Nitrogen Reserves, Spring Regrowth and Winter Survival of Field-grown Alfalfa (Medicago sativa) Defoliated in the Autumn

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Aims The objective of the study was to characterize variations in proline, arginine, histidine, vegetative storage proteins, and cold-inducible gene expression in overwintering roots of field-grown alfalfa, in response to autumn defoliation, and in relation to spring regrowth and winter survival.

Methods Field trials, established in 1996 in eastern Canada, consisted of two alfalfa cultivars (‘AC Caribou’ and ‘WL 225’) defoliated in 1997 and 1998 either only twice during the summer or three times with the third defoliation taken 400, 500 or 600 growing degree days (basis 5°C) after the second summer defoliation.

Key Results The root accumulation of proline, arginine, histidine and soluble proteins of 32, 19 and 15 kDa, characterized as alfalfa vegetative storage proteins, was reduced the following spring by an early autumn defoliation at 400 or 500 growing degree days in both cultivars; the 600-growing-degree-days defoliation treatment had less or no effect. Transcript levels of the cold-inducible gene msa CIA, encoding a glycine-rich protein, were markedly reduced by autumn defoliation in ‘WL 225’, but remained unaffected in the more winter-hardy cultivar ‘AC Caribou’. The expression of another cold-inducible gene, the dehydrin homologue msoCIG, was not consistently affected by autumn defoliation. Principal component analyses, including components of root organic reserves at the onset of winter, along with yield and plant density in the following spring, revealed that (a) amino acids and soluble proteins are positively related to the vigour of spring regrowth but poorly related to winter survival and (b) winter survival, as indicated by plant density in the spring, is associated with higher concentrations of cryoprotective sugars in alfalfa roots the previous autumn.

Conclusions An untimely autumn defoliation of alfalfa reduces root accumulation of specific N reserves such as proline, arginine, histidine and vegetative storage proteins that are positively related to the vigour of spring regrowth but poorly related to winter survival.

Key words: Medicago sativa, amino acids, vegetative storage proteins, cold-inducible genes, autumn defoliation, spring regrowth, winter survival.

INTRODUCTION

Persistence of field-grown alfalfa (Medicago sativa) in northern regions does not only rely on the capacity of the plant to acquire freezing tolerance during autumn acclimation and to withstand subfreezing temperatures during winter (McKenzie et al., 1988), but also depends on the accumulation of root organic reserves to sustain spring regrowth (Volenc et al., 1996). Autumn defoliation, especially the regrowth interval between the last summer defoliation and the one in the autumn, is a determinant factor of alfalfa persistence because of its effect on key organic reserves essential to winter hardening and spring regrowth (Sheaffer et al., 1986; Bélanger et al., 1992; Dhont et al., 2002, 2003). The accumulation of growing degree days (GDD; 5°C threshold) after the last summer defoliation has been proposed as a criterion to estimate the minimum duration of the regrowth interval between the last two defoliations (Bélanger et al., 1992). Under both unheated greenhouse and field conditions, spring regrowth of alfalfa was reduced by autumn defoliation taken at 400 or 500 GDD (Dhont et al., 2002, 2004). Furthermore, autumn defoliation associated with harsh winter conditions induced severe winter damage in a 3-year-old alfalfa stand, significantly compromising its field persistence (Dhont et al., 2004).

It is widely recognized that alfalfa regrowth is not only affected by the availability of C reserves but also depends on the mobilization of organic N from storage organs and their translocation to new growing leaves (Ourry et al., 1994; Volenc et al., 1996; Avice et al., 1997). Amino acids such as asparagine and aspartic acid, as well as specific soluble proteins of 15, 19, 32 and 57 kDa, harbouring characteristics typical of vegetative storage proteins (VSPs), are thought to be involved in alfalfa regrowth (Hendershot and Volenc, 1993a; Avice et al., 1996; Cunningham and Volenc, 1996). Using plants acclimated to winter conditions in an unheated greenhouse, Dhont et al. (2003) recently reported positive correlations between both concentrations and pools of root N reserves and alfalfa spring regrowth.

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Cold acclimation of alfalfa is characterized by significant biochemical changes, including modifications in soluble sugars, amino acids, protein composition and gene expression (McKenzie et al., 1988; Castonguay et al., 1997a). An increasing body of evidence indicates that endogenous N pools that accumulate in taproots during autumn hardening could also play important roles in the overwintering potential of alfalfa. For instance, free proline concentrations markedly increase during autumn acclimation and have been correlated with freezing tolerance in alfalfa (Paquin and Pelletier, 1981). It has also been suggested that alfalfa VSPs could be involved in cold adaptation, based on their higher abundance in winter-hardy perennials than in annual species of Medicago (Cunningham and Volene, 1996) and on their accumulation in the autumn in response to a decreasing photoperiod (Noquet et al., 2001).

Differential accumulation of cold-inducible transcripts and cold-inducible gene products in alfalfa cultivars of contrasting winter hardiness suggests a close relationship between the acquisition of freezing tolerance and changes in gene expression (Mohapatra et al., 1989; Monroy et al., 1993; Castonguay et al., 1997b; Ferrullo et al., 1997). Although knowledge of the molecular bases of cold tolerance in alfalfa has improved (reviewed by Castonguay et al., 1997a), the impact of autumn defoliation on the expression of cold-inducible genes remains to be investigated.

The recently reported relationship between root N reserves and alfalfa regrowth as well as the potential role of some root N reserves in cold acclimation have raised new questions on the potential effects of autumn defoliation on spring regrowth and winter survival of alfalfa. With plants acclimated to winter conditions in an unheated greenhouse, Dhont et al. (2003) reported that the amounts of amino acids, including proline, arginine and histidine, and soluble proteins, in roots of alfalfa were reduced by an autumn defoliation. In the light of these recent findings, further investigations were needed to assess the link between root organic reserves, productivity and longevity of alfalfa under field conditions. Therefore, the objective was to characterize variations in N components (proline, arginine, histidine and VSPs) and cold-inducible gene expression in overwintering roots of field-grown alfalfa, in response to autumn defoliation taken at increasing intervals after the last summer defoliation, and in relation to spring regrowth and winter survival.

### MATERIALS AND METHODS

#### Experimental sites and plant material

A field experiment was conducted from 1996 to 1999 at two sites located in different agroclimatic regions in Quebec, Canada: Normandin (Labare silty clay; 48°50′N and 72°32′W; 1333 GDD, basis 5°C); Pintendre (Kamouraska clay loam; 46°45′N and 71°08′W; 1653 GDD, basis 5°C). Seeds of alfalfa (Medicago sativa L.) ‘AC Caribou’ and ‘WL 225’ were inoculated with a commercial inoculant (Nitragin Inc., Brookfield, WI, USA) of Sinorhizobium meliloti before seeding on 27 May 1996 at Normandin and on 5 Aug. 1996 at Pintendre. Application of fertilizers, weed control and overwintering soil temperatures are reported in Dhont et al. (2004).

#### Defoliation treatments and root sampling

For two years (1997 and 1998) following alfalfa establishment, plants were defoliated at a 7-cm height twice during the summer at the early flowering stage (Table 1). Four defoliation treatments were applied in the autumns of 1997 and 1998: no additional defoliation (two-defoliation treatment) or a third defoliation (three-defoliation treatment) at increasing intervals from the second summer defoliation, i.e. at 400, 500 or 600 GDD cumulated after the second defoliation (Table 1). The GDD were calculated according to Bélanger et al. (1992) by subtracting 5°C from the average of daily maximum and minimum air temperatures recorded at a nearby weather station and accessed daily through an Environment Canada online service. At each defoliation date, dry matter (DM) yield and plant density were determined as described in Dhont et al. (2004).

### TABLE 1. Dates of summer and autumn defoliations, and root samplings at the two experimental sites (Pintendre and Normandin) from 1997 to 1999

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<td>Pintendre</td>
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<td>Normandin</td>
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<td>Second summer defoliation</td>
<td>6 Aug.</td>
<td>5 Aug.</td>
<td>–</td>
<td>30 Jul.</td>
<td>30 Jul.</td>
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<tr>
<td>Autumn defoliation treatments</td>
<td>400 GDD*</td>
<td>9 Sept.</td>
<td>10 Sept.</td>
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<td>12 Sept.</td>
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<td>(381 GDD)†</td>
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<td>(601 GDD)</td>
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<td>(581 GDD)</td>
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*Targeted intervals between the second summer defoliation and the autumn defoliation expressed in growing degree days (GDD), basis 5°C.
† Actual intervals between the second summer defoliation and the autumn defoliation expressed in growing degree days (GDD), basis 5°C.
Approximately the first 20-cm of taproots and the remaining shoots were sampled in a randomly selected quadrate (0.09 m²) from each plot in 1997, 1998 and 1999 (Table 1). At each sampling date, roots were washed free of soil under a stream of cold water. Remaining shoots were removed, and crowns were separated from roots. The fresh weight (f. wt) of the entire roots was measured. Roots were then cut into small segments of 4–5 mm. A sub-sample of 5–10 g f. wt from each replicate was oven dried for 48 h at 55 °C to determine root DM concentration, and to calculate root dry weight. A sub-sample of 1 g f. wt from each replicate was kept frozen at −40 °C in methanol–chloroform–water (12 : 5 : 3; v/v/v) until soluble sugar and amino acid extractions were carried out (Dhont et al., 2003). A sub-sample of 5 g f. wt from each replicate was pooled into 20 g f. wt of root tissue. Approximately 2 g and 5 g f. wt of this pooled sample of roots were stored at −80 °C for soluble protein analysis and RNA extraction, respectively. Due to high mortality at Normandin, no biochemical determinations were made in spring 1999 in plants defoliated three times. Data for starch, sucrose and the raffinose oligosaccharide family (RFO) were previously reported by Dhont et al. (2004).

Proline, arginine and histidine determinations

Total amino acids were extracted in 15 mL of methanol–chloroform–water (12 : 5 : 3; v/v/v) and analysed with the aqueous phase resulting from phase separation with water and chloroform as previously described (Dhont et al., 2003). Arginine and histidine were separated and quantified by HPLC, while proline was analysed by spectrophotometry (Dhont et al., 2003). Amounts of amino acids per plant (mg plant⁻¹) were obtained by multiplying their concentration by the total root dry weight per plant (Dhont et al., 2004).

Soluble protein and VSP analysis

Soluble proteins were extracted and analysed by SDS–PAGE according to the method described by Dhont et al. (2003). Protein samples were load adjusted to a constant amount of 150 µg of protein per lane. The VSPs were subsequently analysed by Western blotting as follows. Electrophoretic transfers of proteins from SDS–PAGE gels onto PVDF membranes (Immobilon-P, Millipore, Billerica, MA, USA) were performed with a tank transfer system (TRANS-BLOT-CELL, Bio-Rad Laboratories Ltd, Mississauga, ON, Canada) under cooling conditions (circulating water coil placed into the tank) at 100 V for 1 h. The transfer buffer was adapted from Towbin et al. (1979) for PVDF membrane: 10 mM TRIS, 100 mM glycine, 0.005 % (w/v) SDS and 5 % (v/v) methanol. After electroblootbing, membranes were incubated for 90 min with a mix of primary antibodies (affinity-purified polyclonal anti-32-, anti-19- and anti-15-kDa protein (Volene et al., 1996) diluted in TRIS buffer saline (TBS) containing Tween-20 (TBS: 10 mM Tris, 150 mM NaCl, 0.15 % (v/v) Tween-20; dilutions 1 : 10,000, 1 : 10,000 and 2 : 10,000 for the anti-32, anti-19 and anti-15, respectively). Membranes were washed in TBST and TBS, and subsequently treated with the goat anti-rabbit secondary anti-body conjugated to alkaline phosphatase (Bio-Rad Laboratories Inc., Marne la Coquette, France; dilution 1/12 000 in TBST). Secondary antibody detection was carried out according to the protocol described in Blake et al. (1984). Optical densities of the VSP bands were subsequently measured on immunoblots using the One-Dscan Image Analysis System (Scanalytics Inc., Billerica, MA, USA). The poor quality of the 19-kDa transfer and hybridization did not allow a densitometry analysis of the band.

RNA extraction and northern analysis

Total RNA was extracted from roots sampled in autumn 1998 and spring 1999 at Pintendre, by the method previously described by De Vries et al. (1988) after reducing samples to a fine powder in liquid N₂ with a mortar and a pestle. Extracted RNA was then dissolved in TE buffer (10 mM TRIS–HCl, pH 7.4; 1 mM EDTA) and quantified by UV absorption at 260 nm. Total RNA was transferred (5 µg per well) by vacuum blotting to nylon membranes (Immobilon-Ny⁺, Millipore, Billerica, MA, USA) using 96-well BIO-DOT microfiltration apparatus (Bio-Rad Laboratories Inc., Mississauga, ON, Canada). The cDNA of two cold-inducible genes: msacCIA encoding a glycine-rich protein (Laberge et al., 1993) and msacCIG encoding a dehydrin-like protein (Wolfraim and Dhindsa, 1993), were radiolabelled by random priming (Rediprime™III, Random Prime Labelling System, Amersham Pharmacia Biotech, Baie d’Urfe, QC, Canada) using [γ-³²P]dCTP, and used as probes for hybridization at 68 °C in 2X SSC (0.6 M NaCl; 300 mM Na citrate) containing 0-25 % (w : v) low-fat powder milk (Carnation Inc., Toronto, ON, Canada). Membranes were washed free from probes with 2X SSC. Blots were then exposed to Kodak O-Mat XAR films for autoradiography at −80 °C. Dot blots were analysed with the One-Dscan Image Analysis System (Scanalytics Inc., Billerica, MA, USA).

Analysis of variance

The experiment at each site was a factorial combination of the two alfalfa cultivars and four defoliation treatments arranged in a randomized complete block design, with four replicates, for a total of 32 plots per site. Separate analyses of variance were done on data from each sampling date. A priori contrasts were used for comparison of defoliation means and cultivar × defoliation means at each site. Standard errors of the mean (s.e.m.) were calculated for each sampling date. Statistical significance was postulated at P < 0.05. Statistical analyses were performed by SAS statistical procedures (SAS Institute, 1996).

Principal component analysis (PCA)

To identify which organic reserve components or combination of components are more closely related to spring regrowth and to winter survival, PCA were performed on treatment means using the correlation matrix method to give equal weight to all variables (Genstat 5 Committee, 1993). The variables involved in the first set of PCA were concentrations and pools of organic reserves including proline, arginine, histidine, total soluble proteins, starch, sucrose,
RFO, total amino acids and root dry weight measured in autumn 1997, the plant density and the DM yield measured in spring 1998. The variables involved in the second set of PCA were concentrations and pools of organic reserves (as described above) and root dry weight measured in the autumn 1998, as well as the plant density and DM yield in spring 1999. Data for starch, sucrose, RFO, total amino acids, plant density, and DM yield were previously reported by Dhont et al. (2004). The PCA results are presented as plots of the loadings, i.e. distribution of the linear coefficients of the variables used to construct the scores in each dimension (Fig. 7A and C), and plots of the scores, i.e. distribution of experimental factors as linear combination of the variables in each dimension (Fig. 7B and D).

RESULTS

Proline, arginine and histidine

The amounts (mg plant\(^{-1}\)) of proline, arginine and histidine in alfalfa roots increased markedly with plant ageing from autumn 1997 to autumn 1998 (Figs 1–3). In November 1997 and April 1998, the amounts of proline in roots of plants defoliated with the shortest regrowth intervals of 400 or 500 GDD in the autumn of 1997 were significantly reduced at both sites and for both cultivars (Fig. 1). In April 1998, there was no significant difference in proline amounts between plants defoliated only twice and plants defoliated a third time at 600 GDD in the preceding autumn. At Pintendre, a repeated application of the defoliation treatments in the autumn of 1998 significantly decreased the proline amounts in November 1998 and April 1999, regardless of the regrowth intervals (Fig. 1A and B). A similar response was observed in Normandin (Fig. 1C and D), with the exception that proline amounts of ‘WL 225’ defoliated at 400 GDD did not significantly differ from those of plants defoliated only twice in the summer of 1998 (Fig. 1D). Plants that had been defoliated a third time in the autumn of 1998 at Normandin were almost completely killed off in the winter and no regrowth was observed in the spring of 1999.

The amounts of arginine were significantly reduced in November 1997 and April 1998 at both sites and for both cultivars defoliated in autumn 1997 at 400 or 500 GDD as
compared with plants defoliated only twice in the summer or a third time at 600 GDD (Fig. 2). In November 1998, amounts of arginine were decreased by a repeated application of the 400-GDD defoliation in the autumn of 1998; the 500- or 600-GDD defoliation treatments had no significant effect (Fig. 2). At Pintendre, autumn defoliation at 400, 500 or 600 GDD after the summer defoliation in 1998 significantly lowered arginine amounts in spring 1999 (Fig. 2A and B).

At Pintendre, the amounts of histidine at each sampling date were significantly reduced in plants of both cultivars that were defoliated at 400 GDD in the autumns of 1997 and 1998 (Fig. 2A and B). In contrast, amounts of this amino acid were not affected by autumn defoliation under the longest regrowth interval of 600 GDD. Plants defoliated at 500 GDD in the autumns of 1997 and 1998 had lower histidine amounts in the following springs. At Normandin, amounts of histidine measured in November 1997 and April 1998 were reduced in plants defoliated a third time in the autumn of 1997 regardless of the regrowth intervals (Fig. 3C and D). A repeated application of the autumn defoliation treatments under the longer regrowth intervals of 500 and 600 GDD in autumn 1998 did not significantly reduce the amounts of histidine assessed in November 1998 as compared with plants that were defoliated only twice (Fig. 3C and D).

Soluble protein profiles and VSPs

During both the sampling periods of 1997–98 (Fig. 4A) and 1998–99 (Fig. 4B), the soluble proteins of 32, 19 and 15 kDa were clearly the most abundant in roots of alfalfa. Hybridization with a mix of the three alfalfa VSP antibodies confirmed the hypothesis that these major proteins observed at 32, 19 and 15 kDa on SDS–PAGE are homologous to alfalfa VSPs previously identified (Fig. 4C). The defoliation treatments at 400, 500 or 600 GDD in autumn of 1997 markedly reduced the levels of the 19-kDa protein (Fig. 4A), and those of the 32- and 15-kDa VSPs (Fig. 5A and B) in late autumn (19 Nov. 97) and early
The accumulation of the 19-kDa protein (Fig. 4B), and that of the two VSPs of 32 and 15 kDa (Fig. 5C and D) were also strongly reduced in late autumn (19 Nov. 98) and spring (21 Apr. 99) following the repeated application of the 400- or 500-GDD defoliation treatments in autumn of 1998. Except in ‘AC Caribou’ in autumn 1997 and spring 1998, and in ‘WL 225’ in spring 1999, there was a noticeable tendency for a reduction of the 32- and 15-kDa VSP levels by a third defoliation taken at 600 GDD (Fig. 5A and B).

**Cold-inducible genes**

In ‘WL 225’, transcript levels of the cold-induced *msa* CIA genes in November 1998 were reduced by autumn defoliation, regardless of the regrowth interval. In contrast, *msa* CIA transcript levels in ‘AC Caribou’ were only slightly reduced in plants defoliated at 600 GDD when compared with those defoliated twice, and they were even higher in plants defoliated at 400 GDD (Fig. 6A). The expression of the dehydrin homologue *msa*CIG gene was not noticeably affected by autumn defoliation treatments (Fig. 6B), highlighting differential effects of autumn defoliation on genes associated with the cold acclimation process. As expected, levels of transcripts of these two genes markedly decreased in spring of 1999 as a result of the deacclimation process (Fig. 6A and B).

**Relationship between organic reserve components, spring regrowth and plant density**

In the sampling period of 1997–98, the first component of the PCA explained 62.1% of the variance while the second component explained 15% (Fig. 7A). The first component indicates that the concentration of sucrose in autumn 1997 was positively related to plant density in the spring of 1998 (Fig. 7A). High plant density in spring 1998 and a high concentration of sucrose in autumn 1997 were associated with the 400- and 500-GDD defoliation treatments in autumn 1997 at Pintendre and to a lesser extent at
Normandin (Fig. 7B). In contrast, the DM yield in spring 1998 was positively related to concentrations and pools of histidine, total amino acids, arginine, total soluble proteins and starch in autumn 1997 (Fig. 7A); high values of those variates were associated with the two-defoliation and 600-GDD defoliation treatments at Pintendre and Normandin (Fig. 7B).

In the sampling period of 1998–99, the first PCA component explained 51.7% of the variance, while the second component explained 22.1%. The first component indicates that concentrations of sucrose and RFO in autumn 1998 were positively related to plant density in spring 1999 (Fig. 7C). High plant density in spring 1999 and high concentrations of sucrose and RFO in autumn 1998 were associated with the 400- and 500-GDD defoliation treatments at Pintendre and Normandin (Fig. 7B).

In both 1997–98 and 1998–99, the variation explained by the first component of the PCA was mainly attributable to autumn defoliation treatments, whereas the second component was primarily driven by site differences (Fig. 7B and D). Overall, when considering the effect of both defoliation treatments and sites, the PCA reveals a close relationship between plant density in spring and concentrations of sucrose and RFO in the previous autumn (Fig. 7A and C) whereas components of root N reserves were related to DM yield in the spring.
Field studies on the effects of autumn defoliation on N reserves in alfalfa taproots in relation to spring regrowth and winter survival are scarce. Dhont et al. (2004) reported recently that the spring regrowth of field-grown alfalfa was significantly reduced when alfalfa was defoliated in the previous autumn, particularly when the interval between the second summer defoliation and a third one in the autumn

**DISCUSSION**

**Fig. 5.** Relative optical densities of the 32- and 15-kDa VSPs as determined by Western blot analysis. For each sampling period of 1997–98 and 1998–99, relative optical densities were calculated by dividing the optical densities in the autumn and in the spring by the optical density of the two-defoliation treatment measured in the autumn. Relative optical densities are presented for Pintendre and for the two cultivars ('AC Caribou' and 'WL 225') defoliated either only twice during the summer (2 Defoliations) or three times with the third defoliation (3 Def.) taken in the autumn of 1997 and 1998 at 400, 500 or 600 GDD after the second summer defoliation.

**Fig. 6.** Expression of the cold-inducible genes *msaCIA* and *msaCIG*, in November 1998 and April 1999 at Pintendre, in roots of alfalfa cultivars 'AC Caribou' and 'WL 225' defoliated either only twice during the summer (2 Def.), or three times with the third defoliation taken in the autumns of 1997 and 1998 at either 400, 500, or 600 GDD after the second summer defoliation.
was less than 500 GDD. This reduction in spring regrowth was associated with a marked reduction in the amounts of carbohydrate and global N reserves (total amino acids and soluble proteins) in roots of overwintering alfalfa (Dhont et al., 2004). The present study gives a more detailed analysis of the N reserves including specific amino-N components and VSPs, as well as the expression of cold-inducible genes in taproots of overwintering alfalfa defoliated in the autumn. This work also assessed the link between changes in the levels (concentrations and amounts) of organic reserves in the autumn and the vigour of the regrowth and plant density in the following spring.

Free amino acids are extensively mobilized to sustain shoot regrowth after a defoliation and in the spring growth (Hendershot and Volenec, 1993a, b; Ourry et al., 1994). Even though asparagine is the single most abundant amino acid present in taproots of alfalfa during winter, combined amounts of proline, arginine and histidine accounted for as much as 30–40% of total amino acids in plants defoliated only twice (data not shown). They therefore constitute an important source of readily mobilizable N, and a reduction in the amounts of these three amino acids by...
an early autumn defoliation could have important consequences for spring regrowth of alfalfa. In a previous experiment under unheated greenhouse conditions, the amounts of these three amino acids in overwintering roots were significantly correlated with the regrowth potential of alfalfa (Dhont et al., 2003). In the present field study, large pools of amino acids, especially histidine, arginine and proline, measured in the autumn in plants defoliated twice or a third time at 600 GDD, were positively associated with a high DM yield in spring (Fig. 7). A role for arginine and histidine in N sequestration is particularly appealing if their low C:N ratio, which minimizes the requirements of C skeletons per unit of N stored, is considered. Kuznetsov et al. (1999) proposed that under unfavourable conditions, enhanced amidation (synthesis of arginine) could indeed be an economical system to salvage N.

This field study confirms that the three major soluble proteins of 32, 19 and 15 kDa observed on SDS–PAGE correspond to the alfalfa VSPs identified by Cunningham and Volenec (1996), which are preferentially mobilized during regrowth of alfalfa (Cunningham and Volenec, 1996; Li et al., 1996; Avice et al., 1997). As previously observed under unheated greenhouse conditions (Dhont et al., 2003), the abundance of these specific proteins was reduced in 2- or 3-year-old alfalfa stands by an early autumn defoliation at 400 or 500 GDD. This reduced accumulation of soluble proteins in plants defoliated early in the autumn (400 or 500 GDD) was related to a lower DM yield in the following spring (Fig. 7). Haagenson et al. (2003) also observed that a reduction in root soluble proteins and VSPs by a mid-October defoliation of field-grown alfalfa was associated with decreased spring vigour. The close association between amino acids, soluble proteins and DM yield supports the hypothesis that reduced accumulation of N reserves by autumn defoliation is a determinant factor of alfalfa productivity in the following year.

**N reserves and winter survival**

In a previous study conducted under natural hardening conditions in an unheated greenhouse, Dhont et al. (2003) reported that three specific amino acids (proline, arginine and histidine) strikingly accumulated in alfalfa taproots during the autumn. In this field study, the accumulation of proline, arginine and histidine in roots of field-grown plants was reduced by autumn defoliation, with the shorter intervals of 400 or 500 GDD causing the larger reductions (Figs 1–3), as previously reported under unheated greenhouse conditions by Dhont et al. (2003). After 600 GDD, this was not the case. This reduction could be expected to have consequences for winter survival of alfalfa since free histidine, along with proline and arginine, can play an osmoregulatory role in the plant response to stresses (Rai, 2002). Proline is a major compatible solute that accumulates in plants in response to abiotic stresses (Franco and Melo, 2000) and its accumulation has been related to cold acclimation in several plant species, including the perennial weeds Cichorium intybus and Taraxacum officinale (Cyt et al., 1990), Arabidopsis thaliana (Nanjo et al., 1999) and alfalfa (McKenzie et al., 1988). However, its role in the acquisition of cold tolerance remains unclear as the increase in freezing tolerance sometimes precedes its accumulation (Paquin, 1984; Wanner and Junttila, 1999). Kim et al. (2004) suggested an alternative role of proline for excess N detoxification in water-stressed plants of white clover (Trifolium repens). A possible association of arginine with the acquisition of cold tolerance has also been documented in many species, including pine (Pinus radiata) (Coker, 1991), bilberry (Vaccinium myrtillus) (Lähdesmaki et al., 1990), and white clover (Svenning et al., 1997). Arginine can act as a precursor in the biosynthesis of polyamines, a group of low molecular weight amines that have been involved in protection against many environmental stresses (Bouchereau et al., 1999). Contrary to expectations, the variations in the levels (concentrations and pools) of these three amino acids caused by autumn defoliation were not found to be closely associated with plant density in the following spring (Fig. 7). Although these results do not preclude the possibility that the accumulation of proline, arginine and histidine is part of a general adaptive response of plants to environmental stresses, they clearly suggest that the levels of these amino acids are more closely related to the vigour of spring regrowth than to winter survival.

On the other hand, the principal component analysis revealed a consistent relationship between the concentration of root cryoprotective sugars in late autumn and plant density in the following year. Accumulation of soluble sugars is a common acclimation response to low temperatures and these metabolites are thought to play important adaptive roles (Pearce, 1999). Although the close relationship between concentrations of RFO in overwintering crowns and taproots of alfalfa and freezing tolerance has been previously established using contrasting genetic material (Castonguay and Nadeau, 1998; Cunningham et al., 2003), this is the first report of such a relationship in alfalfa as a result of autumn defoliation under field conditions. Interestingly, however, the high plant density in spring, along with the high concentrations of sucrose and RFO the previous autumn, resulted from the more intensive autumn defoliation treatments (400 and 500 GDD). Autumn defoliation stress appears to favour the accumulation of cryoprotective sugars and the maintenance of a greater plant density, but this is done at the expense of the spring vigour of those plants as indicated by their lower DM yield in spring.

In addition to its effects on amino-N components, autumn defoliation has been shown to decrease the levels of soluble proteins and VSPs in roots of field-grown alfalfa. Reduction of these N-reserve components by a mid-October defoliation was shown to be associated with increased winter injury (Haagenson et al., 2003), supporting the hypothesis that VSPs play additional roles beyond their contribution as a source of N, including the plant defence against pathogen aggressions and tolerance to freezing temperatures (Avice et al., 2003). However, in this field study, the lack of association between total soluble proteins in late autumn and plant density in the following spring (Fig. 7) does not support the hypothesis of a significant contribution of VSPs to winter survival as affected by an early autumn defoliation.
Meuriot et al. (2004) recently reported that the 32-kDa VSP from alfalfa possesses high homology with class III chitinases, a group of pathogenesis-related proteins induced by leaf wounding and pathogen infection (Collinge et al., 1993). Observations on the root pathogen infection of plants used in this study revealed that the severity of Fusarium root and crown rot symptoms increased significantly with an early autumn defoliation at 400 or 500 GDD (Couture et al., 2002). Whether this is attributable to the reduced accumulation of VSPs with putative pathogenesis-related functions such as the 32-kDa chitinase homologue is a question that deserves further scrutiny.

Cold-inducible genes

Several alfalfa cold-inducible genes have been isolated and characterized in the last 10–15 years (Castonguay et al., 1997a). Little information, however, is available concerning the impact of alfalfa defoliation in the autumn on the expression of these cold-inducible genes. The present study assessed the effect of autumn defoliation on the expression of two alfalfa cold-inducible genes (msaCIA and msCIG). The effect of autumn defoliation on cold-inducible gene expression varied with cultivars and genes (Fig. 6). For instance, the levels of transcripts encoded by the msaCIA gene was strongly reduced by autumn defoliation in the cultivar ‘WL 225’ but were unaffected in the more winter-hardy cultivar ‘AC Caribou’. Two glycine-rich peptides homologous to MSACIA that were isolated from the roots of Capsella bursa-pastoris exhibited strong antimicrobial activity against Gram-negative bacteria and fungi (Park et al., 2000). If the peptide encoded by msaCIA has a similar role in alfalfa, this would provide further indications that autumn defoliation management could reduce the ability of some cultivars to withstand aggressions by pathogens during the winter. On the other hand, the expression of the dehydrin homologue msCIG was unaffected by cultivars and defoliation treatments. Haagenson et al. (2003) recently reported that the expression of the cold-inducible genes cas15 (homologue to msaCIB; Monroy et al., 1993), cas17 and cas18 (homologue to msCIG) was not affected by a mid-October defoliation in six alfalfa cultivars contrasting in their winter hardness and autumn dormancy. There were, however, a few exceptions, with at least two cultivars harbouring higher levels of cas15 transcripts when defoliated in October. The observations presented here, based only in one sampling period (1998–99), indicate that the expression of some cold-inducible genes may be affected by autumn defoliation, with potential consequences on the cold acclimation process. However, these molecular changes occurring in the autumn, particularly following defoliation, as well as their effect on winter survival are still poorly known and should be investigated further.

Conclusions

It is widely recognized that autumn accumulation of organic reserves in roots of alfalfa and other perennial crop species is important for spring regrowth and winter survival. In the present study, autumn defoliation treatments resulted in large variations in the accumulation of several components of root organic reserves. This is one of the first field studies to clearly demonstrate the respective roles of specific N and C reserves in spring regrowth and winter survival. These results confirm the important role of root N reserves, in the form of free amino acids and soluble proteins, for the spring regrowth of field-grown alfalfa. However, they do not indicate that those specific components of N reserves are involved in winter survival. The multivariate analysis also confirms previous reports of an important adaptive role for soluble sugars in the capacity of field-grown alfalfa to survive winters. These results further indicate that autumn defoliation could interfere with molecular changes associated with the hardening process, and thus affect winter survival.

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LITERATURE CITED


