The effect of the Russian wheat aphid on cold-hardiness of acclimating winter wheat seedlings
by Eric William Storlie

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Agronomy
Montana State University
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Abstract:
Affects on cold-hardiness of winter wheat, Triticum aestivum L., seedlings was investigated after an infestation by the Russian Wheat Aphid, Diuraphis noxia (Mordvilko). Osmotic potential measurement, carbohydrate analysis and field survival estimation were engaged as indicators of coldhardiness. An average infestation of 147 aphids per plant, under simulated hardening conditions, increased the osmotic potential of Froid and Brawny seedlings by 2.36 and 3.45 bars, respectively. A natural field infestation averaging 97 aphids per ten plants, under natural hardening conditions, reduced fructan contents of Froid and Brawny seedlings by 2.26 and 5.91 mg/100 mg dry weight, respectively; similiar trends occurred after a natural freeze period. Field survival and yield results indicate that an autumn infestation reduced the winter-survivability of Brawny by 54 percent and reduced grain yields by 17.77 bushels per acre; survival and yield of infested Froid were not significantly affected. PI 372129, a resistant winter wheat line, was not affected by D. noxia in regard to these parameters.

# THE EFFECT OF THE RUSSIAN WHEAT APHID ON COLD-HARDINESS OF ACCLIMATING WINTER WHEAT SEEDLINGS

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## ABSTRACT

Affects on cold-hardiness of winter wheat, *Triticum aestivum* L., seedlings was investigated after an infestation by the Russian Wheat Aphid, *Diuraphis noxia* (Mordvilko). Osmotic potential measurement, carbohydrate analysis and field survival estimation were engaged as indicators of cold-hardiness. An average infestation of 147 aphids per plant, under simulated hardening conditions, increased the osmotic potential of Froid and Brawny seedlings by 2.36 and 3.45 bars, respectively. A natural field infestation averaging 97 aphids per ten plants, under natural hardening conditions, reduced fructan contents of Froid and Brawny seedlings by 2.26 and 5.91 mg/100 mg dry weight, respectively; similiar trends occurred after a natural freeze period. Field survival and yield results indicate that an autumn infestation reduced the winter-survivability of Brawny by 54 percent and reduced grain yields by 17.77 bushels per acre; survival and yield of infested Froid were not significantly affected. PI 372129, a resistant winter wheat line, was not affected by *D. noxia* in regard to these parameters.

# INTRODUCTION

The Russian Wheat Aphid, *Diuraphis noxia* (Mordvilko), was first sighted in a Montana grain field in September 1987[24]. Its ability to survive and proliferate in cold climates, and its destructive feeding habits aroused concern for Montana grain production. A possible effect after an autumn *D. noxia* infestation on winter grain seedlings may be a reduction of cold-hardiness. This thesis sought to determine if the winter-survivability of three winter wheat types - Froid, Brawny and PI 372129 - may be affected by an infestation. Winter-survival is a pertinent concern for grain producers in Montana, since winterkill is a potential outcome for most winter grains. Death is typically attributed to an inherent vulnerability of plants. If winterkill is partly attributable to a *D. noxia* infestation, the problem may be partly remedied with control strategies during autumn or with resistant winter wheat.

# LITERATURE REVIEW

The ability of *Diuraphis noxia* to survive on winter grain seedlings through fall acclimation periods in Montana has prompted concern for its effects on winter wheat cold-hardiness. Survival and natality studies suggest that *D. noxia* survived temperatures between -4° and -25°C[5] and bore nymphs at least to 5°C[36]. *D. noxia* adjusts reproduction rates and generation periods according to temperature and food availability - reproduction rates decrease and generation periods increase when temperatures are low and small grain growth is minimal[1,35,36].

*Diuraphis noxia* putatively injects a toxin while probing plant tissues for food. Symptoms which lead to a toxin-theory are intervenal chlorotic yellow and purple streaks on the leaf surface and organelle damage at the ultrastructural level[13]; analysis of chloroplasts from wheat, *Triticum aestivum* L., tissue treated with an aphid extract revealed chloroplast membrane disintegration[13]. Thus, implications of an infestation are not only a loss of carbohydrates from ingestion but also irreversible damage to the organelles necessary for sugar production.

Since soluble carbohydrates have been associated with

winterhardiness in small grains[2,41,45], the question of a *D. noxia* influence on the hardiness of winter grains may be pertinent.

There are several approaches for testing the coldhardiness of winter grains. Fowler et al.[14] evaluated 34 possible tests and correlated some tests with a Field Survival Index (FSI). The tests most highly correlated with FSI were $LT_{50}$, tissue water content, plant erectness, crown phosphorus content and total sugar content. DeNoma et al.[9] indicate a linear relationship existed between crown osmotic potential and field survival scores for five winter wheat varieties during two winter seasons. Lower osmotic potentials of crown tissue were associated with increased winterhardiness. The results of Thomas and Taylor[49] corroborate the results of DeNoma et al. with a study that suggests a more winterhardy variety, Froid, had a lower osmotic potential than a less winterhardy variety, Brawny, after a freeze period.

When the cell-dessication theory of freeze-injury is considered, the results of DeNoma et al.[9] may correspond with the negative correlation between crown moisture and FSI observed by Fowler et al.[14] Since freeze-injury is believed by some to be a result of intracellular dehydration, a mechanism of hardiness may involve osmoregulation. By increasing its osmotic concentration, a cell may achieve vapor pressure equilibrium with the extracellular

environment during cold periods when the extracellular solution may freeze[27,46]. In regard to this theory, a more negative osmotic potential may be explained by an increase in solutes and proportionate decrease in moisture content, which would prevent plasmolysis and mitigate the concentration of toxic solutes.

Osmoregulation is, at least in part, a function of soluble carbohydrate concentrations[17,27,39]. A compelling candidate for a carbohydrate that can adjust the osmotic potential of a cell is fructan, a storage polymer of fructose which is found in at least 36,000 species representing ten families of plants[7]. A fructan molecule consists of two or more (2->6 or 2->1)-B-D-fructofuranans bound to one D-glucopyranose residue. Molecules may exist as linear pleins, with (2->6) linkages; inulins, with (2->1) linkages; or branched, with (2->6 and 2->1) linkages[7,15,33,40]. Fructans of wheat consist mostly of plein backbone linkages with (2->1 and 2->6) linked, branch residues[7].

Suzuki and Nass[47] enumerated the advantages of fructan over starch as a cold temperature storage carbohydrate: fructan has high solubility in water, it is resistant to crystallization at subzero temperatures, and fructan biosynthetic pathways are insensitive to low temperatures. The advantages of fructan, however, do not suggest that starch is replaced in cool-season grasses. Chatterton et al.[8] defined fructan as an ancillary form of carbohydrate

storage; its accumulation in the vacuole allows photosynthesis to continue in leaves during cool periods when other storage pools are saturated.

Two enzymes are implicated in fructan synthesis in cereals: sucrose: sucrose fructosyl-transferase (SST) has been investigated in small grains[22,23,50,53]; fructan: fructan fructosyl-transferase (FFT) has not been investigated in small grains. SST transfers a fructose moiety from one sucrose molecule to another, producing a ketose and a glucose molecule[53]. In Jerusalem artichoke, *Helianthus tuberosus* L., FFT transfers single terminal B-D-fructofuranosyl residues to the same position of another fructan molecule[11,12]. A putative FFT in small grains is speculated to perform an analagous role as in *H. tuberosus*[53].

Depolymerization of fructan molecules is accomplished by the activity of fructan hydrolase, according to studies on *H. tuberosus*, *Festuca arundinacea* (Schreb.) and *T. aestivum*[12,25,43,50]. Tognetti et al.[50] and Jeong and Housley[23] suggested that fructan hydrolase activity decreased during cold acclimation and subsequently increased during warm acclimation in wheat.

The role of fructans in osmoregulation is conceivable in consideration of the fructan-specific enzymes and the capability of fructan polymers to exist in various degrees of polymerization (DP). FFT may redistribute fructose

residues from a larger polymer to a smaller one and consequently increase the concentration of lower molecular weight fructans. This mechanism allows for rapid changes in solute concentrations of cells and consequently influences the osmotic potential[40].

A high $K_m$ value for SST suggests sucrose must accumulate to a relatively high concentration before fructan is synthesized and begins to accumulate[6,19]. Pollock[38] provides evidence from a study on *Lolium temulentum* L. that sucrose concentrations rapidly increased before fructan accumulated. Bancal and Gaudillere[2] and Wagner et al.[53] show similiar results in *T. aestivum*. Similiarly, Labhart et al.[25] conclude that a threshold level of low DP fructans and sugars were necessary for the synthesis of high DP fructans in *Festuca pratensis* (Huds.).

Chatterton et al.[8] evaluated fructan accumulation in 185 Gramineae accessions and suggests that total nonstructural carbohydrate (TNC) accumulation was more closely related to fructan accumulation than sucrose; a threshold value of 15% TNC was postulated as necessary for fructan accumulation. Livingston et al.[29] present results from a study on *Hordeum vulgare* L. that corroborate the results of Chatterton et al.[8]. In any case, it appears fructan accumulation is induced by a changed ratio of carbon utilization to carbon fixation[41].

Several studies on the relationship between fructan

accumulation and cold-hardiness of winter cereals suggest that significant correlations existed between fructan content and hardiness[30,47,49,51,57]. Tognetti et al.[51] indicate an association occurred between hardiness in winter wheat and sucrose and fructan accumulation at 4°C. Livingston[30] analyzed the accumulation of each DP fructan in *Avena sativa* L., *H. vulgare*, *T. aestivum* and *Secale cereale* L. after a 2°C hardening period. Results of this study suggest that the least winterhardy cereal, *A. sativa*, accumulated as much sucrose and DP3-DP7 as the other, hardier species. However, *A. sativa* accumulated significantly less DP>9 fructan than the other cereals[30]. Suzuki and Nass[47] present similiar results and suggest that high DP fructans were more closely associated with hardiness than low DP fructans.

Thomas and Taylor[49] studied the relationship between crown osmotic potential, fructan content and winterhardiness in Froid and Brawny winter wheat. After a simulated hardening period, their results indicate that Froid, the more winterhardy variety, accumulated more fructans and had a lower osmotic potential than Brawny, the less winterhardy variety. Furthermore their results suggest a negative correlation occurred between fructan content and osmotic potential for Froid and Brawny, after a hardening period. The negative correlation of this study provides evidence for the role of fructan in osmoregulation.

Though the mechanisms of fructan involvement in freeze-resistance remain tenuous[51], an association between fructan accumulation, cold temperatures and relative cold hardiness seem apparent in consideration of the above cited research. Several investigations used fructan levels to infer effects on winterhardiness caused by pests[21,31,54,55,56]. Holmes et al.[21] suggest that a greenbug, *Schizaphis graminum* (Rondani), infestation in *T. aestivum* reduced the level of high DP fructans and may consequently have reduced freezing resistance. Livingston and Gildow[31] studied affects of Barley yellow dwarf virus on fructan concentrations in *A. sativa* crowns and suggest that fructan levels of infected oats were significantly less than the controls.

# MATERIALS AND METHODS

## Cultivars

The winter wheat types used in the growth chamber and field experiments were Froid, with a high winterhardy score of 4.5 (on a scale of 1 to 5)[9]; Brawny, with a low winterhardy score of 2.0[9], and PI 372129, a *D. noxia* resistant accession with an undocumented hardiness[42].

## Growth Chamber Experiment

### Experimental Design

The growth chamber experiment provided plant samples for osmotic potential measurement. It was designed as a split-plot randomized complete block with four blocks. Each plot either was infested with *D. noxia* or was not infested (control), and each subplot consisted of 28 subsamples of one winter wheat type. Four subsamples were used to measure the osmotic potential for a hardening and a freezing period (N = 96). The primary interest in this study was a comparison of the osmotic potential for the infested and control treatments of each variety (treatment combinations).

Seeds for each of the samples were planted two cm deep in sand-filled, 1.5" diameter cone-tainers (Supercell Cone-

tainer). Two blocks were grown at a time in a customized environmental growth chamber for the duration of a growing and hardening period.

Growing Conditions

For the first 33 days of growth, temperatures were 16°C during the 12 hour light period and 14°C during the 12 hour dark period. Light was emitted from four 400 watt, multivapor and four 400 watt, luclox lamps (General Electric) with a photosynthetic-photon-flux-density of 1200 $\mu$mol/m$^2$ min. Plants were watered daily, on alternate days with Hoagland's full strength nutrient solution.

Infestation. Ten days after planting, when seedlings consisted of two leaves, the infested plots were inoculated with three aphids per plant using a damp artist's paint brush. Cages for confining the aphids were made of veil fabric on the sides and a plexiglas cover. The final aphid population per plant was determined from counts of five individual plants of each variety from the four blocks (N = 60).

Hardening And Freezing. The following hardening and freezing procedures were adapted from Gullord et al.[18]. The hardening period began 33 days after planting and entailed reducing growth chamber temperatures to a continuous 4°C setting. Additionally, the luclox lamps were switched off and the multivapor lamps set for continuous lighting with a photosynthetic-photon-flux-density of 280 $\mu$mol/m$^2$ min.

These conditions were maintained for 21 days.

After 21 days of hardening, four subsamples from each subplot were removed and cut to a one cm, soil-free crown section. The crown was immediately placed into a 1 ml microcentrifuge tube and froze in liquid nitrogen for two minutes and subsequently stored at -70°C. Concurrently, four subsamples from each subplot were prepared for a freeze period. Plants were cut to 5 cm sections - including root, crown and leaf tissue. These sections were immediately placed into slots of a moist cellulose sponge which was pressed onto a nipple and flange. Samples were placed into a Lo-Cold freezer (ScienTemp) with no light and a temperature of -2°C for a 56 hour prefreeze. The temperature was monitored with a Thermocouple Thermometer (Omega); five thermocouple wires were randomly distributed to sites adjacent to crowns. After the -2°C prefreeze, temperatures were reduced 2° per hour until -7°C was reached. Samples were removed after two hours at this temperature and immediately cut to 1 cm crown sections, placed in 1 ml microcentrifuge tubes and froze in liquid nitrogen for two minutes before storing at -70°C.

## Theory Of Osmotic Potential Measurements

Theoretically, the freezing and eventual thawing of plant tissue results in disruption of cellular membranes; a process that prevents the generation of turgor, so only the

energy of osmotic potential is measured[3]. The components of water potential are described by the following simplifified equation[3]:

$$\Psi_w = \Psi_o + \Psi_p$$

where o and p represent osmotic and pressure forces, respectively. When the pressure component is canceled by freezing and thawing, the equation reduces to two factors[3]:

$$\Psi_w = \Psi_o$$

Therefore, measurement of water potential approximates the osmotic potential.

Osmotic Potential Measurements

While frozen, crown samples were transferred to 1.1 cubic cm sample and calibration chambers; chambers were immediately fastened to Leaf In-situ thermocouple psychrometers (J.R.D. Merrill). Depth of the psychrometer end window was adjusted to avoid contact with the crown tissue. The psychrometer and 30 cm of lead wire were immersed in a 25°C water bath for a two hour equilibration period; this period facilitates vapor pressure equilibrium, when the water potential of the tissue and vapor pressure of the chamber are in dynamic equilibrium[4]. The water bath prevents severe temperature gradients that may cause water

vapor to migrate from warmer to cooler sectors of the chamber[4].

After equilibration, a Viking connector (J.R.D. Merrill) at the terminus of the lead wire was connected to a HR-33 microvoltmeter (Wescor). A current of about 5 ma was transmitted through the psychrometer unit for 30 seconds. The current was passed through the lead wires to a chromel and constantan sensing junction, and the Peltier effect cooled the junction slightly below the dewpoint temperature of the enclosed atmosphere[52]. This resulted in water condensing on the sensing junction. When the current was terminated, the condensed water evaporated at a rate that was a function of the equilibrium vapor presure[52]. Evaporative cooling reduced the temperature of the sensing junction, and the difference in temperature between the reference and sensing junctions produced an electromotive force (emf) output which was registered by the microvoltmeter[52].

Calibration. Lang[26] documented the relationship between water potentials and NaCl solutions at temperatures between 0° and 40°C. Based on this relationship, calibrating each of the thermocouples involved measuring four concentrations of NaCl - 0.30, 0.50, 0.60 and 0.80 molal - on filter paper at an equilibration temperature of 25°C. Calibration curves of the microvolt outputs vs. waterpotential, in bars, were plotted. Finally, regression equations were computed on

MSUSTAT[32] to express the curvilinear relationship between microvolt output and water potential for each thermocouple psychrometer.

Estimation Theory. The method of estimating osmotic potentials is an inference from the vapor pressure produced by crown tissue in the sample and calibration chamber. Vapor pressure is quantitatively related to the water potential by the following equation:

$$\Psi_w = -\frac{RT}{V_w} \ln \frac{e_w}{e_o}$$

where R is a gas constant, T is temperature (Kelvin), $V_w$ is partial molal volume of water ($m^3\ mol^{-1}$), $e_w$ is vapor pressure and $e_0$ is saturated vapor pressure. Therefore, estimates of $e_w$ will provide estimates of water potential if the temperature is fixed and a thermodynamic equilibrium exists, since all factors other than $e_w$ will remain constant under these conditions[3].

## Field Experiment

### Experimental Design

Planting. The field experiment was planted at the Fort Ellis research plots on September 5, 1990. It provided winter wheat samples for carbohydrate analysis, field survival estimation and grain yield estimation. This

experiment was designed as a split-plot randomized complete block with four blocks. Each plot was either infested with *D. noxia* or was sprayed with insecticides (controlled); each subplot consisted of six rows of one variety, planted at a density of 15-20 seeds per square foot for ten foot rows. There was one sample measurement for each of the subplots for each parameter (N = 24). The primary interest in this study was a comparison of infested and controlled treatments of each variety (treatment combinations).

Infestation. Infested plots were naturally infested with *D. noxia*. Populations per ten plants were estimated on September 30 by counting aphids on ten randomly selected plants from each subplot (N = 24).

Insecticide Applications. The controlled plots were sprayed with Asana (EI-Dupont) at a rate of 0.035 pound active ingredient per acre on September 19; Lorsban (DowElanco) was applied at a rate of 0.50 pound active ingredient per acre on September 27.

## Sample Preparation

On October 30, after a hardening period (Table 3, Appendix), plants were randomly selected from the second and fifth rows of each subplot and immediately trimmed to 1 cm crown sections. The crowns were immersed in liquid nitrogen and stored on dry ice until the samples could be lyophilized in a freeze-drier (Virtis) for 24 hours. On December 10, after a freeze period (Table 3, Appendix), a second

sampling was removed from the frozen soil with a pick-hammer and processed the same as the preceeding samples.

Carbohydrate Extraction. The dried crown samples were ground to a powder with a mortar and pestle and sifted through a 30 mesh tea strainer. Samples of 50 mg were weighed for carbohydrate extraction. Each 50 mg sample was placed in a 5 ml plastic test tube. As adapted from Smith and Grotelueschen[44], three ml of 80% ethanol was added to each sample, vortexed and placed in a 70°C water bath with vigorous agitation. After 15 minutes of agitation, the tissue had settled sufficiently to allow decanting without centrifuging. The ethanol extraction was repeated another time before replacing ethanol with distilled, deionized (ddi) water for three cycles. The 80% ethanol extracts glucose, fructose, sucrose and lower DP fructans from the tissue, and water at 70°C extracts all fructans as well as sucrose, glucose and fructose[44].

Sample preparation and High Performance Liquid Chromatography (HPLC) methods were adapted from Livingston et al.[29]. Supernatants from the extractions were passed through a column of 4cc Amberlite MB-3A (Aldrich) ion-exchange resin, and the column flushed with 20 mls ddi water into 30 ml beakers. All beakers were placed in a 60°C evaporating oven until each sample was completely evaporated. A micropipette was used to add 1.25 mls of ddi water to each beaker to resuspend the carbohydrates and to

transfer the solutions into 1.50 ml microcentrifuge tubes. Samples were centrifuged at 11,750 x g for 15 minutes. The supernatant was decanted with a micropipette and transferred to a new microcentrifuge tube. Samples were vacuum dried in a Speedvac Concentrator (Savant) until completely dehydrated. Subsequently, samples were resuspended in ddi water to produce a 1 ml solution. These carbohydrate solutions were stored at -20°C.

Carbohydrate Analysis

High Performance Liquid Chromatography. The HPLC system was composed of a Spectroflow 400 pump (Kratos), an injector (Rheodyne) with a 20 ul sample loop, a Carbo-C guard column (BioRad), a HPX-42C carbohydrate column (BioRad), a Differential R401 refractometer (Waters), and a Data Module M730 integrator (Waters). The column was heated to 85°C using a water jacket and water bath.

Standard Preparation. Carbohydrate standards for sucrose, glucose and fructose were prepared from reagent-grade, commercial stocks. A fructan standard was prepared from a dried, ground mixture of crowns from cold-hardened Froid, Brawny and PI 372129. The ethanol and water extraction procedure was used to concentrate fructans from fifteen 100 mg samples. After the extraction, solvents were evaporated from 30 ml beakers in a 65°C oven. Subsequently, carbohydrates in each beaker were concentrated into a 1.25 ml solution by successively resuspending each sample with

an original 1.25 ml ddi water. The 1.25 ml carbohydrate solution was centrifuged and vacuum dried as previously described for the sample-extraction procedure. A white precipitate was visible at this stage. The precipitate was washed with 500 µl 80% ethanol and centrifuged 2 minutes at 16,000 x g. The supernatant was discarded and the precipitate washed two more times with 500 µl of 80% ethanol to remove all detectable glucose and fructose and most sucrose. Any remaining detectable sucrose was calculated on the HPLC with a sucrose standard and factored into the final sucrose standard. Some of the lower, 3-5 DP fructans were removed by the ethanol. However, all DP fructans were grouped as one fraction for quantification, and the most prominent fraction, DP>6, was fully retained during the extraction process (Figure 1). A 5 ml carbohydrate standard was prepared with 50 mg fructan, 15mg sucrose, 10 mg glucose and 10 mg fructose for calibrating the integrator each day before a series of sample injections.

Sample Hydrolysis. To confirm that the observed fructan peaks were polymers containing a terminal glucose residue linked to various DPs of fructose residues, 500 µl of a carbohydrate solution, extracted from a hardened Brawny, was hydrolyzed with 500 µl of 0.3 molar HCl for two hours at 80°C. Then the sample was neutralized with 0.3 molar NaOH and passed through an ion-exchange resin. The resin was flushed with 25 ml of ddi water into a 30 ml

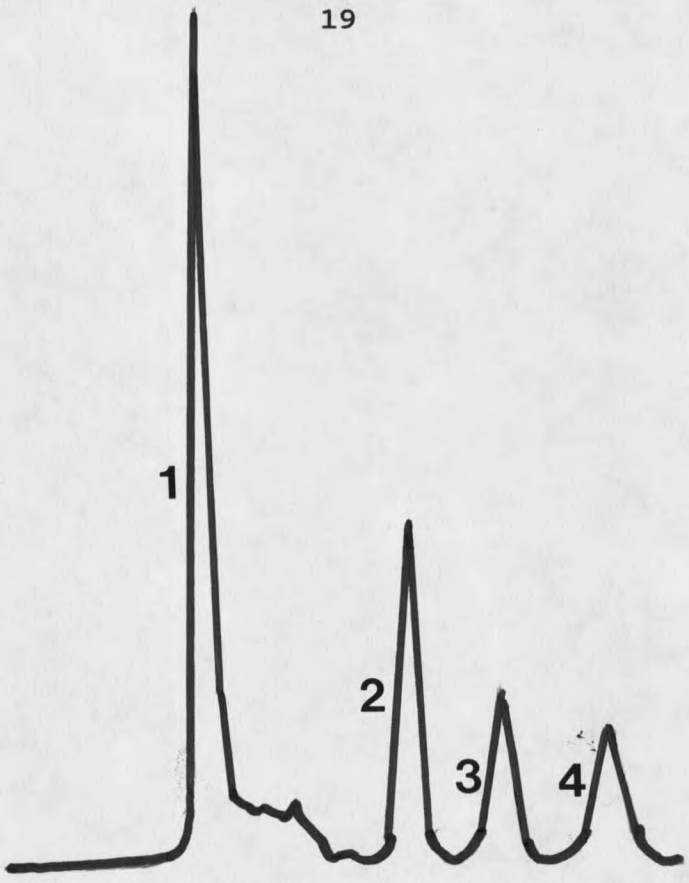


Figure 1. HPLC chromatogram of carbohydrate standard. Peaks are fructan (1), sucrose (2), glucose (3), fructose (4).

beaker with the hydrolyzed sample. Subsequently the solvents were completely evaporated in a 60°C oven and the sample carbohydrates resuspended to a 500 $\mu$l solution with ddi water. The solution was filtered through a 0.45 micron filter before injection into the HPLC. Hydrolysis was not complete, though a comparison of the ratio of concentrations between hydrolyzed and nonhydrolyzed samples revealed that the putative fructan peaks were composed of glucose and fructose residues, with a higher proportion of fructose (Figure 2). The ratio of glucose to fructose was 1:2 before hydrolysis and 1:3 after hydrolysis.