

Apoplastic Carbohydrates do not Account for Differences in Freezing Tolerance of Two Winter-Oat Cultivars that have been Second Phase Cold-Hardened

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SUMMARY

Concentrations of soluble carbohydrates were compared in crown tissue of two oat (*Avena sativa*) cultivars which differed in their response to second-phase cold hardening. The apoplastic fluid from plants which had been hardened at above freezing temperatures (first phase cold hardening) contained 2% of the total crown carbohydrate. After one day of exposure to second phase hardening the carbohydrate percentage in the apoplast increased to 15% of total carbohydrates. Apoplastic carbohydrate concentrations in the two cultivars, however, were not significantly different from each other at any time period. An increase in apoplastic sugars may be a ubiquitous mechanism of winter cereals which allows them to survive below freezing temperatures where other mechanisms provide tolerance to more extreme temperatures.

INTRODUCTION

Exposing winter cereals to below freezing but non-injurious temperatures, known as the second-phase of cold hardening (2PH), has been shown to induce cold hardiness beyond that achieved when plants are exposed to low non-freezing temperatures, or first-phase cold hardening (1PH). This phenomenon has been observed in wheat (*Triticum aestivum*) (Trunova, 1965; Steponkus, 1978), in rye (*Secale cereale*) and barley (*Hordeum vulgare*) (Olien, 1984) and in oats (*Avena sativa*) (Livingston, 1996). The additional hardiness observed in 2PH is probably the result of physiological mechanisms that differ from those in 1PH.

Olien (1984) reported that sugars (sucrose, glucose and fructose, S, G, F, respectively) accumulated in the apoplast of barley and rye crowns during 2PH and proposed that the sugars prevented or inhibited adhesive stress. He suggested that this mechanism allowed plants to survive to temperatures of about -10°C. Livingston and Henson (1998), using a different technique than Olien, reported an increase in apoplastic carbohydrates including fructan in oat crowns after 3 days of 2PH. They also reported an increase in the activity of

invertase and fructan exohydrolase in apoplastic fluid after 3 days of 2PH.

Livingston (1996) reported a difference between 2 oat cultivars in the effect of 2PH on freezing tolerance. With no 2PH, the LT50 of the cultivar 'Wintok' was 3.6°C lower (more hardy) than the cultivar 'Fulghum'. After 7 days of 2PH the LT50 of Wintok was 7.7°C lower than Fulghum.

The purpose of this study was to see if the difference in the response to 2PH of Wintok and Fulghum could be explained by differences in carbohydrate concentrations in apoplastic fluid from crown tissue.

MATERIALS AND METHODS

Plant culture: Seeds of the oat cultivars Wintok and Fulghum were grown in Scotts Metromix 220 (Scotts-Sierra Horticultural Products Co., Marysville, OH) in plastic tubes (2.5 cm diameter X 16 cm height) with holes in the bottom to allow drainage. The tubes were suspended in a grid which held 100 tubes. Plants were watered three times weekly with a complete nutrient solution (Livingston, 1991) and flushed three times weekly with tap water. Plants were grown in a growth chamber for 5 weeks at 13°C day and 10°C night with a 12-h photoperiod at light levels of 250 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photosynthetically active radiation (PAR) (80% cool fluorescent and 20% incandescent). Five weeks after planting, plants were transferred to a chamber at 3°C with a 12-h photoperiod at 250 $\mu\text{mol m}^{-2}\text{s}^{-1}$ of PAR for 3 weeks; this constituted 1PH.

Second phase hardening: After 1PH, plants were removed from tubes and washed free of planting medium in ice water. Roots were trimmed to 2 cm and shoots to 10 cm. Trimmed plants were placed in plastic bags, inoculated with ice and placed in a freezer at -3°C in the dark for 6h, 1 day, 3 days, 7 days. Thermocouples placed next to plants were used to monitor temperatures. Temperatures varied by less than 0.2 °C. 1PH plants were harvested in an identical manner and processed at the same time as the 1 day, 2PH treatment. Treatments in the growth chamber were arranged as a split-plot with days as the main plot and cultivars as the sub-plot.

Extraction of whole tissue: 1PH and 2PH plants were prepared for extraction of whole tissue and apoplast fluid in an identical manner. Six crowns (the tissue remaining after removing leaves and stems to within 2 cm of the stem base) were bulked and ground for approximately 30 s using a stainless-steel grinder (Livingston, 1990). The ground tissue was rinsed into a beaker with 10 mL of HPLC-grade water at 1°C. A 2 mL aliquot was filtered through a 0.45 μm filter and placed on dry ice. These aliquots were used for determining the activity of malate dehydrogenase (MDH) and the concentration of glucose-6-phosphate (G6P). The remaining extract was heated to 95°C for 10 min to inactivate enzymes in preparation for carbohydrate analysis. The lapsed time from plant harvest to either freezing on dry ice or heating to 95°C was approximately 3 min. Heat-treated solution was filtered through a 0.45 μm filter in preparation for HPLC analysis. No changes in carbohydrate composition were observed for 36 h at 2°C in filtered, heat-treated samples.

Extraction of apoplastic fluid: Intact crowns from fifteen plants were placed in the bottom 3 cm of a 50 mL syringe barrel. An HPLC insert vial (1 mL) was placed on the syringe tip to collect apoplastic solution. The syringe barrel containing crown tissue with the 1 mL vial attached was placed in a 50 mL centrifuge tube and centrifuged at 500 g for 10 min.

Higher speeds resulted in increasingly higher MDH activity (data not shown), suggesting increased cellular rupture.

An aliquot of the apoplast fluid was used to determine the concentration of G6P and the activity of MDH. The remaining solution was diluted, heated for 10 min at 95°C and analyzed by HPLC.

Carbohydrate separation and quantification: Fructans were separated according to degree of polymerization (DP) using a modified, Bio-Rad (Richmond, CA, USA) Aminex HPX-42A analytical HPLC column. Separated fructan, S, G and F were detected by a Waters (Milford, MA, USA) 410 refractive index (RI) detector and quantitated using Class VP Chromatographic Software (Shimadzu, Tokyo, Japan). Details of the HPLC system as well as the structure of the fructan isomers quantified were described previously (Livingston and Henson 1998). Carbohydrate data were analyzed using analysis of variance and least significant difference (LSD) values were determined to separate means.

MDH and G6P: Malate dehydrogenase activity and G6P concentration were measured to quantify cellular lysis. NAD-malate dehydrogenase (MDH, EC 1.1.1.37) activity was assayed by the method of Li et al. (1989). The oxaloacetate-dependent oxidation of NADH was measured at 340 nm on a recording spectrophotometer (model UV-1601PC, Shimadzu, Tokyo, Japan), at 25°C. Malate dehydrogenase activities (calculated from initial rates) are expressed as μmoles of product formed per gram of tissue fresh weight.

Glucose-6-phosphate (G6P), was determined spectrophotometrically by its NAD-dependent oxidation, using *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49, Sigma Chemical Co., St. Louis, MO, USA) (Burkey, 1999). The reaction mixture, in a final volume of 1 mL, consisted of 50 mmol of HEPES-KOH (pH 6.9), 1.5 mmol MgCl_2 , 0.2 mmol NAD^+ and up to 0.1 mL of sample. The baseline was monitored at 340 nm and the reaction was initiated by the addition of 1 IU (in 10 μL) of G6PDH on a cuvette stirrer (Fisher Brand 14-386-22). G6P concentration was calculated from the increase in absorbance, which was usually complete in 2 min when G6P was present.

Respiratory rates: Crowns were wrapped in a moist paper towel with the ends kept open and placed inside a plastic 50 mL centrifuge tube with a rubber stopper. Teflon tubing was inserted through holes in the rubber stopper. The longer tubing (outlet) reached the bottom of the centrifuge tube and the shorter tubing (inlet) was 1 cm below the rubber stopper. Compressed air (20.06% O_2 , 314 ppm CO_2 , remainder N_2) was forced through the centrifuge tube at a flow rate of 10.2 mL min^{-1} . Centrifuge tubes containing crown tissue were placed in a freezer at 3°C and taken to -3°C within 2 days. CO_2 evolution was calculated as the difference between the baseline value (314 ppm \pm 2%) and the value when flow was directed through the centrifuge tube. Measurements were taken daily at the same time using a Li-Cor (Lincoln, NE, USA) model LI-6252 CO_2 analyzer.

RESULTS and DISCUSSION

Apoplastic MDH/G6P: MDH is commonly used as an indicator of cellular integrity because its activity is found inside cells as long as the plasmalemma remains intact. It has been used as an indicator of cellular integrity in pea (Beers and Duke, 1988), barley (Tetlow and Farrar, 1993), *Brassica napus* (Husted and Schooerring, 1995), and oat (Livingston and Henson 1998). However, MDH is a relatively large molecule compared to the carbohydrates being studied here and we wanted an indicator of cellular rupture that would be more typical

of smaller molecules. We used G6P because it is also found inside intact cells and has been used as an indicator of cellular integrity in apoplastic studies of snap beans (Burkey, 1999). The percentage of G6P in the apoplast was roughly twice that of MDH (Table 1) which suggests that smaller compounds leak into the apoplast to a greater extent than larger compounds during extraction of apoplastic fluid from 2PH plants.

Table 1 Carbohydrate concentrations in apoplastic fluid from two winter oat cultivars before (Day 0) and during second phase hardening. Also shown is the percentage (%) of the total concentration that was found in the apoplast. Percentages were calculated by dividing the concentration in the apoplast by the concentration in whole tissue (not shown). Each value is the mean of 4 replicates.

Cultivar	Day	MDH		G6P		Fructan dp>2		Sucrose		Glucose		Fructose	
		mmol NADH min ⁻¹ g ⁻¹ FW	%	nmol NADH g ⁻¹ FW	%	mg g ⁻¹ FW	%	mg g ⁻¹ FW	%	mg g ⁻¹ FW	%	mg g ⁻¹ FW	%
Fulghum	0	0.19	0.64	3.0	0.82	0.44	1.1	0.06	1.1	0.07	2.9	0.03	2.7
	0.25	0.58	1.6	15	3.4	1.8	4.1	0.35	5.8	0.41	13	0.19	10
	1	1.2	3.0	24	8.0	4.0	9.1	0.75	13	0.62	23	0.34	15
	3	0.95	1.9	20	6.9	3.0	4.4	1.0	12	0.62	20	0.41	13
	7	1.2	3.8	29	9.2	5.4	13	1.7	23	0.91	26	0.91	19
Wintok	0	0.19	0.92	2.8	0.53	0.49	1.2	0.09	1.4	0.10	3.2	0.05	3.6
	0.25	0.3	1.1	9.9	2.1	1.7	3.0	0.37	5.3	0.47	11	0.22	11
	1	0.48	1.5	11	3.2	2.7	4.7	0.66	8.3	0.72	18	0.32	13
	3	0.77	2.2	18	4.8	3.5	6.2	0.98	9.8	0.76	17	0.49	14
	7	0.83	2.9	17	5.9	4.0	7.5	1.4	13	1.1	20	0.89	15
LSD (0.05)		0.39	1.4	9.9	4.8	2.3	5.9	0.49	7.2	0.31	10	0.24	7.5

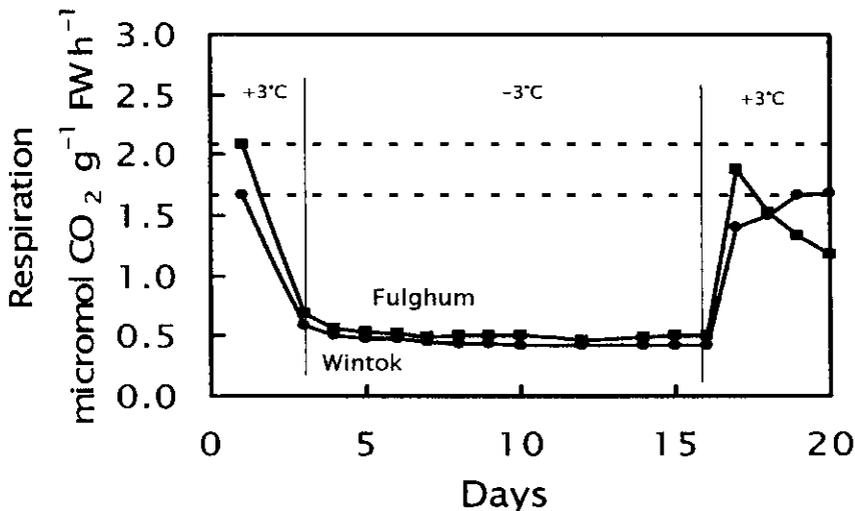
In Fulghum (non hardy), significantly ($P < 0.05$) higher MDH activities and G6P percentages were found after day 1 and they remained near those levels throughout 2PH (Table 1). In Wintok (hardy), both MDH activity and G6P concentration increased throughout 2PH; however, only on day 7 were their values significantly higher than those of 1PH (Table 1). It is possible that the membranes of Fulghum may be more susceptible to injury from ice or desiccation and may partly explain why it is less freezing tolerant than Wintok. Sulc et al., (1991) reported that less winter-hardy alfalfa cultivars had a higher leakage of MDH after freezing than the more hardy ones.

If G6P was in the apoplast as a result of cellular rupture, then the percentage of G6P found in the apoplast could be used to estimate the percentage of cellular rupture. Therefore, percentages of carbohydrates which are above the percentage of G6P could be considered as present in apoplastic fluid as a specific response to -3°C and not from experimentally-induced cellular rupture. A higher percentage of all carbohydrates than can be attributed to cellular rupture was found in the apoplastic fluid of Wintok and Fulghum at every time period except day 3 for fructan DP>2 in Fulghum (Table 1).

Apoplastic carbohydrates: The most rapid change in apoplastic carbohydrate concentrations occurred during the first day of 2PH (Table 1). In fact, the rate of increase of all carbohydrate concentrations during the first 6 hours was higher than at any other time

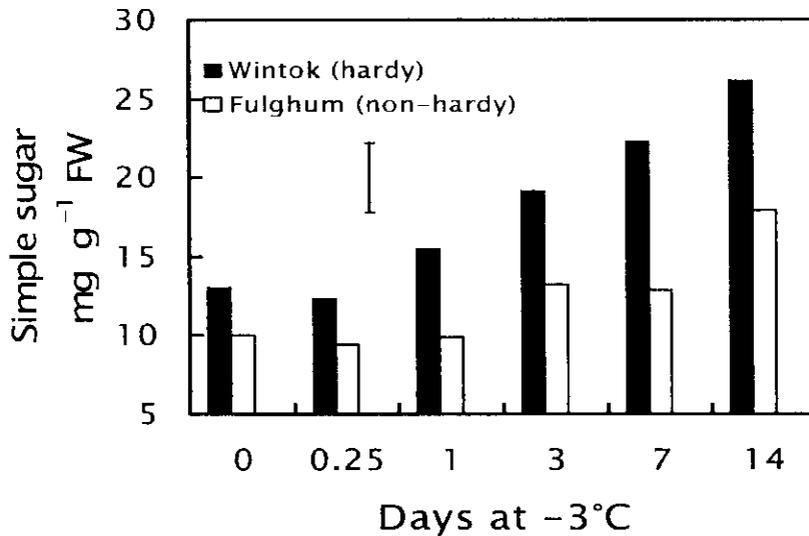
during the 7 day period. The rapid change was probably the result of a sudden change in temperature from 3 to -3°C. The temperature was reduced from 3 to 0°C in one hour and from 0°C to -3°C in approximately 3 hours. In addition, during the first day at -3°C the respiratory rate of whole crowns, as measured by CO₂ evolution, was reduced to 25% of its rate at 3°C (Fig. 1) indicating a rapid change in metabolic activity. The first day was also the period during which the freezing tolerance of Wintok and Fulghum increased to the greatest extent (the LT₅₀ became more negative) (Livingston, 1996).

Fig 1. Respiration rates of Wintok and Fulghum oats before, during and after 14 days of second phase cold hardening at -3°C. Differences between cultivars were not significant at any time period. Each data point is the mean of 3 replicates.



The freezing tolerance of Wintok increased to a greater extent than that of Fulghum during 2PH, particularly after the first day at -3°C (Livingston, 1996). During the first day of 2PH the LT₅₀ of Fulghum was reduced from -9°C to -11.1°C and the LT₅₀ of Wintok was reduced from -12.6°C to -17.9°C. Because apoplastic sugar concentrations also increased during this time period (Livingston and Henson, 1998), and sugars are known cryoprotectants, we hypothesized that the two cultivars which differed in their increase in freezing tolerance during 2PH may also differ in the change in the concentration of apoplastic sugars. Contrary to what was expected, the differences in apoplastic sugar concentration between the two cultivars were not significant ($P=0.05$) at any time period (Tab. 1). In addition, the level of sugars in total ground tissue was not significantly different (not shown). However, when the concentration of symplastic sugars were calculated, by subtracting apoplastic sugars from the total, Wintok had significantly higher concentrations than Fulghum (Fig. 2). This difference increased as 2PH progressed.

Fig 2. Symplastic (total minus apoplastic) simple sugars (Suc+Glu+Fru) in Wintok and Fulghum oat crowns during 2PH. The error bar indicates the LSD at $P=0.05$.



A higher symplastic sugar concentration in the more hardy cultivar, Wintok, may help stabilize membranes from freeze damage. Membrane stabilization by carbohydrates as a freezing protection mechanism has been proposed by several researchers (Sugiyama and Simura, 1967; Santarius, 1973; Uemura and Steponkus, 1998). Uemura and Steponkus (1994) reported that the plasma membrane lipid composition of oat leaves was different from that of rye and suggested that this may be one reason for their difference in freezing tolerance.

It is possible that the apoplastic extraction technique used in this study did not sample fluid from tissues that were critical for survival. For example, it is possible that the centrifugation technique removes apoplastic fluid from the stem tissue directly above the meristematic zone. The tissue which supports the root and stem meristems, and is directly below and adjacent to the meristems, is very dense and the cells are tightly packed together. It is possible that changes in this meristematic support tissue, are not reflected in the centrifugable fluid from stem tissue.

When Wintok plants were exposed to 1°C in the dark for 3 days, the concentration of apoplastic sugars and their level of freezing tolerance were both *lower* (unpublished data). In contrast to 1PH, and the dark, it is possible that an *increase* in apoplastic carbohydrates was necessary for rapid cold hardening at below freezing temperatures in *both* Wintok and Fulghum. To reach the full hardening level observed during 2PH, the more hardy cultivar possibly required higher symplastic carbohydrates or some other mechanism to stabilize membranes, prevent desiccation or some other form of freeze-induced stress.

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