrow). This exotherm appeared for a temperature below –4°C. At the exact time of the exotherm, stem shrinkage was observed for control stem. In contrast, stem swelling was observed for the autoclaved stem.

Figure 4 illustrated the calculation of the percentage injury by the Gelista™ method. Freeze-thaw cycle at each temperature tested was followed by a rapid heat shock cycle at +55°C and by a new freeze-thaw cycle at the same initial temperature tested. Diameter fluctuation for the first freeze thaw cycle presented, in this case, a loss of diameter (a = 116 µm) and after the heat shock, for the second freeze thaw cycle, a maximal loss of diameter (b = 379 µm). In this example, stem segment presented 31 % of injury for the tested temperature of –10°C.

DISCUSSION AND CONCLUSIONS

Results of our LVDT studies can be related to what is currently known of extra-cellular freezing in freezing tolerant plants (Burke et al., 1976). During freezing, plant cells are dehydrated by the formation of ice in the intercellular spaces, causing the protoplasts to shrink and the cell turgor to decrease (Zhu et al., 1989). Our results provide evidence that the stem diameter changes in winter with freezing-induced shrinkage and thaw-induced expansion. During the summer season, we have used LVDT devices to observed diurnal and seasonal changes in stem diameter that related to variations in water content (Améglio and Cruiziat, 1992; Simonneau et al., 1993; Zweifel et al. 2000).

In this process, extra-cellular water freezes first since it has a lower solute concentration than intracellular vacuolar and cytoplasmic water. 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 1969; Loris et al., 1999). Consequently water diffuses from the cells through the plasma membrane to the ice crystals in the extracellular matrix and, as a consequence, cell water is lost. However, this water lost from cells by freezing in extracellular spaces should increase by about 9% in volume by changing state. Why, in these conditions, do we observe a decrease in the thickness of the bark? We suppose that the structure of the bark is very porous.

Reversible stem shrinkage was obtained for a living tissue, but not for autoclaved tissue. In this case, swelling was observed with the freeze and this swelling could be explained by bark alone, and due to water expansion with the liquid solid phase change.

Similar swelling was observed during early November (Figure 1a). By comparison with autoclaved treatment, we analysed this result in terms of cell death due to non-acclimated trees. Our results showed non reversible shrinkage of bark and water lost after one cycle at -10°C. In each case, freezing injury on bark, with discoloration of tissues, was observed after a few cycles at the same temperature.

Given that the diameter fluctuation patterns were dramatically different for acclimated versus non-acclimated plants, and for living versus autoclaved tissues LVDT sensors could represent a novel, non-invasive approach to testing cold hardiness. We defined the practical conditions to test a temperature freeze event with the Gelista™ method. It presents the advantage of using limited plant material, allowing its use in breeding programs.

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Figures

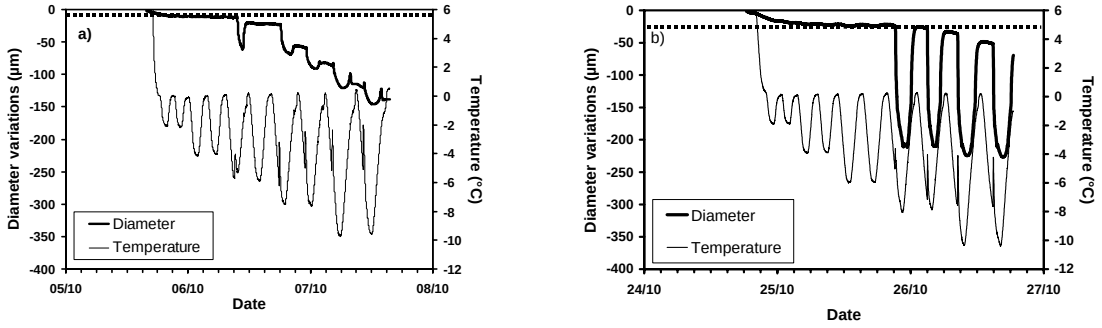

Fig. 1. Stem diameter for progressive freeze-thaw cycles (2 cycles at the same temperature for each time). We observed stem shrinkage only when stem temperature presented an exotherm (≈-6°C). a) in early November 1999, the diameter fluctuations were not entirely reversible and repeated freeze/thaw cycles resulted in progressive loss of diameter. b) in late November, we observed large and reversible diameter fluctuations.

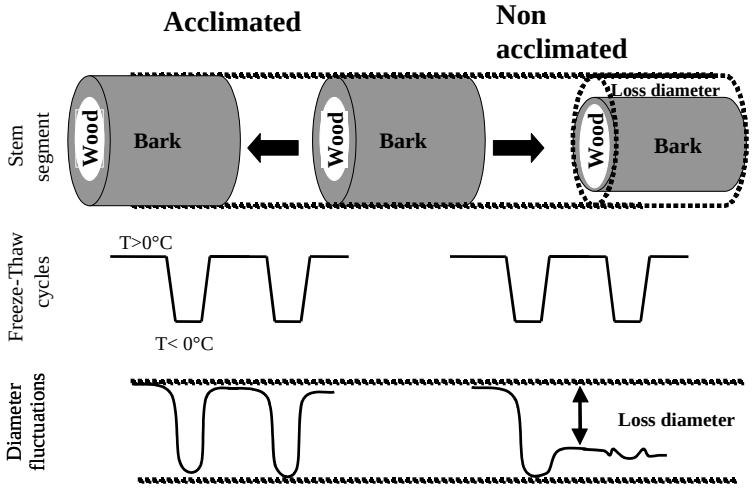

Fig. 2. Schematic responses with LVDT sensors during freeze-thaw cycles: For acclimated stems, diameter fluctuations were entirely reversible. For non acclimated stems, diameter fluctuations was not entirely reversible and repeated freeze-thaw cycles resulted in progressive loss of diameter with positive spikes associated with the exotherm and endotherm.

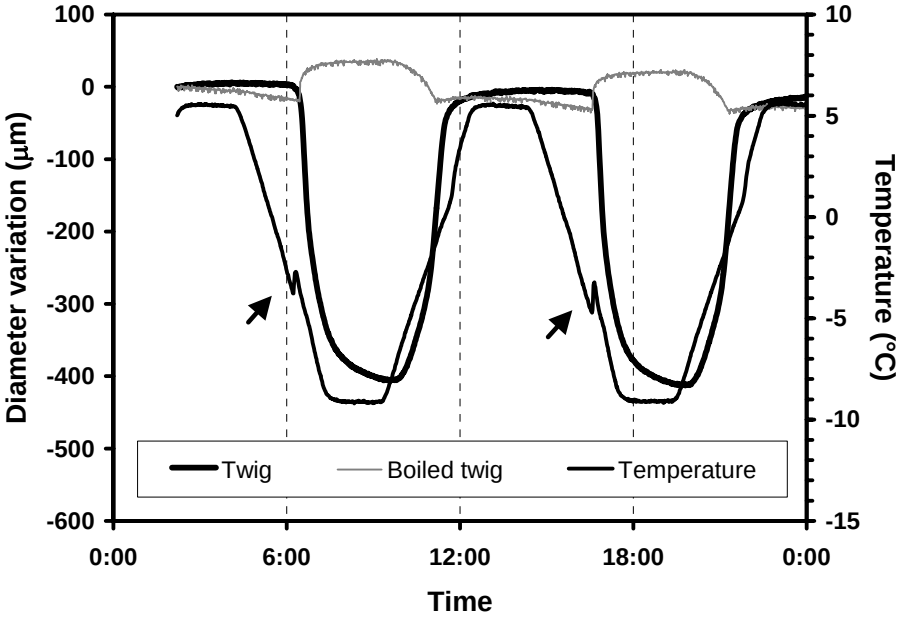

Fig. 3. Diameter variations during 2 freeze-thaw cycles for an acclimated twig segment and for an autoclaved twig segment. Arrow indicated an exotherm on stem temperature. At the exact time of the exotherm, stem shrinkage was observed for the control twig. In contrast, diameter swelling was observed for the boiled twig.

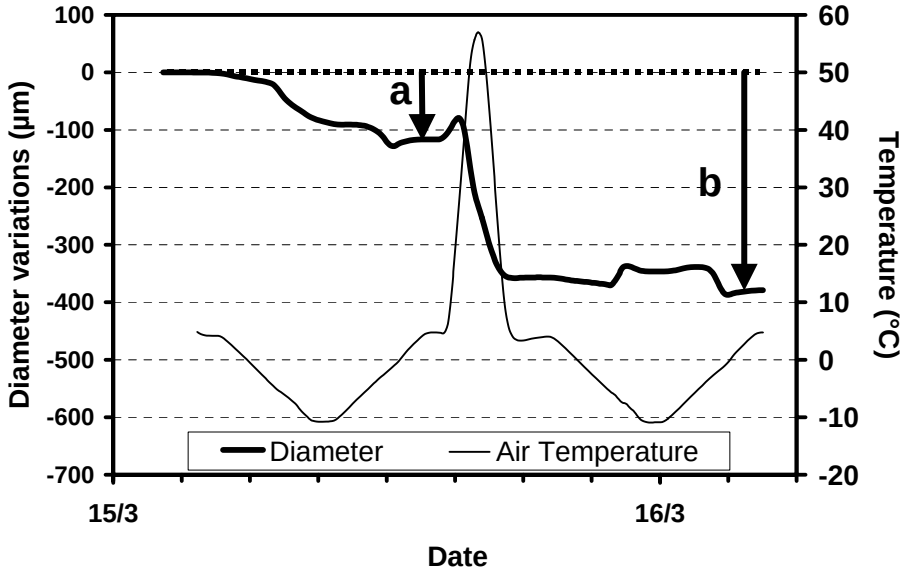

Fig. 4. Gelista™ method: To determine the percentage of injury at a given temperature, the loss of diameter after one cycle of freeze/thaw to this temperature (a) is compared to the maximal diameter loss (b) after one heat shock cycle (T°C >55°C).