Root Physiology of Less Fall Dormant, Winter Hardy Alfalfa Selections

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ABSTRACT

The physiological mechanisms causing fall dormancy (FD)-induced differences in alfalfa (*Medicago sativa* L.) shoot growth in autumn and winter hardiness are not understood. The objective of this research was to examine root physiology of experimental germplasms selected for decreased FD that also were selected simultaneously for high winter hardiness. Dormant and semi-dormant cultivars and germplasms had high root sugar concentrations that were positively associated with winter hardiness. Root amino N and protein levels in December were greater for germplasms selected for decreased FD and increased winter hardiness than for cultivars with comparable levels of winter hardiness. Among the five most fall dormant cultivars Vernal incurred the greatest amount of winter injury and it had lower root amino-N concentrations when compared with three of the other four dormant cultivars and germplasms. Germplasm 98-132 with an intermediate FD, incurred relatively low winter injury, similar to that of fall dormant Vernal, when compared with other intermediate dormancy cultivars and germplasms. This germplasm had root sugar concentrations that were similar to plants with FD ratings of 1 to 3. Creation of even less FD germplasms that possess high winter hardiness would facilitate our understanding of the physiological and molecular mechanisms controlling these two very important agronomic traits of alfalfa.

The extensive genetic and phenotypic variation found in alfalfa permits its cultivation in diverse climates. Fall dormancy has an especially large impact on alfalfa adaptation to particular environments because of its association with winter survival (McKenzie et al., 1988). Fall dormant cultivars produce short, prostrate shoots in autumn, exhibit slow rates of shoot elongation after harvest in summer, and possess high winter hardiness. In contrast, nondormant alfalfa plants grow extensively in autumn producing tall, erect shoots, resume rapid shoot elongation after defoliation in spring and summer, but nondormant cultivars are not winter hardy (Zaleski, 1954; Smith, 1961; Busbice and Wilsie, 1965; Stout and Hall, 1989; Sheaffer et al., 1992).

The specific physiological, biochemical, and molecular mechanisms causing FD-induced differences in shoot growth in autumn and their association with poor winter hardiness is not completely understood. The improved winter survival exhibited by fall dormant cultivars is closely associated with sugar accumulation in roots and crown buds (Graber et al., 1927; Bula and Smith, 1954; Volenec et al., 1991; Castonguay et al., 1995; Cunningham and Volenec, 1998; Cunningham et al., 2001). In addition, Castonguay et al. (1995) reported a close association of raffinose family oligosaccharide (RFO) accumulation with improved alfalfa winter survival. In contrast, root and bud starch concentrations were higher in nondormant, nonhardy cultivars when compared with fall dormant plants (Volenec, 1985; Boyce and Volenec, 1992; Castonguay et al., 1995; Cunningham and Volenec, 1998). Therefore, accumulation of sugars, and especially RFOs, rather than root total nonstructural carbohydrate (TNC) levels in general, may influence alfalfa winter hardiness.

The impact of root N on growth and stress tolerance of alfalfa has come under study recently (Volenec et al., 1996). Changes in amino and non-amino N concentrations have been observed during alfalfa cold acclimation (Bula et al., 1956; Wilding et al., 1960; Hendershot and Volenec, 1993). Unlike non-hardy cultivars that exhibited little change in amino and non-amino N concentrations between August and December, winter hardy cultivars increased root amino and non-amino N levels by 20 and 31%, respectively (Wilding et al., 1960). Bula et al. (1956) found a positive correlation between increased levels of soluble protein in autumn and enhanced winter hardiness of dormant alfalfa cultivars. Hendershot and Volenec (1993) reported that soluble amino-N and buffer-soluble protein increased in autumn and early winter, and declined in spring as herbage growth resumed, with nondormant genotypes having decreased amino N and protein concentrations when compared with dormant genotypes. Three vegetative storage proteins (VSPs) were identified whose concentration in roots increased markedly during autumn cold acclimation. These proteins were extensively utilized as an N source for initial shoot growth in spring and during shoot regrowth after defoliation in summer (Hendershot and Volenec, 1993; Avice et al., 1996; Barber et al., 1996; Cunningham and Volenec, 1998; Noquet et al., 2001). Roots of fall dormant, winter hardy cultivars and populations accumulate more protein in their roots in December when compared with roots of nondormant plants that do not survive winter (Li et al., 1996; Cunningham et al., 1998; 2001). In addition, several cold-induced polypeptides appear in roots and crown buds of winter hardy cultivars during cold acclimation that are not present in roots and buds of nondormant plants that winter kill (Castonguay et al., 1993; Cunningham et al., 1998).

Investigators have identified several alfalfa cold hardiness genes (Mohapatra et al., 1987; Mohapatra et al., 1989; Wolfraim et al., 1993). Northern blot analyses revealed a positive association between transcript levels for these genes and alfalfa FD and winter survival. Although these differentially regulated transcripts have been identified in roots of cultivars possessing increased

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Abbreviations: *car*, cold acclimation responsive; FD, fall dormancy; RFO, raffinose family oligosaccharide; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; VSP, vegetative storage protein.

FD and winter hardiness in autumn, little is known about the function of their protein products *in planta*.

One approach to improve alfalfa forage yield has been to simultaneously select for winter hardiness and reduced FD. Busbice and Wilsie (1965) and more recently Brummer et al. (2000) have suggested that these two traits exhibit independent inheritance, and gain from selection for higher yielding less fall dormant, winter hardy cultivars should be possible. In contrast, Cunningham et al. (1998, 2001) showed that selection for greater FD using CUF 101 (nondormant, non-hardy) as a parent resulted in a germplasm with decreased fall height and improved winter hardiness. This confirmed the strong association between FD and winter survival reported in other studies (Smith, 1961; Stout and Hall, 1989; Sheaffer et al., 1992). The objective of this research was to examine root physiology of experimental germplasms selected for decreased FD that also were selected simultaneously for high winter hardiness. We expected to find less dormant plants possessing winter hardiness comparable with those of known fall dormant, winter hardy cultivars. In addition, we expected to detect specific alterations in root carbohydrate and protein metabolism that are closely associated with improved winter hardiness in these less fall dormant germplasms.

MATERIALS AND METHODS

Plant Culture and Sampling

Plant materials used in this experiment included five experimental germplasms provided by Forage Genetics International (West Salem, WI): 98-132, 98-141, 98-142, 98-148, and 98-157. In addition, six alfalfa cultivars were included as controls representing a broad range in FD and winter hardiness. These included: 'Vernal' (fall dormant, FD = 2), 'Dart' (fall dormant, FD = 3), 'G2852' (semi-dormant, FD = 4.2), 'Archer' (semi-dormant, FD = 5), 'Sutter' (nondormant, FD = 7), and 'CUF 101' (nondormant, FD = 9). Seedlings were established at the Agronomy Research Center, Purdue University, West Lafayette, IN, in early May of 1998 and 1999. Seeds were sown in 3-m long rows spaced 92 cm apart in a randomized complete-block with four replicates. Resultant plant populations were approximately 60 plants m^{-1} of linear row. The soil was a Starks-Fincastle silt loam (fine-silty, mixed, superactive, mesic, Aeric Endoaqualfs and Epiaqualfs) that was fertilized and limed according to soil test for high alfalfa yield. Seeds were inoculated with *Rhizobium meliloti* (Liphatech Corp., Milwaukee, WI) before planting. Plots were hand-weeded, and insects controlled as needed. Planting, cutting, and root sampling dates are summarized in Table 1. Plant heights were measured in mid-October at eight randomly selected positions within each plot. Linear regression of FD ratings of the alfalfa control cultivars vs. their mean fall height was used to predict FD ratings of the five experimental germplasms being evaluated. Plant survival was determined when shoot growth of winter hardy cultivars resumed in April by excavating 15 to 30 plants per plot and scoring the winter injury of each plant using the following scale: 1 = uninjured; 2 = injured; and 3 = completely dead. A weighted average (by count and injury category) was calculated for each plot and used for statistical analysis.

Table 1. Timetable of crop management activities.

Activity	1998 to 1999		1999 to 2000	
Planted	6	May	11	May
Initial defoliation	10	July	8	July
Second defoliation	13	Aug.	9	Aug.
Third defoliation	17	Sept.	14	Sept.
Fall height measured	12	Oct.	12	Oct.
Initial root sampling	12	Oct.	12	Oct.
Final root sampling	3	Dec.	2	Dec.
Winter survival counts	13	Apr.	5	Apr.

Root tissues for laboratory analyses were collected in mid-October and again in early December. Roots were dug to a soil depth of approximately 20 cm, and were washed free of soil using cold water. The uppermost 5 cm of each taproot was removed, diced into small pieces, and packed with solid CO_2, (for protein and carbohydrate analyses) or immersed in liquid N_2 (RNA analyses). The upper 5 cm of taproots were selected for analyses because this was a morphologically well-defined tissue region that minimized variation due to rooting depth, degree of branching, and other genetic differences in root morphology that could confound results obtained by analyzing the entire root system. Tissues for protein and carbohydrate analyses were lyophilized, ground to pass a 1-mm screen and stored at −20°C. Tissues for RNA analyses were stored at −80°C.

Carbohydrate, Amino-N, and Protein Analyses

Starch and ethanol-soluble sugars were assayed by the methods described in Li et al. (1996). Protein analysis was conducted at 4°C unless otherwise stated. Soluble proteins were extracted by suspending 30 mg of freeze-dried root tissue in 1 mL of 100 m*M* sodium phosphate buffer (pH 6.8) containing 1 m*M* phenylmethylsulfonylfluoride and 10 m*M* 2-mercaptoethanol. Tissue suspensions were vortexed four times for 30 s at 5-min intervals and then centrifuged at 14 000 × *g* for 10 min. The supernatants were retained. Protein concentrations were determined by the protein dye-binding assay as reported previously (Cunningham et al., 1998). Amino-N in the supernatant was determined using ninhydrin with glycine as a standard (Rosen, 1957). For sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, proteins were separated in 0.75-mm-thick gels containing 12% (w/v) acrylamide (Laemmli, 1970), and stained with Coomassie Brilliant Blue R-250.

RNA Isolation and Northern Blot Hybridization Analysis

Total RNA was isolated using hot phenol, and RNA (20 μg) was separated on a 1.5% (w/v) agarose-formaldehyde gel as described previously (Gana et al., 1998). A cold acclimation responsive (*car*) cDNA clone from alfalfa, *bN-1 12a3* [similar to *cas17* (GenBank Accession L13415)] was labeled with ^{32}P-dCTP using random priming (Feinberg and Vogelstein, 1983) and used to probe blots for steady-state transcript levels for this gene. Hybridization and washing of membranes were done as described by Gana et al. (1997). Membranes were exposed to x-ray film at −80°C.

Statistical Analysis

The experimental design was a randomized complete block with four replicates arranged as a split-plot. The experiment was replicated four times in each of 2 yr, 1998 and 1999. Years and replicates were treated as random effects and germplasm (cultivar) and root sampling times (October, December) were treated as fixed effects. Variances over years were found to be homogenous using Bartlett's test (Steel and Torrie, 1980); therefore, data are presented as means averaged across both years. Data were analyzed using analysis of variance (SAS

Institute, 1999). An *F*-protected LSD ($P \leq 0.05$) was calculated for comparisons of main effect means. Significant differences ($P \leq 0.05$) between means of two way and higher order interactions were determined as twice the standard error of the mean (Snedecor and Cochran, 1980). Regression was used to examine the relationship of shoot height in autumn and winter injury of alfalfa.

RESULTS AND DISCUSSION

Plant Height and Winter Survival

Soil and air temperatures during both years of the study at the Agronomy Research Center are shown in Fig. 1. In 1998, a killing freeze (defined as ambient air temperature of −4°C or 25°F) occurred on 5 November, and in 1999 occurred on 15 November. Plant height in October (an estimate of FD) of the control cultivars differed in the expected manner (Fig. 2A). Averaged across years, height of fall dormant Vernal averaged 17 cm, while height of nondormant CUF 101 approached 37 cm with remaining cultivars intermediate in height. Linear regression of FD (FD) rating of the control cultivars Vernal, Dart, G2852, Archer, Sutter, and CUF 101 vs. mean autumn plant height was significant and resulted in the following equations for each year: Year 1, $y = 2.77(FD) + 16.77$; Year 2, $y = 2.36(FD) + 7.04$; $r^2 \geq 0.95$. These regression equations were used to predict FD ratings of the experimental germplasms within each of the respective years, and these FD estimates were analyzed using analysis of variance. Averaged across years, 98-141 and 98-157 were the most fall dormant germplasms, followed by 98-148 and 98-142, with 98-132 being the least fall dormant (Fig. 2A).

As expected winter injury of the cultivars differed ranging from 1.3 and 1.1 for the dormant cultivars Vernal and Dart, respectively, to 2.3 for the nondormant cultivar CUF 101. Germplasms also differed in winter injury with 98-141, 98-157, and 98-148 having significantly less winter injury than Vernal, and the germplasms 98-142 and 98-132 (Fig. 2B). The germplasm 98-148 and Dart both were less fall dormant than Vernal, but each exhibited better winter hardiness. Likewise, germplasm 98-132 had a FD value similar to Archer, but a winter injury rating similar to Vernal, a winter hardy check cultivar used in many variety performance trials. Averaged over both years, a significant increase in winter injury was observed as autumn shoot height increased (Fig. 3). Winter injury increased slightly in the interval from 10 to 25 cm, but increasing shoot height in October beyond 25 cm resulted in a substantial increase in winter injury. These results agree with previous reports documenting the positive relationship between fall height and winter injury (Cunningham et al., 1998; Cunningham et al., 2001). Although a close relationship between FD and decreased winter injury was observed, data for three cultivars/germplasms (98-148, Dart, and

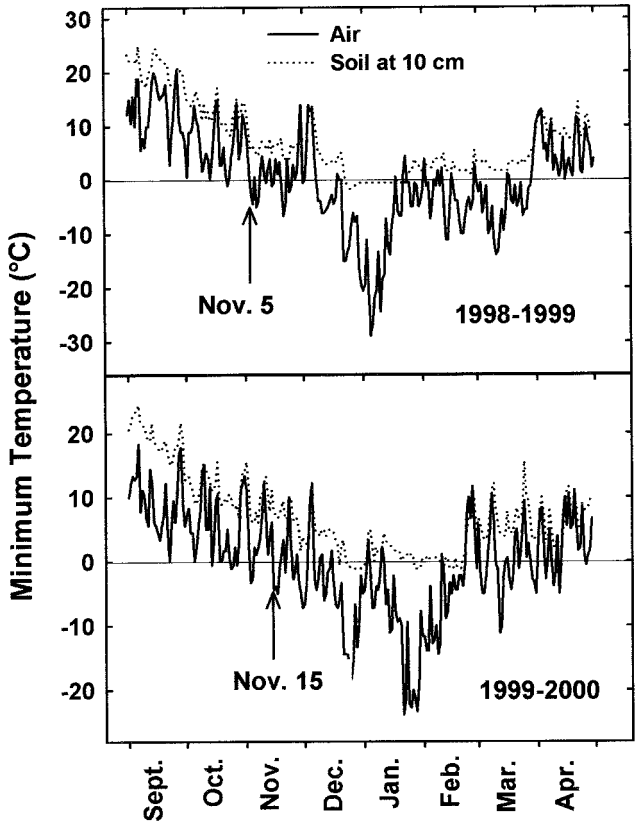

Fig. 1. Minimum air and soil (10-cm depth) temperatures at the Agronomy Research Center during the 2 yr of the study. Dates of the first killing freeze (−4°C) were 5 Nov. 1998 and 15 Nov. 1999.

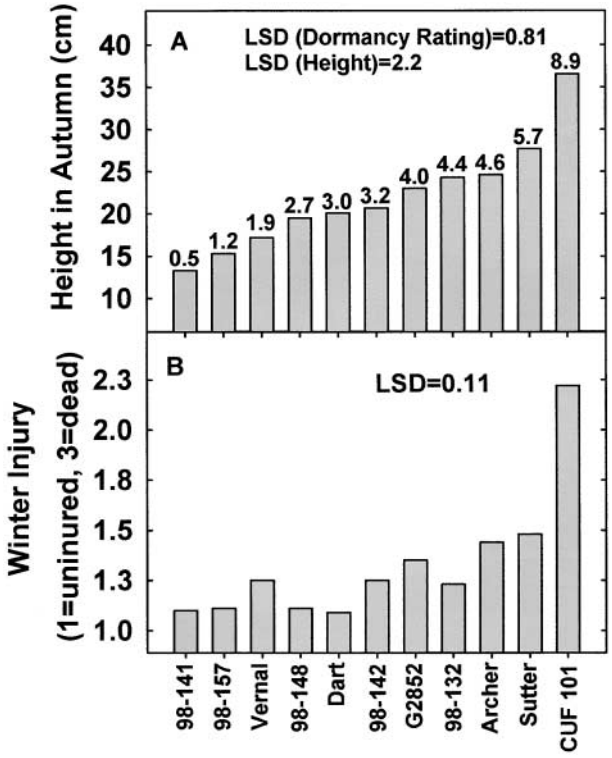

Fig. 2. (A) Shoot height in autumn and (B) winter injury of alfalfa cultivars and germplasms. Six alfalfa cultivars; Vernal, Dart, G2852, Archer, Sutter, and CUF 101 with known fall dormancy and winter hardiness were compared to five experimental germplasms; 98-141, 98-157, 98-148, 98-142, 98-132. Shoot height was measured in mid-October and winter injury was assessed in April. Data were averaged over 2 yr. The least significant difference (LSD, $P \leq 0.05$) is provided.

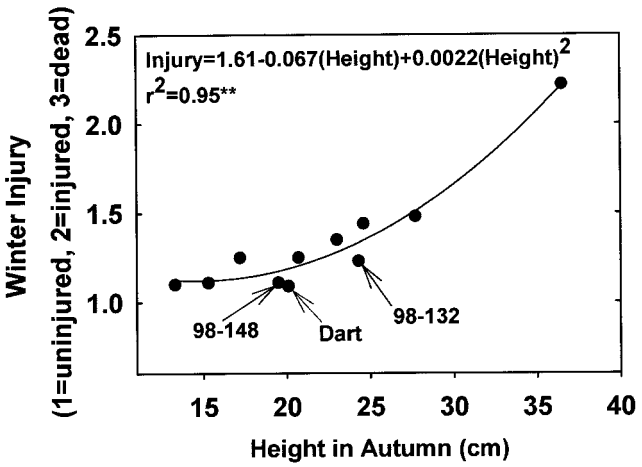

Fig. 3. Relationship of shoot height in autumn to winter injury of alfalfa. Data are means averaged over both years. Responses of the germplasms 98-148 and 98-132, and the cultivar Dart are identified because these data points were positioned below the regression line. Asterisks (**) indicate significant quadratic relationship at $P \leq 0.01$.

98-132) was below the regression line indicating that they exhibited less winter injury than predicted from their shoot heights when compared with other cultivars/germplasms in this study. We hoped that detailed analysis of the root physiology of these germplasms/cultivars, when compared with plants with similar FD but poorer winter survival, would reveal attributes associated with improved winter hardiness.

Root Sugar and Starch Concentrations

Root sugar concentrations were influenced by both cultivar or germplasm, and the date of root sampling. Root sugar concentrations in October averaged 88 mg g^{-1} and were slightly lower in 98-142 when compared with the dormant cultivars and germplasms. When compared with plants sampled in October, root sugar concentrations increased an average of 35% in December, except for CUF 101, which exhibited decreased root sugar concentrations in December (Fig. 4A). Root sugar concentrations in December were closely associated with FD. Sugar concentrations were highest in roots of the very fall dormant 98-141 (155 mg g^{-1}) and lowest in the nondormant, CUF 101 (58 mg g^{-1}). Root sugar accumulation in late autumn has been positively correlated with enhanced alfalfa winter survival (Graber et al., 1927; Grandfield, 1943; Bula and Smith, 1954; Volenec et al., 1991; Castonguay et al., 1995; Cunningham and Volenec, 1998). Germplasm 98-132 had higher root sugar concentrations in December when compared with Archer, a factor associated with its greater winter hardiness. However, the reduced winter injury of 98-148 and Dart, when compared with Vernal, could not be attributed to differences in root sugar concentrations. This agrees with recent findings (Dhont et al., 2002; Haagenson et al., 2003) where mowing alfalfa in autumn, which reduced winter survival, consistently increased root sugar concentrations when compared with plants left uncut during autumn. Dhont et al. (2002) suggested the amount of root carbohydrate reserves in roots in autumn

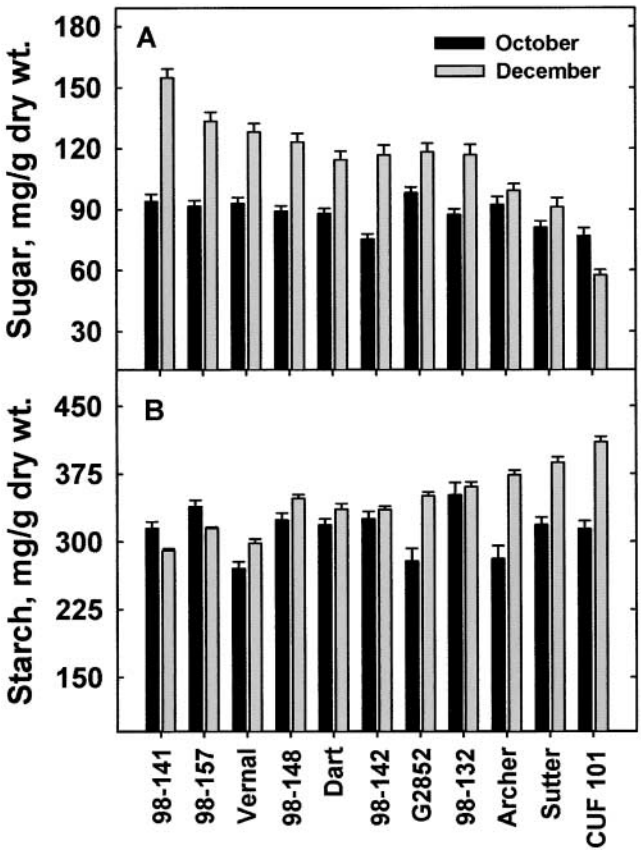

Fig. 4. (A) Sugar and (B) starch concentrations of alfalfa taproots sampled in mid-October and early December. Six alfalfa cultivars; Vernal, Dart, G2852, Archer, Sutter, and CUF 101 with known fall dormancy and winter hardiness were compared to five experimental germplasms; 98-141, 98-157, 98-148, 98-142, 98-132. Data were averaged over 2 yr. Error bars represent one standard error of the mean (n = 8).

had a stronger correlation with shoot regrowth potential in spring than does the root carbohydrate concentration. In our study, root carbohydrate reserves were reported on a concentration basis, and examination of root mass and carbohydrate amounts among 98-132, 98-148, Dart, and Vernal may help explain their differing winter survival.

Root starch concentrations in October differed among cultivars and germplasms (Fig. 4B). Vernal, the least winter hardy of the five most fall dormant cultivars/germplasms had lower root starch concentrations in October than the other fall dormant plants. Similarly, 98-132, which incurred less winter injury than G2852 and Archer, had higher root starch concentrations in October than did these check cultivars. In December, root starch concentration increased in G2852, Archer, Sutter, and CUF 101; cultivars that exhibited the greatest degree of winter injury. The positive association between winter injury and late-season starch accumulation agrees with previous research (Castonguay et al., 1995; Cunningham and Volenec, 1998). In addition, sugar accumulation in roots of these cultivars was relatively low when compared with roots of other cultivars/germplasms in this study indicating that partition of C between starch and sugar pools differs in December in

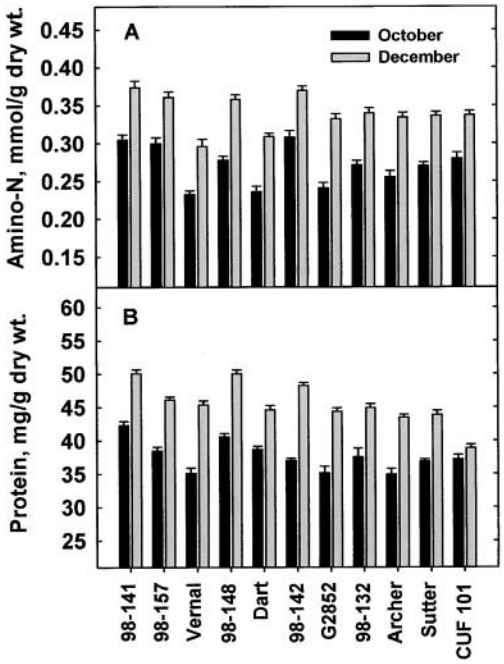

Fig. 5. (A) Amino N and (B) buffer-soluble protein concentrations of alfalfa taproots sampled in mid-October and early December. Six alfalfa cultivars; Vernal, Dart, G2852, Archer, Sutter, and CUF 101 with known fall dormancy and winter hardiness were compared with five experimental germplasms; 98-141, 98-157, 98-148, 98-142, 98-132. Data were averaged over 2 yr. Error bars represent one standard error of the mean (n = 8).

roots of dormant versus nondormant alfalfa cultivars. An exception to this was the semi-dormant germplasm 98-132 that accumulated high concentrations of both sugars and starch in roots in December and exhibited good winter survival. While starch accumulation in December may not directly reduce winter hardiness, its impact on root sugar accumulation in Archer, Sutter, and CUF 101 (Fig. 4A), may contribute to the poorer winter survival of these cultivars.

Root Amino-N and Protein Concentrations

Amino-N concentrations varied with germplasm and sampling date (Fig. 5A). Averaged over all cultivars/germplasms, root amino-N concentrations were 26% higher in December when compared with October. In October, root amino-N concentrations were lowest in Vernal, Dart, and G2852 and were higher in the germplasms 98-141, 98-157, 98-148, and 98-142. In December, these germplasms maintained high root amino-N concentrations, whereas roots of Vernal and Dart had the lowest amino N concentrations. With the exception of Dart, there was a positive association between root amino-N concentration in December and winter hardiness among the five most fall dormant cultivars/germplasms. Vernal had low root amino-N concentrations and incurred the greatest amount of injury. However, variation in winter injury among 98-132, G2852, and Archer was not explained by changes in amino-N concentrations in October or December, which were similar among these cultivars/germplasms. In a previous study using older cultivars, higher amino-N concentrations were observed in roots of nondormant alfalfa cultivars when compared with fall dormant cultivars (Cunningham and Volenec, 1998). Vernal and Dart, both older cultivars, had lower root amino-N concentrations in December when compared with Archer, Sutter, and CUF 101. However, the new dormant germplasms 98-141, 98-157, 98-148, and 98-142 all possessed higher root amino-N levels than even the nondormant cultivars used in this study and at FD ratings <3, had excellent winter hardiness.

Like amino N, both germplasm and sampling date influenced root soluble protein concentrations (Fig. 5B). In October, the dormant experimental germplasms 98-141 (42 mg g^{-1}) and 98-148 (41 mg g^{-1}) had the highest root protein concentrations, with protein concentrations of the remaining cultivars and germplasms averaging 37 mg g^{-1}. Root protein concentrations in December also were highest for the experimental germplasms: 98-141, 98-148, and 98-142. High root protein concentrations in December have been positively correlated with increased FD and enhanced winter survival in previous studies (Cunningham et al., 1998; 2001). However, variation in root protein concentration could not explain variation in winter injury among the fall dormant cultivars/germplasms (FD $\leq$ 3). Although Vernal had lower root protein concentrations in December than 98-141 and 98-148 and had greater injury than these germplasms, its root protein levels were similar to those of 98-157 and Dart, both of which incurred less injury than Vernal. Likewise, root protein concentrations of G2852, 98-132, Archer, and Sutter were similar in both October and December, even though 98-132 demonstrated superior winter survival. The SDS-PAGE analysis of polypeptides revealed small seasonal and germplasm-related changes that were not consistently associated with dormancy or winter hardiness (data not shown).

Expression of Cold Acclimation Responsive Genes

Previous studies have shown that fall dormant, winter hardy cultivars have increased expression of cold hardiness genes during autumn, whereas nondormant, nonhardy cultivars have low transcript levels for these genes (Mohapatra et al., 1987; Mohapatra et al., 1989; Wolfraim et al., 1993; Cunningham et al., 1998; Cunningham et al., 2001). In agreement with these studies, abundance of a cold acclimation responsive (*car*) transcript in roots of the nondormant, severely injured CUF 101 was below our detection limits in October (Fig. 6). Low *car* transcript levels also were detected in October in roots of G2852, which incurred more winter injury than germplasms 98-142 and 98-132, which had similar FD (Fig. 2) but had higher *car* transcript levels. Between October and December, transcript abundance increased to similar levels in roots of all cultivars and germplasms irrespective of FD and winter survival. This is consistent with previous work where a closer association between expression of *car* genes and genetic differences in winter injury is observed in October and November than in December when plants are completely dormant (J. Vo-

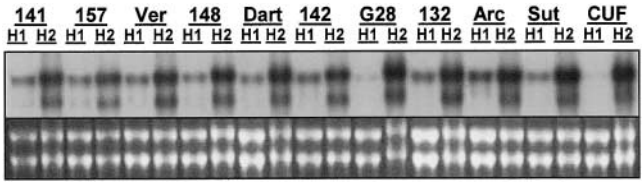

Fig. 6. RNA gel blot analysis showing steady state transcript levels of a cold acclimation-responsive gene in roots of six alfalfa cultivars and five experimental germplasms in October (H1) and December (H2). Twenty micrograms of total RNA was loaded per lane and blots were hybridized with a ^{32}P-labeled cold acclimation-responsive cDNA (bN-1 12a3) whose steady state transcript levels were previously shown to be high in winter hardy cultivars. The alfalfa cultivars and germplasms are listed in order of decreasing fall dormancy and include: 98-141 (141); 98-157 (157); Vernal (Ver); 98-148 (148); Dart; 98-142 (142); G2852 (G28); 98-132 (132); Archer (Arc); Sutter (Sut); CUF 101 (CUF). The lower panel is an ethidium bromide stained gel showing RNA loading levels.

lenec, unpublished data, 2002). Transcript abundance of Vernal was similar to that of the other fall dormant germplasms and cultivars despite its higher winter injury. Likewise, the improved winter survival of 98-132 was not reflected in greater abundance of this *car* transcript in either October or December when compared with Archer and Sutter, both of which exhibited greater winter injury.

Germplasms 98-148 and 98-132, and the cultivar Dart exhibited slightly higher levels of winter hardiness than expected given their FD ratings. Sugar concentrations exceeded 120 mg g^{-1} in roots of these germplasms, and root starch concentrations in December were high. However, variation in root amino-N and protein concentrations, and *car* gene expression patterns could not be consistently associated with the improved winter hardiness of these three alfalfas. Development and analysis of germplasms with even less FD, but which retain high levels of winter hardiness, would allow us to more easily ascertain the physiological and molecular mechanisms controlling these two very important agronomic traits of alfalfa.

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REFERENCES

Avice, J.C., A. Ourry, J.J. Volenec, G. Lemaire, and J. Boucaud. 1996. Defoliation-induced changes in abundance and immuno-localization of vegetative storage proteins in taproots of *Medicago sativa.* Plant Physiol. Biochem. 34:561–570.

Barber, L.D., B.C. Joern, J.J. Volenec, and S.M. Cunningham. 1996. Supplemental nitrogen effects on alfalfa regrowth and nitrogen mobilization from roots. Crop Sci. 36:1217–1223.

Boyce, P.J., and J.J. Volenec. 1992. Taproot carbohydrate concentrations and stress tolerance of contrasting alfalfa genotypes. Crop Sci. 32:757–761.

Brummer, E.C., M.M. Shah, and D. Luth. 2000. Reexamining the relationship between fall dormancy and winter hardiness in alfalfa. Crop Sci. 40:971–977.

Bula, R.J., and D. Smith. 1954. Cold resistance and chemical composition in overwintering alfalfa, red clover, and sweetclover. Agron. J. 46:397–401.

Bula, R.J., D. Smith, and H.J. Hodgson. 1956. Cold resistance in alfalfa at two diverse latitudes. Agron. J. 48:153–156.

Busbice, T.H., and C.P. Wilsie. 1965. Fall growth, winterhardiness, recovery after cutting and wilt resistance in F_2 progenies of Vernal × Dupuits alfalfa crosses. Crop Sci. 5:429–432.

Castonguay, Y., P. Nadeau, and S. Laberge. 1993. Freezing tolerance and alteration of translatable mRNAs in alfalfa (*Medicago sativa* L.) hardened at subzero temperatures. Plant Cell Physiol. 34:31–38.

Castonguay, Y., P. Nadeau, P. Lechasseur, and L. Chouinard. 1995. Differential accumulation of carbohydrates in alfalfa cultivars of contrasting winterhardiness. Crop Sci. 35:509–516.

Cunningham, S.M., J.A. Gana, J.J. Volenec, and L.R. Teuber. 2001. Winter hardiness, root physiology, and gene expression in successive fall dormancy selections from 'Mesilla' and 'CUF 101' alfalfa. Crop Sci. 41:1091–1098.

Cunningham, S.M., and J.J. Volenec. 1998. Seasonal carbohydrate and nitrogen metabolism in roots of contrasting alfalfa (*Medicago sativa* L.) cultivars. J. Plant Physiol. 153:220–225.

Cunningham, S.M., J.J. Volenec, and L.R. Teuber. 1998. Plant survival and root and bud composition of alfalfa populations selected for contrasting fall dormancy. Crop Sci. 38:962–969.

Dhont, C., Y. Castonguay, P. Nadeau, G. Belanger, and F.-P. Chalifour. 2002. Alfalfa root carbohydrates and regrowth potential in response to fall harvests. Crop Sci. 42: 754–765.

Feinberg, A.P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6–13.

Gana, J.A., N.E. Kalengamaliro, S.M. Cunningham, and J.J. Volenec. 1998. Expression of β-amylase from alfalfa taproots. Plant Physiol. 118:1495–1505.

Gana, J.A., F. Sutton, and D.G. Kenefick. 1997. cDNA structure and expression patterns of a low-temperature-specific wheat gene *tacr7.* Plant Mol. Biol. 34:643–650.

Graber, L.F., N.T. Nelson, W.A. Luekel, and W.B. Albert. 1927. Organic food reserves in relation to the growth of alfalfa and other perennial herbaceous plants. Univ. of Wis. Agric. Exp. Stn. Res. Bull. 80.

Grandfield, C.O. 1943. Food reserves and their translocation to the crown buds as related to cold and drought resistance in alfalfa. J. Agric. Res. 67:33–47.

Haagenson, D.M., S.M. Cunningham, B.C. Joern, and J.J. Volenec. 2003. Autumn defoliation effects on alfalfa winter survival, root physiology, and gene expression. Crop Sci. 43:1340–1348. (this issue).

Hendershot, K.L., and J.J. Volenec. 1993. Taproot nitrogen accumulation and use in overwintering alfalfa (*Medicago sativa* L.). J. Plant Physiol. 141:68–74.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 222:680–685.

Li, R., J.J. Volenec, B.C. Joern, and S.M. Cunningham. 1996. Seasonal changes in nonstructural carbohydrates, protein, and macronutrients in roots of alfalfa, red clover, sweetclover, and birdsfoot trefoil. Crop Sci. 36:617–623.

McKenzie, J.S., R. Paquin, and S.H. Duke. 1988. Cold and heat tolerance. p. 259–301. *In* A.A. Hanson (ed.) Alfalfa and Alfalfa Improvement. Agron. Monogr. 29. ASA, Madison, WI.

Mohapatra, S.S., R.J. Poole, and R.S. Dhindsa. 1987. Changes in protein patterns and translatable messenger RNA populations during cold acclimation of alfalfa. Plant Physiol. 84:1172–1176.

Mohapatra, S.S., L. Wolfraim, R.J. Poole, and R.S. Dhindsa. 1989. Molecular cloning and relationship to freezing tolerance of cold-acclimation specific genes of alfalfa. Plant Physiol. 89:375–380.

Noquet, C., J.C. Avice, A. Ourry, J.J. Volenec, S.M. Cunningham, and J. Boucaud. 2001. Effects of environmental factors and endogenous signals on N uptake, N partitioning and taproot vegetative storage protein accumulation in *Medicago sativa.* Aust. J. Plant Physiol. 28:279–287.

Rosen, H. 1957. A modified ninhydrin colorimetric analysis for amino acid. Arch. Biochem. Biophys. 67:10–15.

SAS Institute. 1999. The SAS system for Windows. Release version 8.1. SAS Inst., Cary, NC.

Sheaffer, C.C., D.K. Barnes, D.D. Warnes, W.E. Lueschen, H.J. Ford, and D.R. Swanson. 1992. Seeding-year cutting affects winter sur-

vival and its association with fall growth score in alfalfa. Crop Sci. 32:225–231.
Smith, D. 1961. Association of fall growth habit and winter survival in alfalfa. Can. J. Plant Sci. 41:244–251.
Snedecor, G.W., and W.G. Cochran. 1980. Statistical Methods, 7th Ed. p. 593. Iowa State University Press, Ames, IA.
Steel, R.G.D., and J.H. Torrie. 1980. Principles and procedures of statistics: A biometrical approach. 2nd Ed. McGraw-Hill, New York.
Stout, D.G., and J.W. Hall. 1989. Fall growth and winter survival of alfalfa in interior British Columbia. Can. J. Plant Sci. 69:491–499.
Volenec, J.J. 1985. Leaf area expansion and shoot elongation of diverse alfalfa germplasms. Crop Sci. 25:822–827.
Volenec, J.J., P.J. Boyce, and K.L. Hendershot. 1991. Carbohydrate metabolism in taproots of *Medicago sativa* L. during winter adaptation and spring regrowth. Plant Physiol. 96:786–793.
Volenec, J.J., A. Ourry, and B.C. Joern. 1996. A role for nitrogen reserves in forage regrowth and stress tolerance. Physiol. Plant. 97:185–193.
Wilding, M.D., M.A. Stahmann, and D. Smith. 1960. Free amino acids in alfalfa as related to cold hardiness. Plant Physiol. 35:726–732.
Wolfraim, L.A., R. Langis, H. Tyson, and R.S. Dhindsa. 1993. cDNA sequence, expression, and transcript stability of a cold acclimation-specific gene, *cas18*, of alfalfa (*Medicago falcata*) cells. Plant Physiol. 101:1275–1282.
Zaleski, A. 1954. Identification and classification of lucerne 'varieties' and 'strains'. J. Agric. Sci. 44:199–220.