Winter Hardiness, Root Physiology, and Gene Expression in Successive Fall Dormancy Selections from ‘Mesilla’ and ‘CUF 101’ Alfalfa

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ABSTRACT

Fall dormancy is positively associated with alfalfa (Medicago sativa L.) winter survival, but the physiological bases for this association are not understood. Our objective was to determine how incremental changes in fall dormancy due to genetic selection influenced autumn height and winter survival, root physiology, and expression of a cold acclimation responsive gene family. Seed from each of three cycles of selection for contrasting (greater or less) fall dormancy using ‘Mesilla’ and ‘CUF 101’ as parents were planted in rows in the field (Starks-Fincastle, fine-silty, mixed, mesic, Aeric Ochraqualf) in West Lafayette, IN, in May 1997 and 1998. Plant height was measured in October and roots were sampled in December. Plant survival was determined in March of the year following seeding. Fall dormancy (reduction in shoot height in October) increased in a linear manner over the three cycles of selection for both Mesilla and CUF 101. A positive linear relationship was observed between fall height and winter injury in both years. Root sugar and protein concentrations increased as fall dormancy increased in populations derived from both Mesilla and CUF 101. Expression of the cold acclimation-responsive gene, RootCAR1, was positively associated with winter survival, and may be useful as a molecular marker for identifying winter hardy plants among semi-dormant or nondormant alfalfa germplasm in December of the seeding year.

Fall dormant alfalfa cultivars produce short, decumbent shoots in autumn, whereas fall nondormant plants possess tall, upright shoots in autumn (Smith, 1958; Barnes et al., 1979; Sheaffer et al., 1992; Cunningham et al., 1998). Non-fall dormant alfalfa cultivars are desirable because nondormant plants produce more herbage in autumn, resume shoot growth earlier in spring, and initiate shoot regrowth quickly after harvest in summer (Zaleski, 1954; Busbice and Wilse, 1965). Once shoot initiation occurs, shoot elongation rate of nondormant alfalfa is up to twice that of fall-dormant germplasm pools, and results in large differences in leaf area expansion and mass per shoot (Volenec, 1985), factors known to be correlated with high forage yield (Volenec et al., 1987).

The major constraint preventing widespread use of nondormant alfalfas in temperate regions is their poor winter hardness. Sheaffer et al. (1992) examined in spring-planted alfalfa the interactions between seeding-year harvest management, fall dormancy, and winter survival in Minnesota. They reported that fall dormancy influenced alfalfa winter hardness more than any other feature they measured. Nondormant alfalfa cultivars died during winter irrespective of harvest management or location, whereas fall-dormant cultivars had good winter survival. These findings are supported by other studies (Smith, 1961; Barnes et al., 1979; Stout, 1985; Stout and Hall, 1989), and have resulted in fall dormancy routinely being used to predict alfalfa winter hardness.

The mechanisms controlling the close positive association between fall dormancy and winter hardness are not clearly understood. Genetic differences in alfalfa winter survival have been associated with several physiological changes in overwintering organs. Accumulation of starch and sugar in taproots has been positively associated with alfalfa winter survival (Grabber et al., 1927; Grandfield, 1943; Smith, 1964). Accumulation of soluble sugars in roots is thought to enhance tolerance to low temperatures and other stresses associated with winter (Bula et al., 1956; Ruelke and Smith, 1956). Castonguay et al. (1995) and Castonguay and Nadeau (1998) recently reported that the accumulation of raffinose and stachyose was more closely associated with winter survival than accumulation of starch or sucrose. Other studies indicate that N-containing compounds also accumulate in roots during winter hardening, and serve as a source of N when shoot growth is initiated in spring and for regrowing shoots after harvest (Volenec et al., 1991, 1996; Hendershot and Volenec, 1993; Avice et al., 1996). Differential gene expression and accumulation of their gene products occur during cold acclimation, and these changes also have been positively associated with ge-

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Abbreviations: LSD, least significant difference; PMSF, phenyl methyl sulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TNC, total nonstructural carbohydrate.
MATERIALS AND METHODS

Plant Culture and Sampling

Populations from each of three cycles of selection for contrasting fall dormancy from ‘Mesilla’ (semi-dormant, fall dormancy rating of 6.5) and ‘CUF 101’ (nondormant, fall dormancy rating of 8.8) were studied (Putnam et al., 1999). More fall dormant populations were designated as L1, L2, and L3 for populations resulting from the first, second, and third cycles of selection, respectively. Less fall dormant populations were designated as H1, H2, and H3 for populations resulting from the first, second, and third cycles of selection, respectively. Parent cultivars were designated as CUF 101-O and Mesilla-O. Details of the selection process have been published recently (Cunningham et al., 1998). ‘Norseman’ (highly fall dormant; fall dormancy rating of 5), ‘Saranac’ (semi-dormant, fall dormancy rating of 4), ‘Lahontan’ (semi-dormant, fall dormancy rating of 6), and ‘Wadi Qurayat’ (highly nondormant, fall dormancy rating >10) were included as additional controls representing the present range of fall dormancy reactions in cultivated alfalfa.

Seedlings were established at the Agronomy Research Center, Purdue University, West Lafayette, IN, in early May of 1997 and 1998. Seeds were sown in 3-m-long rows spaced 92 cm apart in a randomized complete-block with four replications. Resultant plant populations were approximately 20 plants/m of linear row. The soil was a silt loam of the Starks-Fincastle series that was fertilized and limed according to soil test for high alfalfa yield. Seeds were inoculated with *Rhizobium meliloti* (Liphatech Corp., Milwaukee, WI) prior to planting. Plots were hand-weeded, and insects controlled as needed. Planting and sampling dates are summarized in Table 1. Plant height was measured at eight randomly selected positions within each row, and the average used as an indication of fall dormancy for that plot. Plant survival was determined when shoot growth of winter hardy cultivars resumed in April by excavating 15 to 30 plants and scoring the winter injury of each plant using the following scale: 1 = uninjured; 2 = injured; and 3 = completely dead. A weighted average was calculated for each plot and used for statistical analysis. Air and soil temperatures were recorded daily each year using National Weather Service Standard maximum and minimum thermometers (for air temperature) and a mercury thermometer for soil temperature at a 10-cm depth under bare soil (Fig. 1).

Roots dug to a soil depth of approximately 20 cm 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 CO₂ for protein and carbohydrate analyses or immersed in liquid N₂ for RNA analyses. The upper 5 cm of taproots were selected for analysis because this was a well-defined tissue region and minimized variation due to rooting depth, degree of branching, and other root morphological differences that could confound results obtained by using the entire root system for analysis. Tissues for protein and carbohydrate analyses were lyophilized, ground to pass a 1-mm screen and were stored at −20°C. Tissues for RNA analyses were stored at −80°C.

Sugar, Starch, and Protein Analyses

Details of these analyses have been recently published (Cunningham et al., 1998). Sugars were extracted with 800 ml/L ethanol, microfuged, and the sugar concentration of the supernatant determined with anthrone (Van Handel, 1968) using glucose as a standard. Starch in the ethanol-extracted residue was gelatinized, and starch digested by adding 0.2 U of amyloglucosidase (Sigma Chemical Co., St. Louis, MO; product A3514 from *Aspergillus niger*) and 40 U of α-amylase (Sigma Chemical Co., St. Louis, MO; product A0273 from *A. oryzae*). Tubes were centrifuged and glucose in the supernatant was determined using glucose oxidase (Glucose Trinder, Sigma Chemical Co., St. Louis, MO; product 315-100). Starch concentration was estimated as 0.9 × glucose concentration. Protein extraction was conducted at 4°C. Proteins were extracted from parallel samples using 100 mM imidazole-HCl buffer (pH 6.5) containing 10 mM 2-mercaptoethanol and 1 mM PMSF. Suspensions were centrifuged, and soluble protein in the supernatant was estimated using a protein dye-binding technique (Bradford, 1976).
Fig. 1. Minimum air and soil (10-cm depth) temperatures at the Agronomy Research Center.

RNA Isolation and Northern Blot
Hybridization Analysis

Tissues were ground in liquid N₂ using a mortar and pestle, and total RNA isolated using hot phenol (Ougham and Davis, 1990) with minor modifications (Gana et al., 1998). Total RNA (20 mg) was separated on a 1.5% (w/v) agarose-formaldehyde gel (Lehrach et al., 1997). The RNA was transferred by capillary action to a Zeta-probe membrane (BioRad) after electrophoresis. The membranes were pre-hybridized for 4 h at 42°C (Stewart and Walker, 1989) with slow shaking. A cold acclimation responsive cDNA clone from alfalfa, RootCAR1 (GenBank Accession AF072932), was labeled with ³²P-dCTP using random priming (Feinberg and Vogelstein, 1983). 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 experiment was replicated four times in each of 2 yr. Years were treated as random effects and germplasm pools as fixed effects. Data were analyzed as a randomized complete block design with a mixed model using analysis of variance (SAS Institute, Cary, NC). Where F-tests were significant (P ≤ 0.05 unless otherwise stated), an LSD was calculated for mean comparisons. Regression was used to examine the relationships among variables.

RESULTS

Plant Height and Winter Survival

Soil and air temperatures in both years of the study (Fig. 1) did not deviate markedly from the long-term averages at the Agronomy Research Center. The interval from December 1998 to January 1999 was colder than this same interval the previous winter; however, plant survival was similar both years.

Plant height in October (estimate of fall dormancy) of the cultivars used as controls differed in the expected manner (Fig. 2A). Averaged across years, height of Norseman averaged 8 cm, while height of Wadi Qurayat approached 50 cm, with Saranac and Lahontan intermediate between these. For each year linear regression of fall dormancy ratings of Norseman, Saranac, Mesilla, Lahontan, and Wadi Qurayat vs. mean fall plant height (hgt) was significant and resulted in the equations: Year 1, y = −0.6 + 4.1hgt; Year 2, y = 8.8 + 3.9hgt; r² ≥ 0.96. These regression equations were used to predict fall dormancy ratings of the selected populations within each of the respective years, and these estimates were analyzed using analysis of variance (Fig. 2A). Selection for greater fall dormancy reduced plant heights and fall dormancy ratings for each cycle of selection, CUF 101-L1 through CUF 101-L3. A similar pattern of reduction in height and fall dormancy rating in response to selection for greater fall dormancy occurred in Mesilla, but differences between the L1 and L2 populations, and the L2 and L3 populations were not statistically significant. Selection for less fall dormancy in CUF 101 had no effect on height and fall dormancy rating of this already fall nondormant cultivar, whereas only Mesilla H3 was significantly taller and less fall dormant than Mesilla-O, -H1, and -H2 (Fig. 2A).

Response to selection for contrasting fall dormancy was determined using linear regression of fall height vs. cycle of selection. Regression analysis revealed that response to selection for less fall dormancy was not...
Fig. 3. Relationship between fall height and winter injury of Mesilla and CUF 101 alfalfa populations presented in Fig. 2. Data are presented separately for each year and include data for Norseman, Saranac, Lahontan, and Wadi Qurayat. Asterisks (**) indicate statistically significant linear relationship at $P \leq 0.01$.

Fig. 4. Root sugar (A) and starch (B) concentrations of alfalfa populations selected for contrasting fall dormancy (see Fig. 2 for population descriptions). Data are means averaged across both years. The least significant difference (LSD, $P = 0.05$) is provided.

Root Sugar, Starch, and Protein

Root tissues were sampled in December to determine how selection for contrasting fall dormancy altered cold acclimation of these alfalfa populations. Averaged over both years root sugar concentrations of Norseman and Saranac exceeded 100 g/kg, which was at least twice that of CUF 101-O and Wadi Qurayat (Fig. 4A). The semi-dormant cultivars Lahontan and Mesilla-O had intermediate root sugar concentrations. Selection for greater fall dormancy resulted in higher root sugar concentrations in Mesilla-L2 and L3 when compared to Mesilla-O, while selection for less fall dormancy reduced root sugar concentrations in Mesilla-H3. Similarly, selection for greater fall dormancy increased sugar concentrations in roots of all CUF 101-L populations, and nearly doubled root sugar concentrations in CUF 101-L3 when compared to CUF 101-O. In contrast, selection for less fall dormancy did not alter root sugar concentrations of the CUF 101-H populations when compared to CUF 101-O.

Regression analysis was used to evaluate the relationship between root sugar concentrations and winter injury. Averaged over both years, a highly significant reduction in injury was observed as root sugar concentrations increased (Fig. 5). The greatest impact of increased root sugar concentration on injury reduction occurred between 40 and 100 g/kg, with little additional reduction in injury as sugar concentrations exceeded 100 g/kg.

Root starch concentrations also differed among cultivars and selected populations. Averaged over both years, root starch concentrations were lowest in Norseman, highest in Wadi Qurayat, with the remaining cultivars having intermediate root starch concentrations (Fig. 4B). Selection for contrasting fall dormancy in Mesilla-O...
did not alter root starch concentration of any of the Mesilla-L and -H populations. Selection for greater fall dormancy did not change root starch concentrations of the CUF 101-L populations, but selection for less fall dormancy resulted in higher root starch concentrations in CUF 101-H2 and -H3 when compared to CUF 101-O. Winter injury increased in a linear fashion (injury = −1.4 + 0.009(starch), $r^2 = 0.69^{**}$) with increasing root starch concentrations (data not shown), indicating that availability of starch reserves did not necessarily enhance cold tolerance and winter survival of these alfalfa cultivars and populations.

Protein concentrations of roots sampled in December differed among cultivars and populations. Averaged over both years roots of Norseman, Saranac, and Lahontan had higher protein concentrations than roots of Mesilla-O, CUF 101-O, and Wadi Qurayat (Fig. 6). When compared to Mesilla-O, selection for greater fall dormancy significantly increased root protein concentrations of Mesilla-L2 and -L3, while selection for less fall dormancy reduced protein concentrations in roots of Mesilla-H3. Likewise, the more fall dormant CUF 101-L2 and -L3 had higher root protein concentrations than CUF 101-O. However, selection for less fall dormancy did not significantly reduce root protein concentrations in CUF 101-H populations, probably due, in part, to the low protein concentrations already present in roots of the parent CUF 101-O. Averaged over both years, regression of winter injury scores vs. root protein concentration revealed a significant negative linear relationship between these characteristics (Fig. 7). Under the conditions of this study increasing root protein concentration in December by 5 g/kg dry wt. did reduce winter injury scores by 0.5, equivalent to the winter injury score difference between the very winter hardy Norseman and Mesilla-O, the latter of which is considered not adapted for use under Midwest growing conditions.

**Expression of Cold Acclimation Responsive (CAR) Genes**

To improve our understanding of the role of gene expression in alfalfa cold acclimation and fall dormancy, we assayed steady-state transcript abundance RootCAR1 [GenBank Accession AF072932; similar to cas 15a (GenBank Accession AAA16927) and cas 15b GenBank Accession AAA16926], a cold acclimation-responsive transcript previously shown to be positively associated with alfalfa winter survival. RootCAR1 transcript abundance was high in Norseman and below our detection limit in Wadi Qurayat, with Mesilla-O and CUF 101-O exhibiting intermediate transcript abundance (Fig. 8). In roots of these cultivars the high abundance of RootCAR1 transcripts was associated with reduced winter injury (Fig. 2B and 8).

In Mesilla, selection for greater fall dormancy resulted in slightly higher RootCAR1 transcript abundance in Mesilla-L1 and -L3, but winter injury was significantly reduced only in Mesilla-L3. Selection for less fall dormancy in Mesilla resulted in slightly lower RootCAR1 transcript abundance in Mesilla-H1 and -H3, when compared to Mesilla-O, and slightly greater winter injury scores in Mesilla-H3. Mesilla-H2 seed was limited...
ment, we studied alfalfa populations derived by three cycles of disruptive selection for contrasting fall dormancy using either CUF 101 or Mesilla as parents. We observed incremental reductions in fall dormancy with each cycle of selection (Fig. 2A) and with these changes reduced winter injury (Fig. 2B). Linear regression revealed a close ($r^2 = 0.81–0.88$) association between fall shoot growth (nondormancy) and winter injury in these populations (Fig. 3). Previous work with CUF 101-O, -L3, and -H3 also showed that CUF 101-L3 was more fall dormant, and much more winter hardy than CUF 101-O (Cunningham et al., 1998). A continuum of variation in fall dormancy in response to selection, however, was not evident in that study because only the third cycle of selection was studied, and the Mesilla populations were not included. While fall dormancy has been associated with cultivar differences in winter survival in previous studies, never before has a set of populations representing consecutive cycles of alfalfa selected exclusively for contrasting fall dormancy been available to evaluate this association. Results from this study and our previous work with these populations (Cunningham et al., 1998) suggest that decreasing alfalfa fall dormancy without increasing susceptibility to winter injury will be difficult. Recently, Brummer et al. (2000) reported little association between fall growth and winter injury in an $F_1$ population derived from a $M. sativa \times M. falcata$ cross. Based on genetic correlations and heritabilities, they suggested that both winter hardness and fall growth can be improved simultaneously in this population. Although possible, the consistent association between fall dormancy and winter hardness observed in our populations in this and a previous study (Cunningham et al., 1998), suggest that it may be necessary to use different germplasms than those we have studied to simultaneously improve fall growth and winter survival.

High concentrations of root total nonstructural carbohydrates (TNC, sugar $+\ starch$) is generally believed to be essential for alfalfa winter survival (Klebesadel, 1971; Sheaffer et al., 1992). Root TNC concentrations (data not shown) mirrored trends in root starch (Fig. 4B), and like starch, were lowest in Norseman the most fall dormant, winter hardy cultivar. The nondormant populations and cultivars had root starch and TNC levels that equaled or exceeded those of Norseman indicating that starch and TNC reserve levels per se cannot be used to predict genetic differences in winter survival.

Root sugar concentrations were positively associated with fall dormancy and limited winter injury (Fig. 4A and 5). Small increases in fall dormancy were accompanied by small, but consistent increases in root sugar concentrations and reduced winter injury in both CUF 101 and Mesilla. This agrees with our previous report where root sugar concentrations of CUF 101-L3 exceeded those of CUF 101-O (Cunningham et al., 1998). Root sugar concentrations in the present study were lower than those previously reported for these populations, possibly due to year-to-year differences in the environment under which plants cold acclimated. Nevertheless, the ranking of root sugar concentrations did
not vary between this and our previous (Cunningham et al., 1998) study.

Other research (Castonguay and Nadeau, 1998), including our own (Cunningham and Volenec, 1998) has shown less consistency in fall dormancy-driven changes in root sugar accumulation during cold acclimation. These studies used an assortment of cultivars that differed in fall dormancy, rather than contrasting fall dormancy populations derived from a single cultivar, so genetic background differences may have confounded the cold acclimation responses. In addition, it has been suggested that accumulation of raffinose and stachyose in crowns may be more important than sucrose accumulation in enhancing alfalfa winter survival (Castonguay et al., 1995; Castonguay and Nadeau, 1998). Work is being initiated to evaluate the composition of sugars found in overwintering organs of these contrasting populations.

Like root sugars, root protein concentrations also systematically increased in response to selection for greater fall dormancy in both CUF 101 and Mesilla (Fig. 6), and these changes were positively associated with reduced winter injury (Fig. 7). We have previously reported greater accumulation of protein in roots of fall dormant alfalfa cultivars (Cunningham and Volenec, 1998), and CUF 101-L3 when compared to CUF 101-O (Cunningham et al., 1998). Electrophoretic analysis revealed that a large proportion of this protein accumulation is vegetative storage proteins (VSPs, Cunningham and Volenec, 1996) that serve as a source of N when shoot growth is initiated in spring. Although cold acclimation-induced polypeptides have been observed in fall dormant populations in previous work (Cunningham et al., 1998), SDS-PAGE analysis did not reveal new polypeptides in root protein extracts of the fall dormant, winter hardy populations in this study. This is not surprising considering that a few hundred of the thousands of polypeptides present in alfalfa roots are resolved using this technique. Changes in the abundance of key proteins associated with greater fall dormancy and enhanced winter survival could easily escape detection.

We used Northern analysis to assay transcript abundance for a gene (RootCAR1) whose expression has been associated with cultivar differences in winter hardiness (Monroy et al., 1993; Volenec, unpublished data, 2000). The high sensitivity of this assay may permit us to detect differences in gene expression that result from selection for contrasting fall dormancy that cannot be determined using SDS-PAGE analysis of proteins. Mesilla-O roots contain moderate RootCAR1 transcript levels, and these increased slightly as fall dormancy increased and winter injury declined in Mesilla-L3. Selection for greater fall dormancy in CUF 101 markedly increased the steady-state transcript abundance of RootCAR1 in roots of CUF 101-L2 and -L3, and with it, greatly reduced winter injury. By comparison, no change in transcript abundance was observed with selection for less fall dormancy in CUF 101-H1, -H2, or -H3, and there was no change in fall dormancy or winter injury of these populations. This positive association between RootCAR1 transcript abundance and alfalfa winter survival is further verified by the high transcript abundance found in the very winter hardy Norseman, and the lack of transcript detected in roots of Wadi Qurayat. Even though we do not yet know the function of the RootCAR1 protein in plants, expression of this gene may serve as a useful marker for identifying winter hardy alfalfa plants in the fall of the seeding year. This would improve selection efficiency by eliminating evaluation at multiple sites over several years to identify winter hardy plants. Work is underway to determine the feasibility of this approach for alfalfa improvement.

REFERENCES


