

Relationships of Soluble Carbohydrates and Freeze Tolerance in Saltgrass

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ABSTRACT

Information is lacking regarding the changes of endogenous soluble carbohydrates of saltgrass [*Distichlis spicata* (L.) Greene] during cold acclimation. The objective of this study was to quantify soluble carbohydrates and their relationships to freezing tolerance in six saltgrass accessions (A65, A29, C66, 32, 55, and 48). The study was performed at monthly intervals under natural acclimation in two consecutive winter seasons (October 1999–April 2000 and October 2000–April 2001) at Fort Collins, CO. Concurrent with LT₅₀ (subfreezing temperature resulting in 50% mortality) assessment, soluble carbohydrates, including sucrose, fructose, glucose, raffinose, and stachyose were measured by gas chromatography (GC). Results indicated significant differences among accessions and sampling dates in LT₅₀ and carbohydrate content. Sucrose was the predominant sugar, but did not show a clear seasonal trend and had no correlation with freezing tolerance. Fructose, glucose, raffinose, and stachyose exhibited clear seasonal changes, reaching highest concentrations during midwinter. In December of both seasons, higher concentration of fructose and glucose were observed in 48 and 55 as compared with other accessions. Accession A29 had the highest concentration of raffinose in December and January in both seasons. A29 also had the highest stachyose content in midwinter of 1999–2000. Higher fructose, glucose, or raffinose concentrations were frequently observed in accessions of 48, 55, and A29, which coincided with their lower LT₅₀ as compared with the other accessions. In contrast, C66 had the lowest sugar concentrations, which related to its sensitivity to low temperatures. These results indicate that fructose, glucose, raffinose, and stachyose may play important roles in saltgrass freezing tolerance.

NONSTRUCTURAL CARBOHYDRATES (NSC), including water soluble carbohydrates (WSC) are thought to serve an important role in freezing tolerance of many plants (Levitt, 1980), including some turfgrasses. Fry et al. (1993) found a positive correlation between sucrose level and surviving stolons in acclimated vs. nonacclimated centipedegrass [*Eremochloa aphiuroides* (Munro) Hack.]. They suggested that common centipedegrass accumulates sucrose during fall acclimation, which contributes to an increase in freezing tolerance of about 2°C for acclimated vs. nonacclimated plants. Rogers et al. (1975) measured the changes in freezing tolerance and sucrose and starch content of zoysiagrass [*Zoysia japonica* (Steud) Meyer] during fall and winter. They found an increase in NSC of both rhizomes and stolons from September to December and a 15% decrease in NSC by mid-March. Dionne et al. (2001) assessed the relationship between freezing tolerance of green-type annual bluegrass (*Poa annua* L.) and levels of fructans, mono- and disaccharides. Their results indicated a positive correlation between freezing tolerance and the lev-

els of fructans and sucrose. Ball et al. (2002) reported that the concentrations of sucrose, fructose, glucose, and raffinose increased during cold acclimation of buffalograss [*Buchloe dactyloides* (Nutt.) Engelm.]. Endogenous WSC was also found to be of importance in freezing tolerance in other species such as grape (*Vitis vinifera* L.) (Hamman et al., 1996), honeysuckle (*Lonicera carerulea* L.) (Imanishi et al., 1998), alfalfa (*Medicago sativa* L.) (Castonguay et al., 1995), eastern white pine (*Pinus strobes* L.), eastern redcedar (*Juniperus virginiana* L.), Leyland cyperus (*Cupressocyparis leylandii* Dallim.), Virginia pine (*Pinus virginiana* L.) (Hinesley et al., 1992), red raspberry (*Rubus idaeus* L.) (Palonen, 1999), and aspen (*Populus tremuloides*) (Cox and Stushnoff, 2001).

In contrast, some studies indicated no correlation or relationship between freezing tolerance and carbohydrate content, especially in some grass species with poor freezing tolerance (Bush et al., 2000; Dunn and Nelson, 1974; Fry et al., 1991; Maier et al., 1994). Fry et al. (1991) found little variability in 'Floritam' St. Augustinegrass freezing tolerance among sample dates on which a change in starch and sucrose levels was recorded. This indicates no effect of those carbohydrates on freezing tolerance. The results of the work done by Maier et al. (1994) on the same grass supported the results of Fry et al. (1991). Sucrose accumulation did not appear to influence the ranking of freezing tolerance among three bermudagrass [*Cynodon dactylon* (L.) Pers.] cultivars during fall accumulation (Dunn and Nelson, 1974). Furthermore, no correlation was found between freezing tolerance and NSC in St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze.] and carpetgrass (*Axonopus affinis* Chase.) (Bush et al., 2000). These contrasting observations suggest that the roles of WSC and NSC in freezing tolerance are not clearly defined and may differ among species.

Inland saltgrass [*Distichlis spicata* var. *stricta* (L.) Greene], indigenous to America and Australia, is a dioecious, rhizomatous, perennial, salt tolerant, warm-season grass. A saltgrass breeding project is on-going at Colorado State University, in cooperation with the University of Arizona, to develop saltgrass cultivars that can be used as turfgrass where soil and water salinity and alkalinity are high. In conjunction with saltgrass breeding efforts, we studied the freezing tolerance of six saltgrass accessions (Shahba et al., 2003). The objectives of this study were to: (i) determine seasonal changes in levels of sucrose, glucose, fructose, raffinose, and stachyose and (ii) relate these changes to the freezing tolerance in saltgrass.

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Abbreviations: NSC, nonstructural carbohydrates; WSC, water soluble carbohydrates; GC, gas chromatography.

MATERIALS AND METHODS

Field Plots Establishment

Saltgrass accessions used in this experiment were collected between 1981 and 1997. Saltgrass accessions A65 and A29 were originally collected from Denver, CO, while C66 was from Humboldt Sink, NV, 32 from Wanship, UT, 55 from Hereford and 48 from Farmingdale, SD. The collected accessions were maintained and increased in the greenhouse through vegetative propagation. In July 1998, these accessions were established via rhizome plugs in 5 by 5 m field plots at the Horticulture Research Center, Fort Collins, CO. The soil in the field was a Nunn clay loam (fine, smectitic, mesic Aridic Argiustoll) with the initial soil N, P, and K content of 23.0, 14.0, and 497 $\mu\text{g g}^{-1}$, respectively. The soil pH was 8.1. Entries were replicated two times in a randomized complete block design. No fertilizers were applied and the field was unmowed during 1999 to 2001 to serve as the breeding nursery. Daily maximum and minimum air temperatures were recorded via the cellular-linked weather station located 100 m east of the study area.

Sample Collection

Two sections in each of the replicated plots were selected. Rhizomes from each section were sampled at monthly intervals from October 1999 through April 2000 and from October 2000 through April 2001. After washing with cold water to remove soil and plant debris, approximately 5 g of the rhizomes was freeze dried (Genesis 25 LL lyophilizer, Virtis, Gardiner, NY) for soluble carbohydrates analysis and the remainder were used to evaluate freezing tolerance.

Freezing Test to Determine Lethal Low Temperature

To determine freezing tolerance, seven to eight fractions of rhizomes were subjected to freezing treatments with a thermo-controlled freezer (Tenny Jr. Programmable Freezer, Tenny Inc., South Brunswick, NJ) as previously described (Qian et al., 2001; Shahba et al., 2003). Each fraction contained at least 10 nodes. The freezing chamber was programmed to cool linearly at 2°C/h after an initial 16 h at 0°C. One fraction of rhizomes was removed when each of the target temperatures (ranging from -8°C to -29°C at 3°C intervals) was reached. Following freezing treatments, individual nodes were planted in commercial potting soil in the greenhouse and recorded for survival by observing regeneration of shoots 4 wk after planting. Saltgrass response to freezing temperature was evaluated on the basis of rhizome survival. The subfreezing temperature resulting in 50% mortality is defined as lethal temperature (LT_{50}).

Analysis of Carbohydrates

After freeze drying, rhizomes sampled from each section of each plot were ground with a Wiley mill, sieved through an 80-mesh screen, and kept in airtight vials at -20°C. Approximately 1 mg of screened sample was weighed and placed into a test tube containing 50 μL of methyl glucopyranoside at 1 mg mL^{-1} as an internal standard. Carbohydrate derivitization was performed according to Cox and Stushnoff (2001) and Sweeley et al. (1963), by adding 400 μL pyridine, 80 μL hexamethyldisilazane, and 40 μL trimethylchlorosilane. Tubes were capped tightly, heated at 80°C for 20 min, then vials were dried under a stream of air. Hexane was added to each tube, and the supernatant was transferred to a clean tube and dried under an air stream. Finally, the dried, derivitized samples were dissolved in 200 μL hexane before injection into the gas chromatograph. As a derivitization check, a standard solution containing 25 μL (1 mg mL^{-1}) each of fructose, sucrose, glucose, raffinose, stachyose, and the internal standard was included and derivitized along with each set of eight samples.

One-microliter samples were injected into an HP 5890 series II gas chromatograph (Hewlett Packard, Boulder, CO.) with a 30-m-long silica capillary column (J&W DB-1, 0.25-mm-inner diam, 0.25- μm -film thickness) and a flame ionization detector. Helium was the carrier gas at a flow rate of 2 mL min^{-1} . Carbohydrates in the samples were identified by comparing retention times with known standards. Carbohydrate quantifications were determined by comparing peak areas to the internal standard area with Peak Simple 1.72 (SRI, Inc., Torrance, CA).

Data Analysis

Data were subjected to analysis of variance to test the effect of accession, sampling date, and their interactions by means of the GLM procedure (Table 1) (SAS Institute, 1991). The two seasons of freezing tolerance and carbohydrate data were presented separately. Monthly means were separated by Fisher's protected least square difference (LSD) at $P \leq 0.05$. Pearson's correlation coefficient among individual carbohydrates and LT_{50} were analyzed by the PROC CORR procedure of SAS for each season. Linear regression analysis by the PROC REG procedure of SAS were done to test the association between LT_{50} and individual carbohydrate concentrations.

RESULTS AND DISCUSSIONS

Freezing Tolerance

Detailed results of freezing tolerance, including monthly LT_{50} and winter survival in the field, among saltgrass accessions were presented in Shahba et al. (2003). Briefly, freezing tolerance of all accessions in-

Table 1. Analysis of variance with mean squares and treatment significance of LT_{50} , sucrose, fructose, glucose, raffinose, and stachyose of six saltgrass accessions from October to April 1999-2000 and 2000-2001 winter seasons at Fort Collins, CO.

Source	Mean square					
	LT_{50}	Sucrose	Fructose	Glucose	Raffinose	Stachyose
			1999-2000			
Accessions (A)	23.0*	98.1*	37.8*	18.0*	0.12*	0.02*
Month (M)	198.7*	204.1*	709.2*	848.4*	0.77*	0.08*
A \times M	8.3*	66.8*	18.1*	22.7*	0.14*	0.02*
			2000-2001			
Accessions (A)	34.8*	139.2*	55.9*	75.4*	0.2*	0.07*
Month (M)	378.7*	460.9*	956.3*	1074.8*	9.3*	0.22*
A \times M	8.5*	98.0*	19.9*	36.1*	0.2*	0.01*

* Significant at $P < 0.0001$.

Table 2. The subfreezing temperature resulting in 50% mortality (LT_{50}) of six saltgrass accessions sampled in January 2000 and 2001.

Accessions	LT_{50}	
	2000	2001
	°C	
48	–20.0a	–26.0a
A29	–20.0a	–20.0c
55	–17.0b	–26.0a
32	–15.5bc	–23.0b
A65	–14.0c	–23.0b
C66	–14.0c	–18.5c

Means within columns followed by the same letter are not significantly different at $P \leq 0.05$ using Fisher's LSD test.

creased in fall, reaching a maximal freezing tolerance in December and January with deacclimation occurring in March. Significant differences in freezing tolerance were observed among accessions in January in both seasons (Table 2). Accessions A29, 48, and 55 were most cold hardy in January 2000, with LT_{50} down to about -20°C . In the second season, 55 and 48 exhibited lowest LT_{50} (best freezing tolerance), with their LT_{50} approaching -26°C . Accession C66 had poor freezing tolerance with the LT_{50} ranging from -14°C to -18°C in January 2000 and 2001.

Soluble Carbohydrates

Sucrose was the predominant sugar. Fructose and glucose followed sucrose in abundance, whereas stachyose

and raffinose were present in low concentrations in all accessions (Fig. 1 and 2). Concentrations of soluble carbohydrates varied significantly with sampling time, accession, and their interactions (Table 1).

All measured soluble carbohydrates except sucrose exhibited a clear trend of seasonal changes, increasing from October to midwinter with fructose and glucose reaching their maximum in December, raffinose reaching its maximum in January, and stachyose reaching its maximum in December and January. All soluble carbohydrate concentrations declined gradually from February to April. Stachyose was completely absent in October and April, with only low concentrations in midwinter months.

Greater differences of fructose, glucose, raffinose, and stachyose among accessions were found during midwinter than during fall or spring. The highest fructose content was observed in accession 48 in December [28.8 to $37.2 \mu\text{mol/g}$ dry weight (DW)] in both seasons. Accession 55 and 48 had a higher glucose content ($34.4 \mu\text{mol/g}$ DW) than other accessions in December 1999. In December 2000, accession 48 had the highest glucose content followed by 55 and A29. A29 produced the highest raffinose content ($1.6 \mu\text{mol/g}$ DW) among all accessions in both seasons and the highest stachyose content ($0.4 \mu\text{mol/g}$ DW) in December 1999 and January 2000. Surprisingly, A65, a relatively cold tender accession had the highest stachyose during the winter of 2000-2001.

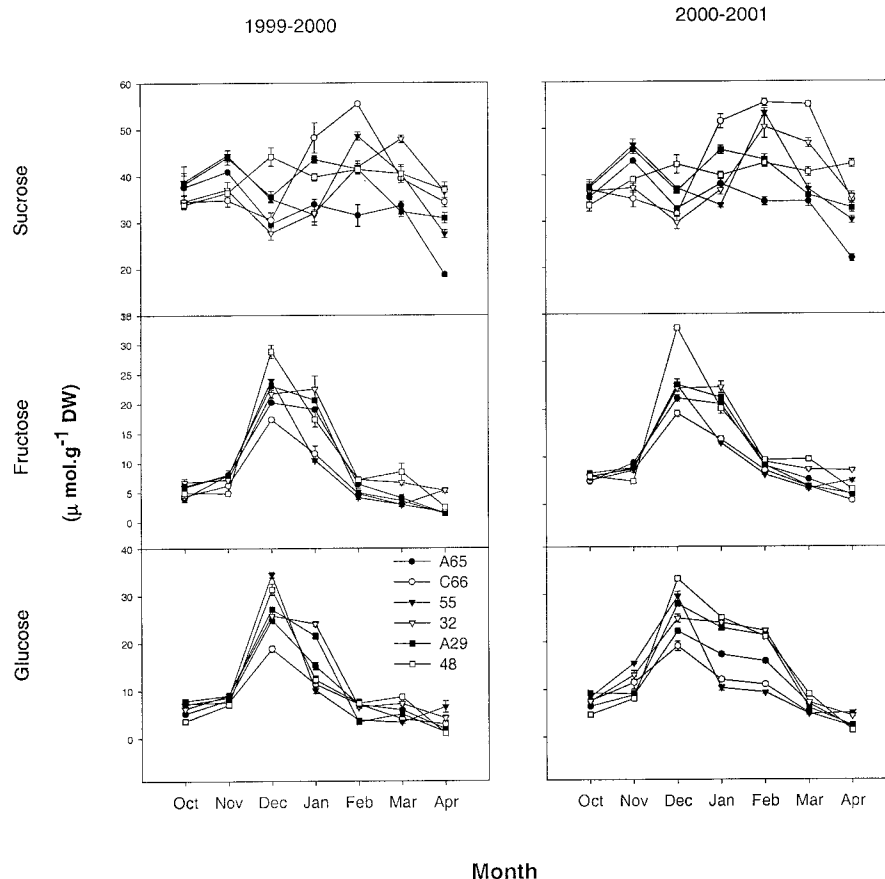


Fig. 1. Mean concentrations of sucrose, fructose, and glucose of six saltgrass accessions from October 1999 to April 2000 (left panel) and from October 2000 to April 2001 (right panel). Vertical bars represent SE.

Higher fructose, glucose, and raffinose concentrations were frequently observed in accessions 48, 55, and A29, which coincided with their better freezing tolerance (i.e., lower LT_{50}) when compared with other accessions. In contrast, among all accessions, C66 exhibited the lowest fructose, glucose, and raffinose levels during midwinter of 1999-2000 and the lowest fructose, glucose, and stachyose levels during the midwinter of 2000-2001. Accession C66, the most cold tender accession (Table 2), was originally collected from Nevada. This area is relatively warm so that it may be genetically adapted to warm climates and carbohydrate concentrations responded to that adaptation.

Sucrose concentration was highly variable showing no clear trend with seasonal changes in either season (Fig. 1). Fry et al. (1993) suggested that sucrose might play a role in the freezing tolerance of 'Oklawn' centipede grass stolons. Dionne et al. (2001) also noticed that an increase in sucrose levels in crowns of annual bluegrass coincided with its maximum freezing tolerance. This does not appear to occur in saltgrass accessions. Dunn and Nelson (1974) also found that sucrose differences among cultivars were slight and not related to winter survival of bermudagrass.

Relationships of Carbohydrate Content and Freezing Tolerance

Regression and correlation analyses demonstrated that sucrose had no relationship with LT_{50} in both sea-

sons (Table 3 and Fig. 3). In the first season, correlations between LT_{50} vs. fructose, glucose, raffinose and stachyose were significant with correlation coefficients of -0.73 , -0.68 , -0.71 , and -0.59 , respectively. The correlations between LT_{50} vs. fructose, glucose, raffinose, and stachyose were significant also in the second season with correlation coefficients of -0.70 , -0.67 , -0.64 , and -0.64 , respectively. Generally, fructose had the highest correlation with LT_{50} in both seasons followed by glucose, raffinose, and stachyose.

These results indicate that fructose, glucose, raffinose, and stachyose may play important roles in saltgrass freezing tolerance. The significant linear relationships of LT_{50} vs. fructose, glucose, raffinose, and stachyose concentrations support this conclusion (Fig. 3). Imanishi (1998) suggested that raffinose and stachyose may play a role in the freezing tolerance of the shoot apices of honeysuckle. Castonguay et al. (1995) related variations in alfalfa freezing tolerance to differences in sucrose, raffinose, and stachyose concentrations of overwintering crowns. Hinesley et al. (1992) related cold hardiness of coniferous trees to raffinose and sucrose. Kandler and Hopf (1980) suggested that the seasonal changes in stachyose and other oligosaccharides enhance frost tolerance in winter hardy plants. Most of these studies stressed the importance of carbohydrates in freezing tolerance but no study described the mechanism or the physiological basis of this action. Nevertheless, it is the general belief that the increase in carbohydrates as sol-

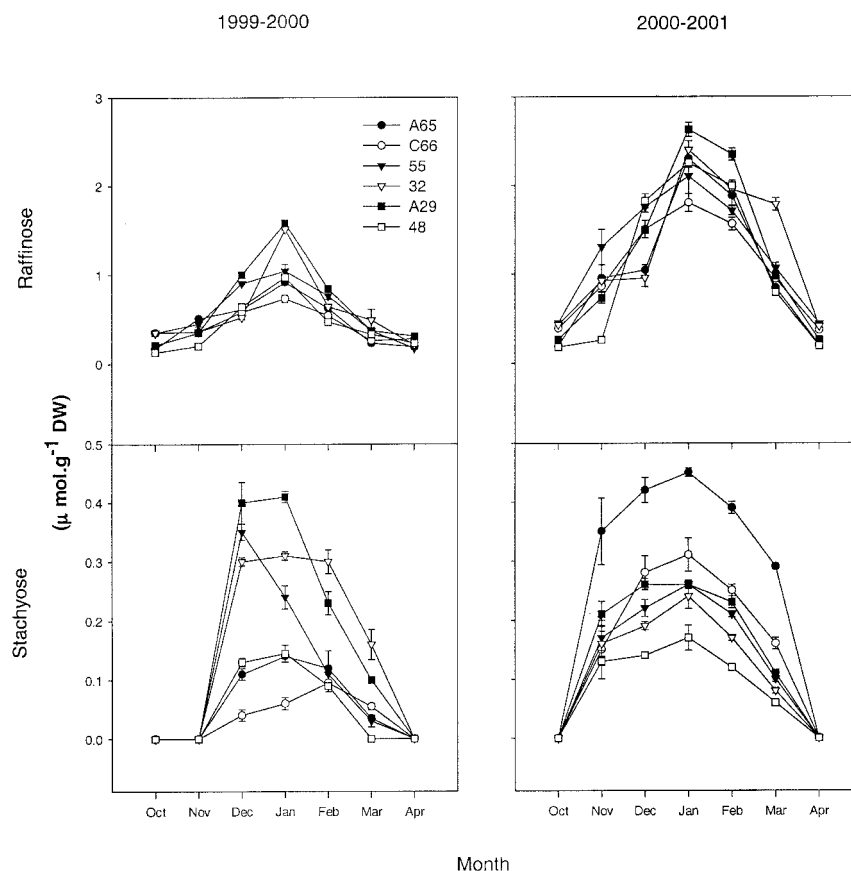


Fig. 2. Mean concentrations of raffinose and stachyose of six saltgrass accessions from October 1999 to April 2000 (left panel) and from October 2000 to April 2001 (right panel). Vertical bars represent SE.

Table 3. Correlation coefficient between freezing tolerance (LT_{50}) and individual soluble carbohydrate content in the rhizome of the tested accessions (A65, A29, 55, 48, C66 and 32) of saltgrass sampled from October 1999 to April 2000 and from October 2000 to April 2001. A negative coefficient indicates that greater freeze tolerance is accompanied with an increase in carbohydrate content.

Parameter	Sucrose	Fructose	Glucose	Raffinose	Stachyose
1999–2000					
LT_{50}	-0.11NS	-0.73***	-0.01NS	-0.71***	-0.59***
Sucrose		-0.01NS	-0.07NS	0.33**	0.22*
Fructose			0.95***	0.49***	0.50***
Glucose				0.44***	0.51***
Raffinose					0.87***
2000–2001					
LT_{50}	-0.11NS	-0.7***	-0.67***	-0.64***	-0.64***
Sucrose		0.02NS	0.16NS	0.43***	0.25**
Fructose			0.85***	0.56***	0.50***
Glucose				0.74***	0.56***
Raffinose					0.68***

NS, Nonsignificant.
 * Significant at $P \leq 0.05$.
 ** Significant at $P \leq 0.005$.
 *** Significant at $P < 0.0001$.

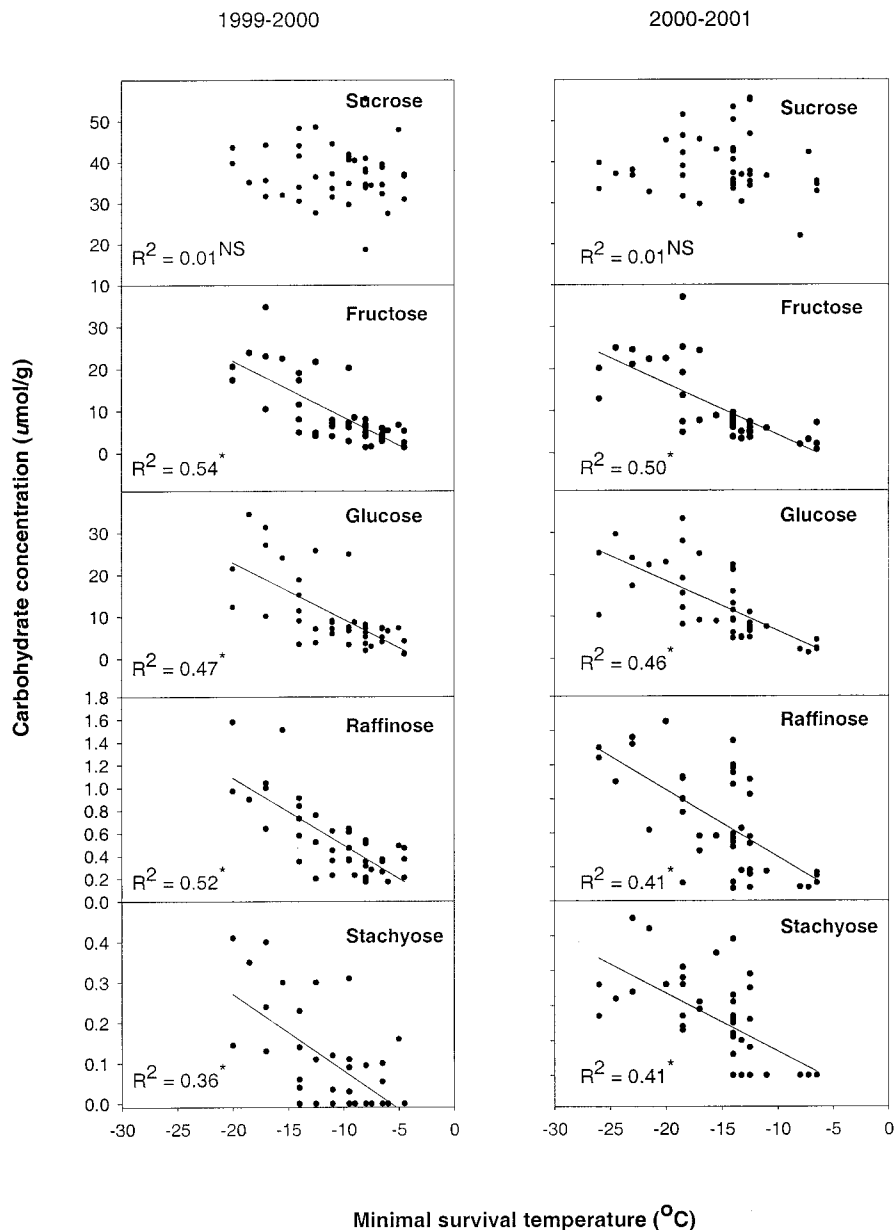


Fig. 3. Relationship between carbohydrate concentrations and 50% survival temperatures of six saltgrass accessions from October 1999 to April 2000 (left panel) and from October 2000 to April 2001 (right panel). NS, * Nonsignificant or significant at $P < 0.001$.

utes with decreasing temperature results in increased osmotic potential, which decreases freezing point and enhances freezing tolerance. Soluble carbohydrates act as cryoprotectants as they prevent ice formation and cell desiccation. Soluble carbohydrates may interact with membrane phospholipids and proteins to stabilize their structures as water is removed during freezing. Koster and Leopold (1988) and Caffrey et al. (1988) suggested that raffinose and other galactose-containing oligosaccharides might confer desiccation tolerance by preventing crystallization of sucrose, thereby facilitating availability of sucrose during the drying of seeds.

Correlation analysis indicated a close interrelation between fructose and glucose ($r = 0.95$ in the first season and 0.85 in the second season). This is expected since these hexoses are the intermediates of sucrose cleavage, which then enters the metabolic pathways of the cell to provide the energy and substrates required for viability and growth. It is well known that sucrose hydrolyzes into fructose and glucose by the catalytic action of invertase isoenzymes or converts into uridine diphosphate-glucose (UDP-glucose) and fructose by sucrose synthase (Avigad and Dey, 1997). Raffinose and stachyose were found to be highly correlated ($r = 0.87$ in the first season and 0.68 in the second). Stachyose coexists with raffinose although it is synthesized at the expense of the pool of raffinose and galactinol (Avigad and Dey, 1997).

In summary, our results suggest that 55, 48, and A29 had the highest soluble carbohydrate concentrations overall, which were associated with their greater freezing tolerance. C66 had low carbohydrate concentrations and correspondingly poor freezing tolerance. Sucrose was the predominant carbohydrate, but had no correlation with freezing tolerance. Fructose and glucose followed sucrose in abundance and correlated well with freezing tolerance. Although raffinose and stachyose concentrations were very low, they correlated significantly with freezing tolerance.

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