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RESEARCH PAPER

VSP accumulation and cold-inducible gene expression during autumn hardening and overwintering of alfalfa*

Catherine Dhont[1], Yves Castonguay[2,†], Jean-Christophe Avice[3] and François-P. Chalifour[1]

[1] *Département de Phytologie, Université Laval, Québec, QC, Canada G1K 7P4*

[2] *Agriculture and Agri-Food Canada, 2560 Boulevard Hochelaga, Sainte-Foy, QC, Canada G1V 2J3*

[3] *Unité Mixte de Recherche INRA/Université de Caen Basse-Normandie, Écophysiologie Végétale Agronomie & Nutritions N, C, S (EVA), F-14032 Caen cedex, France*

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Abstract

This study describes the time-course of the accumulation of total soluble proteins (TSPs) and vegetative storage proteins (VSPs) and of the transcripts of cold-inducible (CI) and VSP-encoding genes in taproots of two alfalfa cultivars (AC Caribou and Europe) during their acclimation to natural autumn hardening and overwintering conditions in eastern Canada. The impact of a defoliation in September on these winter hardening-related changes was also assessed. Both concentrations and pools of VSPs increased significantly between early September and mid-October and remained unchanged thereafter, concomitantly with the disappearance of VSP-encoding transcripts. Other soluble protein constituents continued to increase later in the autumn and early winter and accounted for nearly 60% of taproot TSP pools in winter. As a result, VSP abundance relative to TSPs decreased markedly during the winter. The increase in the levels of CI transcripts was induced by lowering temperatures, and distinct patterns suggest differences in the regulation of their accumulation. RNA analyses revealed that the accumulation of VSP transcripts during the autumn precedes the accumulation of CI transcripts. Autumn defoliation interrupted the accumulation of both TSPs and VSPs during autumn hardening and repressed the transcript levels of two CI genes differentially between cultivars. The well-documented impact of autumn defoliation on the vigour of spring regrowth and long-term persistence of alfalfa could be related to its negative impact on the accumulation of VSPs and TSPs and on the expression of genes encoding CI proteins potentially involved in cold tolerance and pathogen resistance.

Key words: Alfalfa, autumn defoliation, chitinase, cold acclimation, cold-inducible genes, vegetative storage proteins.

Introduction

The accumulation of endogenous carbon (C) and nitrogen (N) reserves in taproots of the perennial forage legume alfalfa (*Medicago sativa* L.) during autumn acclimation is known to determine the vigour of the regrowth in the following spring (Gervais and Girard, 1987; Dhont *et al*., 2006) and to increase the plant capacity to withstand winter stresses (McKenzie *et al*., 1988; Volenec *et al*., 2002). It has been well documented that alfalfa regrowth is not only affected by the availability of C reserves but strongly depends on the translocation of organic N stored in vegetative organs to new growing leaves when N_2 fixation and mineral N uptake are limited (Kim *et al*., 1993*a*, *b*; Hendershot and Volenec, 1993*a*; Ourry *et al*., 1994). Compelling evidence indicates that root soluble proteins of 15, 19, 32, and 57 kDa, which exhibit characteristics typical of vegetative storage proteins (VSPs), are preferentially mobilized during alfalfa shoot growth in the spring or its regrowth after defoliation in the summer (Cunningham and Volenec, 1996; Avice *et al*., 1997; Justes *et al*., 2002). Furthermore, Dhont *et al*. (2003) recently concluded that the total amounts of N reserve

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† To whom correspondence should be addressed. E-mail: castonguayy@agr.gc.ca

Abbreviations: GDD, growing degree-days; C, carbon; CI, cold-inducible; N, nitrogen; PR, pathogenesis related; TSP, total soluble protein; VSP, vegetative storage protein.

components in roots are a better determinant of alfalfa shoot regrowth than their concentrations.

In addition to their involvement as N reserves in alfalfa shoot growth, alfalfa VSPs could play adaptive roles in plant tolerance against biotic and abiotic stresses (Avice *et al*., 2003). For instance, the alfalfa VSP of 32 kDa shares high homology with class III chitinases (Meuriot *et al*., 2004) that have been shown to possess antifreeze activity in other species (Griffith and Yaish, 2004). Moreover, enriched extracts of 32 kDa VSP exhibit *in vitro* chitinolytic activity (Meuriot *et al*., 2004), strongly suggesting a potential antifungal role. However, the alfalfa VSP of 57 kDa was found to be the translational product of a β-amylase gene with no demonstrable starch hydrolytic activity, suggesting a loss of original function (Gana *et al*., 1998). In addition, the progressive increase in total soluble protein (TSP) concentrations in roots of alfalfa from October to December (Hendershot and Volenec, 1993*b*; Li *et al*., 1996), as well as a higher abundance of VSPs in a winter hardy perennial than in the annual species of *Medicago* (Cunningham and Volenec, 1996), also suggest the potential involvement of soluble proteins in the cold-hardening process and long-term persistence of alfalfa. This link is further supported by the positive relationship observed between reduced winter injury and high TSP concentrations in roots of alfalfa selected for higher autumn dormancy (Cunningham *et al*., 1998, 2001).

Environmental and management factors are known to affect the accumulation of VSPs. Short-day (SD) exposure significantly increases the levels of alfalfa VSPs and promotes the accumulation of their encoding transcripts when compared with plants exposed to long days (LD) (Noquet *et al*., 2003). Experiments conducted under environmentally-controlled conditions revealed that although low temperature markedly increased TSP concentrations, there was no concomitant VSP accumulation in cold-treated taproots under both SD and LD conditions (Noquet *et al*., 2001). Preliminary assessments performed under naturally declining temperatures in autumn revealed that steady-state levels of the 32 kDa VSP transcripts are markedly reduced as compared to control plants kept at high temperature (Avice *et al*., 2003). Together, these results suggest that the accumulation of alfalfa VSP in the autumn is mainly induced by the reduction of the photoperiod rather than by low temperature. Moreover, the accumulation of VSPs is also significantly impeded by untimely defoliations in autumn, with a subsequent increased injury and reduced plant vigour in the following spring (Haagenson *et al*., 2003*a*; Dhont *et al*., 2004, 2006).

Understanding of the molecular bases of cold tolerance of alfalfa has been greatly improved by the isolation and characterization of several cold-inducible (CI) genes including *msa*CIA encoding a glycine-rich protein (Laberge *et al*., 1993), *msa*CIB encoding a putative nuclear targeted protein (Monroy *et al*., 1993), *msa*CID encoding a homologue to pathogenesis related (PR) proteins (Castonguay *et al*., 1997*a*), and *msa*CIG encoding a dehydrin-like protein (Wolfraim and Dhindsa, 1993). The accumulation of cold-inducible transcripts and CI gene products in alfalfa cultivars of contrasting winter hardiness has been, in some cases, related to the levels of freezing tolerance (Castonguay *et al*., 1997*b*; Ferullo *et al*., 1997). However, these studies essentially focused on changes occurring in the crowns of plants acclimated to cold under controlled conditions, and do not provide information on the changes in CI gene expression that take place in perennial root tissue under natural autumn acclimation. Moreover, the reduction by autumn defoliation of the transcript levels of the RootCar1 (homologue to *msa*CIB) and *msa*CIA genes recently observed in roots of alfalfa sampled in late fall (Haagenson *et al*., 2003*a*; Dhont *et al*., 2006) raises new questions with regard to the consequences of autumn defoliation management on the accumulation of CI gene products during the acclimation process.

The potential contribution of alfalfa TSPs and VSPs to spring regrowth, overwintering potential, and stress tolerance reinforces the relevance of assessing their accumulation during the winter hardening period. The hypotheses of the study were (i) root alfalfa VSPs accumulate markedly during autumn hardening, (ii) CI gene expression varies between alfalfa cultivars of contrasting winter hardiness, and (iii) autumn defoliation adversely affects soluble protein accumulation and molecular changes during autumn acclimation. Consequently, the objective of the study was to characterize the time-course of the accumulation of TSPs and VSPs and to analyse variations in the expression of CI- and VSP-encoding genes under natural declining temperatures and photoperiod in autumn and winter and in response to autumn defoliation, in roots of two alfalfa cultivars of distinct ecophysiological origins. The *in vitro* chitinase activity of the 32 kDa VSP was also assessed in plant extracts sampled throughout the overwintering period.

Materials and methods

Plant material

Establishment under controlled environment conditions: Two alfalfa (*Medicago sativa* L.) cultivars, AC Caribou and Europe, respectively recommended for growth in eastern Canada and France, were used in order to compare the responses of genetic material adapted to contrasting winter conditions. Plants were initially established under a controlled environment to ensure uniformity and to minimize uncontrolled sources of stress. Seeds were sown on 5 June 2003 in 20 cm diameter and 20 cm deep plastic pots filled with a mixture (10:3, v:v) of top soil/peat moss (Pro-mix BX, Premier Peat Moss, Rivière-du-Loup, QC, Canada) supplemented with a controlled release fertilizer (N: 17% w:w; P: 7.31% w:w; K: 14.1% w:w; 250 g/35 l; Muticote 4, Haifa Chemicals Ltd, Haifa Bay, Israel). Seedlings were thinned to 10 plants per pot on 16 June 2003, and inoculated with 100 ml per pot of an inoculum solution containing 0.765% w:v NaCl and 10% v:v of a *Sinorhizobium meliloti* solution (10^9 cells ml^{-1} each of Balzac and USDA 1002 strains) on 18 June 2003. The cell cultures were grown separately according to Prévost *et al*. (1987) on yeast extract mannitol (Vincent, 1970). Plants were grown for 9 weeks in

an environmentally controlled chamber set to the following conditions: photoperiod, 16 h; day-time temperature, 22 °C; night-time temperature 17 °C. Artificial lighting was provided by a mixture of high pressure sodium and metal halide 400 W lamps (PL Light Systems, Beamsville, ON, Canada) with photosynthetic photon flux density of 600–800 μmol photons m^{-2} s^{-1}. Plants were kept well-watered and fertilization was started on 18 June 2003 with the application twice a week of a nutrient solution without nitrogen containing 0.5 g l^{-1} monopotassium phosphate fertilizer (22.4% w:w P, 28.2% w:w K; Haifa Chemicals Ltd, Haifa Bay, Israel), 0.3 g l^{-1} chelated micronutrient mix (real concentrations: 7% Fe, 2% Mn, 0.4% Zn, 0.1% Cu, 1.3% B, 0.06% Mo; Plant Products Co. Ltd, Brampton, ON, Canada), and 9.5×10^{-4} g l^{-1} $CoCl_2$ (Sigma Chemicals Co., St Louis, MO, USA). Plants were defoliated twice during the summer on 14 July 2003 and on 7 August 2003 at 20% and 10% flower stage of development, respectively (Table 1).

Outdoor and unheated greenhouse conditions: Immediately after the second defoliation (7 August 2003), plants were transferred to an experimental field near Québec City (Latitude 46°49′15″ N; Longitude 71°12′00″ W; Elevation ≈45 m) and pots were buried in the soil. Plants were kept outdoors allowing shoot regrowth and autumn acclimation to occur under the natural irradiance and temperatures that prevail in late summer and early autumn in eastern Canada. On 4 November 2003, pots were dug out and transferred to an unheated greenhouse, where plants remained throughout the winter. The unheated greenhouse was continuously ventilated during the day in order to maintain the inside temperature similar to that of the outside. When the inside air temperature remained permanently below freezing, plants were covered with a layer of insulating fibreglass wool to simulate snow cover (3 December 2003). Air temperatures (outside and inside the unheated greenhouse) and soil temperatures in pots were continuously monitored and recorded from 7 August 2003 to mid-March 2004 (Fig. 1), as described in Castonguay *et al*. (1995). Recorded temperatures at the experimental site were comparable with Canadian climate normals recorded from 1971 to 2000 at the Jean Lesage International Airport of Québec City (latitude 46°48′ N; longitude 71°22′ W; elevation 74.40 m) available at http://www.climate.weatheroffice.ec.gc.ca. The first severe frost (<−5 °C) occurred on 19 October 2003, and induced visual damages to above-ground tissues. Photoperiod was obtained from an online sunset calculator from the National Research Council of Canada (http://www.hia-iha.nrc-cnrc.gc.ca/sunrise_e.html).

Table 1. *Timetable of plant management activities during the season 2003–2004*

Actual GDD indicate the actual growing degree-days (basis 5 °C) cumulated from the second summer defoliation.

	Dates	Actual GDD
Seeding/controlled conditions	5 June 2003	–
First summer defoliation/ controlled conditions	14 July 2003	–
Second summer defoliation/ controlled conditions	7 August 2003	–
Transfer to outdoor conditions	8 August 2003	0
Autumn defoliation treatment: 500 GDD	18 September 2003	545
Transfer to unheated greenhouse	4 November 2003	740
Root samplings	8 August 2003	0
	21 August 2003	226
	29 August 2003	316
	5 September 2003	389
	10 September 2003	437
	18 September 2003	545
	29 September 2003	657
	14 October 2003	728
	4 November 2003	740
	10 February 2004	–
	15 March 2004	–
Beginning of spring regrowth/ controlled conditions	15 March 2004	–
Shoot regrowth assessment	4 April 2004	–

Autumn defoliation treatments

Two defoliation treatments were applied on both cultivars in autumn 2003: no additional defoliation (the two-defoliation treatment), or a third defoliation (the three-defoliation treatment) at 500 growing degree days (GDD; basis 5 °C) cumulated after the second defoliation (i.e. on 18 September 2003; Table 1). The GDD were calculated by subtracting 5 °C from the average of daily maximum and minimum air temperatures (Bélanger *et al*., 1992). Cutting height was about 6 cm, and there was little leaf area remaining.

Root sampling

Samples were collected 11 times from the second defoliation on 7 August 2003 to 15 March 2004 (Table 1). At each sampling date, plants were washed free of soil under a stream of cold water. Shoots were removed and oven-dried for 48 h at 55 °C for shoot dry weight determination. Crowns (transition zone between shoots and roots) were separated from the roots. Roots were weighed fresh and subsequently cut into small segments (about 4–5 mm). A subsample of 5 g FW was oven-dried for 48 h at 55 °C for root dry weight determination. Another subsample of 1.5 g FW. from each replicate was freeze-dried, and ground to an ultra-fine powder (*c*. 100 μm) with a Retsch Mixer Mill MM-2 (Brinkmann Canada Ltd, Rexdale, ON, Canada) for protein analyses. A subsample of 1.5 g FW was frozen and reduced to a fine powder in liquid N_2 with a mortar and a pestle for RNA analysis.

Soluble protein analysis

Total soluble protein extraction was adapted from Meuriot *et al*. (2004) by suspending 150 mg of ground, freeze-dried root sample with 150 mg of polyvinyl polypyrrolidone in 3 ml of extraction buffer (100 mM sodium phosphate, pH 7) at 4 °C. After centrifugation at 3200 *g* for 20 min at 4 °C, supernatants were collected, and pellets were re-extracted with 3 ml of extraction buffer. Supernatants were pooled and centrifuged for 5 min at 1000 *g* at 4 °C. An aliquot of the resulting supernatants was used for TSP quantification by the method of Lowry *et al*. (1951) and VSP quantification by ELISA (see details below). Total amounts of TSPs and VSPs were obtained by multiplying concentration values by the total root dry weight per plant. For chitinase activity determination, another aliquot was adjusted to 0.015% (w:w) deoxycholic acid and incubated for 10 min at room temperature before TSP precipitation in a final concentration of 7.2% (v:v) trichloroacetic acid (Peterson, 1983). The precipitates were collected by centrifugation for 15 min at 12 000 *g* at 4 °C. Supernatants were discarded, pellets were washed with acetone, air-dried, and stored at −20 °C until chitinase assays.

VSP quantification by ELISA

Samples (TSP extracts) were diluted 100-fold in fixation buffer (100 mM bicarbonate, pH 9.6). A freeze-dried pellet containing VSPs of 32, 19, and 15 kDa previously obtained by Noquet *et al*. (2001) was solubilized in fixation buffer, quantified by the Lowry method (Lowry *et al*., 1951), and used for calibration of the ELISA assay (VSP concentrations ranking from 0.107 to 6.139 μg ml^{-1}). The ELISA procedure, adapted from Noquet *et al*. (2001), started by coating wells of microplates (Dynex technologies, Cergy-Pontoise,

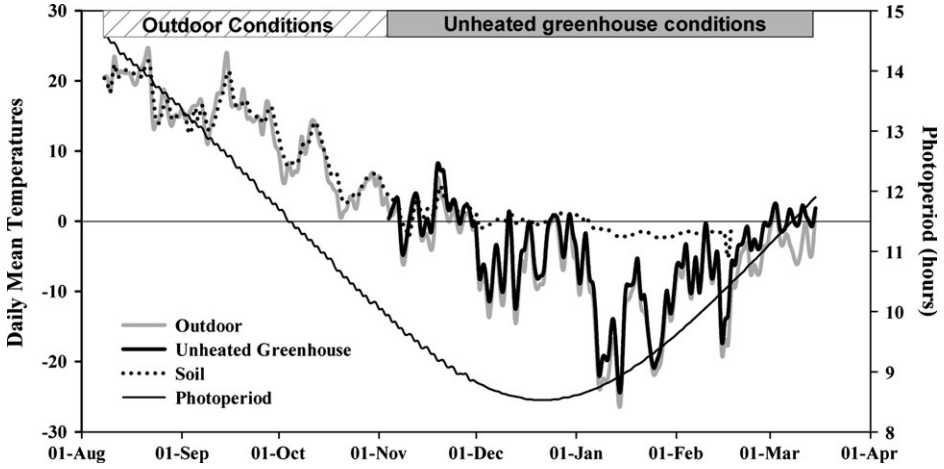

Fig. 1. Agroclimatic conditions of alfalfa growing under natural outdoor conditions and then transferred to an unheated greenhouse: daily average temperatures of the air outside (solid grey line; left axis) and inside the unheated greenhouse (solid black line; left axis), daily average temperatures of the soil in the pots (dotted black line; left axis), and natural photoperiod (fine black line; right axis), from August 2003 to mid-March 2004. When the inside air temperature remained permanently below freezing, plants were covered with a layer of insulating fibreglass wool to simulate snow cover (pot insulation on 3 December 2003).

France) with 50 µl of VSP standards or samples during an overnight incubation at 4 °C. Wells were subsequently washed with phosphate buffer saline (PBS; 150 mM NaCl, 16 mM Na_2PO_4, 8 mM $NaHPO_4$, pH 7.2) before drying at 37 °C. Samples were blocked by rinsing with PBS containing 0.3% gelatine and 0.2% Tween (PGT) buffer. Primary antibody incubation was allowed to proceed for 90 min with a mix of rabbit polyclonal anti-32, anti-19, and anti-15 kDa proteins (Volenec *et al*., 1996) diluted in PGT (1:1000, 1:1000, and 2:1000 for the anti-32, anti-19, and anti-15, respectively). Wells were washed with PGT, and subsequently treated with the goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories Inc., Marne la Coquette, France; dilution 1/1000 in PGT). The antigen–antibody complex was revealed by the addition of *p*-nitrophenyl-phosphate (PNPP; 1 mg ml^{-1} in diethanolamine buffer containing 97 ml l^{-1} diethanolamine and 1 mM $MgCl_2$). After staining for 30 min, absorbencies were read at 410 nm on a microplate reader (Dynex Technologies, Cergy-Pontoise, France). The proportion (ratio) of VSPs in alfalfa roots was determined by dividing root VSP concentrations by root TSP concentrations.

Chitinase activity of 32 kDa VSP

Samples (protein pellets) were solubilized in a buffer adapted from Laemmli (1970) containing 125 mM TRIS, 0.1% (v:v) SDS, 4% (v:v) glycerol, and 1% (w:v) bromophenol blue. Samples were loaded on 15% SDS–polyacrylamide gels containing 0.1% (w:v) glycol-chitin (Trudel and Asselin, 1989). After SDS–PAGE, gels were incubated for 2 h at 37 °C in 100 mM sodium acetate and 1% (v:v) Triton X-100, pH 5, for protein renaturation and chitin lysis. Lytic zones, corresponding to chitinase activity, were stained for 5 min at room temperature with 0.01% (w:v) calcofluor white M2R (Sigma, Aldrich) dissolved in 500 mM TRIS. Gels were washed for 2 h with ultra-pure water before the visualization of lytic zones as non-fluorescent dark bands under UV light.

RNA extraction and analysis

Total RNA was extracted from roots by the method previously described by De Vries *et al*. (1988). Extracted RNA was then dissolved in TRIS-EDTA buffer (TE; 10 mM TRIS–HCl, pH 7.4; 1 mM EDTA) and quantified by UV absorption at 260 nm. For dot blot analysis, total RNA from each replicate was transferred (5 µg per well) by vacuum blotting to nylon membranes (Immobilon-Ny^+, Millipore, Billerica, MA) using a 96-well BIO-DOT micro-filtration apparatus (Bio-Rad Laboratories Inc., Mississauga, ON, Canada). For northern analysis, 10 µg of pooled RNA from the three replicates was separated by electrophoresis on denaturing agarose gels before vacuum-transfer onto Immobilon-Ny^+ membranes. For both dot and northern blots, the cDNA of the CI genes *msa*CIA (Laberge *et al*., 1993), *msa*CIB (Monroy *et al*., 1993), *msa*CID (Castonguay *et al*., 1997*a*), and *msa*CIG (Wolfraim and Dhindsa, 1993), as well as the cDNA of chitinase and β-amylase gene homologues from an alfalfa EST collection (Dr S Laberge, AAFC), were radiolabelled by random priming (Rediprime™II, Random Prime Labelling System, Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada) using [α^{32}]P-dCTP, and used as probes for hybridization at 68 °C in 2× 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 2× SSC. Detection of blot radioactivity was carried out using a phosphorimager BAS-1000 (Fudji, Tokyo, Japan). The resulting optical densities were quantified using Array Gauge v1.2 (Fudji, Tokyo, Japan).

Alfalfa winter survival and spring regrowth

At the time of the last root sampling (15 March 2004), three pots of each cultivar×defoliation treatment combination were transferred to an environmentally controlled chamber for regrowth under the initial establishment conditions described above. After three weeks of regrowth on 6 April 2004, winter survival was determined as a percentage of live plants, and shoots were harvested and oven-dried at 55 °C until constant weight for the determination of spring regrowth.

Data analysis

The experiment was a factorial combination of two cultivars×two defoliation treatments×11 sampling dates, and was conducted using a completely randomized design with three replicates. The experimental unit was a pot containing ten plants. Analyses of variance and multiple comparisons (protected LSD) were performed on cultivar, defoliation treatment, sampling date main effects, and their corresponding first and second order interactions. To assess the relationship between environmental conditions and observed changes in proteins and gene expression, linear regressions were performed between photoperiod and temperature, and biochemical data gathered

from 29 August to 4 November 2003. Statistical significance was postulated at $P < 0.05$. Statistical analyses were performed by SAS statistical procedures (SAS Institute, 1999–2001).

Results

Environmental conditions during autumn acclimation and the overwintering period

Daily mean air temperatures progressively declined from 20 °C at the beginning of August, reaching 0 °C on 5 November, and a minimum of –25 °C in mid-January (Fig. 1; left axis). Air temperatures started to increase thereafter to reach approximately –3 °C by mid-March. Ventilation of the unheated greenhouse throughout the overwintering period was effective since the inside air temperature measurements were close to the ones recorded outdoors (Fig. 1; left axis). Soil temperatures closely followed outdoor temperatures until pot insulation with fibreglass wool (3 December), and then remained near freezing throughout the winter, with a mean value of –0.9 °C from December to March (Fig. 1; left axis). Daylength progressively declined from 14.6 h on 8 August to 8.3 h on 21 December. At the end of the experiment, photoperiod had increased to 11.9 h (Fig. 1; right axis).

Shoot height in the autumn, winter survival, and spring regrowth

In both cultivars, shoot height measured on 21 October was significantly reduced by a third defoliation taken on 18 September, compared with alfalfa defoliated only twice in the summer (Table 2). Regardless of the defoliation treatment, the European cultivar Europe had a greater shoot height in the autumn than the Canadian cultivar AC Caribou.

Winter survival was 100% in both cultivars defoliated only twice during the summer, and in AC Caribou defoliated a third time in the autumn at 500 GDD (Table 2). However, a slight but significant plant mortality was observed with cultivar Europe defoliated a third time in the autumn, as indicated by the 7% reduction of winter survival (Table 2). In both cultivars, spring regrowth was significantly reduced by 34% when a third defoliation was taken in the autumn on 18 September 2003, compared with alfalfa defoliated only twice in the summer (Table 2). The two cultivars did not differ with regard to their regrowth response to the defoliation treatments.

Evolution of TSP and VSP concentrations

Root concentrations of TSPs remained stable from early August to mid-September (Fig. 2A). In roots of plants defoliated only twice during the summer, average TSP concentrations increased from mid-September to mid-February, reaching a maximum of *c.* 45 mg g^{-1} DW. Cultivars differed in their initial response to the third defoliation at 500 GDD in the autumn, with TSP concentrations initially increasing in roots of Europe while they slightly declined in roots of AC Caribou (Fig. 2A). From mid-October to mid-March, root TSP concentrations remained significantly lower in plants of both cultivars defoliated in the autumn, compared with plants defoliated only twice in the summer, with AC Caribou showing the lowest values ($\approx$ 30 mg g^{-1} DW in mid-February) (Fig. 2A). By mid-March, these concentrations had started to decrease in Europe, while they remained unchanged in AC Caribou.

Root concentrations of VSPs significantly declined in Europe within the first 14 d of alfalfa shoot regrowth after the second summer defoliation, from 11.5 mg g^{-1} DW on 8 August to 7.6 mg g^{-1} DW on 21 August (Fig. 2B). Following VSP mobilization, root concentrations of VSPs markedly increased, reaching a maximum of 15 mg g^{-1} DW on 29 September. Changes in VSP concentrations from the end of August until the beginning of October were not significantly related to declining temperatures, but were negatively related to the declining photoperiod (Figs 1, 2B; r=0.74; P=0.003). The third defoliation at 500 GDD decreased VSP concentrations in mid-October by 15 and 10% in Europe and AC Caribou, respectively. Regardless of the autumn defoliation treatment, and in both cultivars, root VSP concentrations remained stable from early autumn until the end of the winter (Fig. 2B). These concentrations declined by mid-March in Europe defoliated three times.

During alfalfa regrowth in August, the relative contribution of VSPs to TSP concentrations (%VSP/TSP) significantly declined by 22% in both cultivars (Fig. 2C).

Table 2. *Shoot height in the autumn, winter survival, and spring regrowth of Europe and AC Caribou, defoliated either only twice during the summer (2nd defoliation on 7 August), or three times with the third defoliation occurring in the autumn 500 GDD after the second one (19 September)*

Values followed by the same letter are not significantly different at $P < 0.05$.

Cultivars	Defoliation treatments	Shoot height (21 October 2003) (cm)	Winter survival (6 April 2004) (%)	Spring regrowth (6 April 2004) (g $plant^{-1}$)
Europe	2 Defoliations	38 a	100 a	3.29 a
	3 Def. 500 GDD	19 c	93 b	2.21 b
AC Caribou	2 Defoliations	30 b	100 a	3.29 a
	3 Defoliation 500 GDD	10 d	100 a	2.12 b
	LSD	6	4	0.64

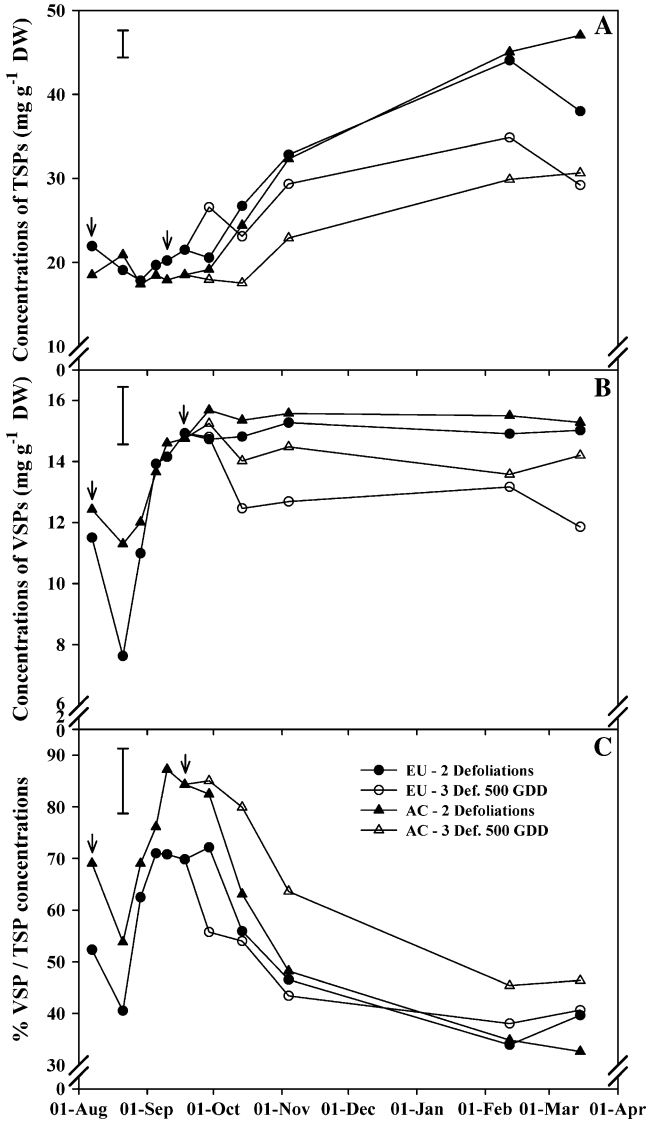

Fig. 2. Time-course analysis from August 2003 to March 2004 of the concentrations of TSPs (A) and VSPs (B), and the percentage of VSPs (C) in the roots of two alfalfa cultivars, Europe (circles) and AC Caribou (triangles), defoliated either twice during the summer (filled symbols), or three times with the third defoliation taken in the autumn at 500 GDD after the second summer defoliation (open symbols). Vertical arrows indicate the second and third defoliations, i.e. 7 August and 18 September 2003, respectively. Values are means of three replicates, and vertical bar indicates LSD.

Subsequently, the %VSP/TSP markedly increased from 21 August to 10 September, with VSPs representing 70–87% of TSPs in Europe and AC Caribou, respectively (Fig. 2C). From the end of August to early November, the decline in %VSP/TSP was positively related to the decrease of temperatures (Figs 1, 2C; r=0.81; P=0.011). The effect of an autumn defoliation at 500 GDD on %VSP/TSP was only transient and differed between cultivars. The %VSP/TSP was significantly lower on 29 September in plants of Europe defoliated in the autumn compared with plants left uncut and, conversely, was significantly higher in autumn-defoliated AC Caribou sampled on 14 October and 4 November (Fig. 2C). By mid-February and mid-March, there was no significant difference among cultivars and autumn defoliation treatments in the proportion of VSPs, which represented approximately 40% of TSPs (Fig. 2C).

Evolution of root dry weight, TSP, and VSP pools

Root dry weight markedly increased from 1.15 mg plant^{-1} on 29 August to 4 mg plant^{-1} on 4 November in plants defoliated only twice during the summer (Fig. 3A). The third defoliation at 500 GDD significantly reduced the root dry weight in both cultivars. Indeed, on 4 November, root dry weight of alfalfa defoliated in the autumn represented only 50% of the roots of plants defoliated twice (Fig. 3A). In both cultivars, and for both defoliation treatments, root DW remained relatively stable from early November to mid-March (Fig. 3A).

Pools of TSPs and VSPs (Fig. 3B, C) markedly accumulated in roots of both cultivars in autumn and winter. Whereas the deposition of VSPs in taproots occurred in late summer to early autumn with a rapid rate of accumulation in September, pools of TSPs continuously increased throughout the winter hardening period. Pools of VSPs increased rapidly from 8 mg plant^{-1} on 21 August, reaching a maximum of 50 and 63 mg plant^{-1} on 14 October in Europe and AC Caribou, respectively (Fig. 3C). By contrast, pools of TSPs in roots of alfalfa defoliated only twice during the summer, progressively increased from ≈15 mg plant^{-1} on 21 August to levels as high as 160 and 200 mg plant^{-1} on 10 February in Europe and AC Caribou, respectively (Fig. 3B). At the end of the overwintering period, roots of AC Caribou defoliated twice had significantly higher pools of TSPs than Europe (Fig. 3B). In both cultivars, a third defoliation in the autumn abruptly interrupted the accumulation of TSPs and VSPs in taproots (Fig. 3B, C). On 4 November, the average pools of TSPs and VSPs for both cultivars were respectively reduced by 60% and 45% in plants defoliated on September 18 compared with plants defoliated only twice in the summer.

Transcript levels of VSP-encoding and cold-inducible genes

The time-course of the accumulation of transcripts encoding homologues to VSPs and CI genes in alfalfa provided relevant information on the regulation of the accumulation of soluble proteins in overwintering taproots. Chitinase and β-amylase transcripts showed two major peaks of accumulation between August 21 and November 4 (Fig. 4A, B), with chitinase transcripts being significantly higher in Europe than in AC Caribou (Fig. 4A). High levels of β-amylase transcripts were positively correlated with the reduction in the duration of the daylength at the end of summer (Figs 1, 4B; r=0.77; P=0.001) while the disappearance of their transcripts in October was significantly

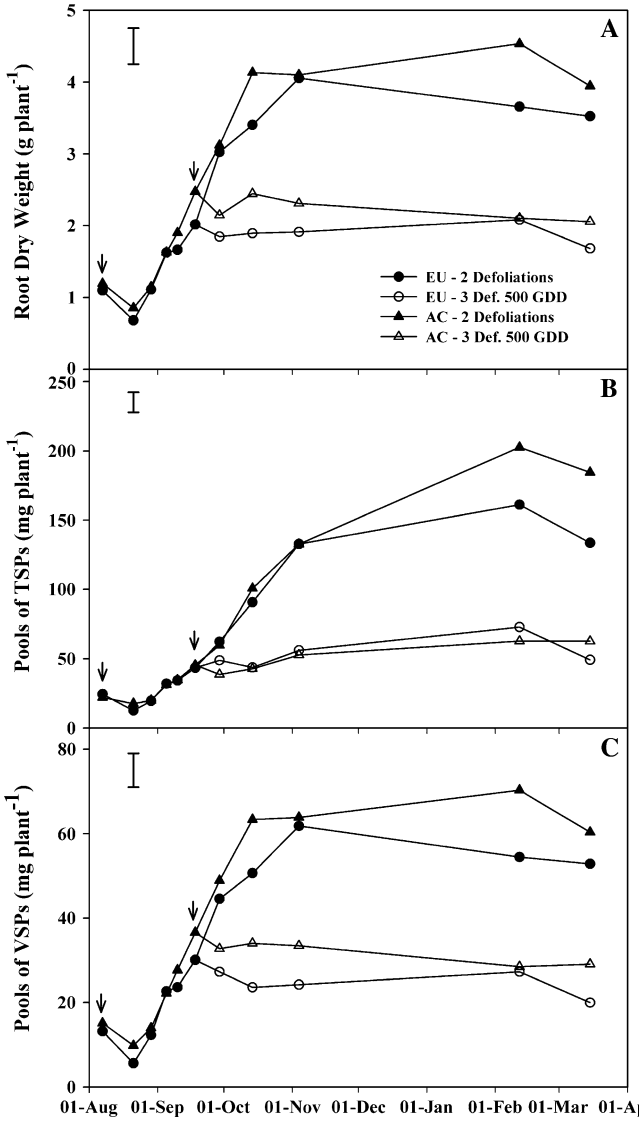

Fig. 3. Time-course analysis from August 2003 to March 2004 of the total root dry weight (A), the total amounts of TSPs (B) and VSPs (C) in the roots of two alfalfa cultivars, Europe (circles) and AC Caribou (triangles), defoliated either twice during the summer (filled symbols), or three times with the third defoliation taken in the autumn at 500 GDD after the second summer defoliation (open symbols). Vertical arrows indicate the second and third defoliations, i.e. 7 August and 18 September 2003, respectively. Values are means of three replicates, and vertical bar indicates LSD.

correlated with rapidly declining temperatures during that period (Figs 1, 4B; r=0.67; P=0.009). Autumn defoliation caused an almost complete disappearance of both chitinase and β-amylase transcripts in the period that immediately followed shoot removal (Fig. 4A, B). The levels of these two transcripts subsequently recovered without, however, reaching those measured in plants defoliated only twice in the summer. For both cultivars and both defoliation treatments, the levels of the chitinase and β-amylase transcripts dropped to baseline values on 4 November and remained low until the end of winter (Fig. 4A, B).

Although CI genes share a number of similarities in their induction during autumn hardening, there were major differences in the pattern of their transcript accumulation. With the exception of the *msa*CID gene, transcripts of the CI genes were almost completely absent from taproot extracts collected between early August and the end of September (Fig. 4C–F). Cold-inducible gene transcripts rapidly increased in early October at the time when temperatures started to fall below 10 °C (Fig. 1) and they reached a maximum level on 4 November for the *msa*CIB, *msa*CID, and *msa*CIG gene while *msa*CIA transcripts peaked later in mid-February. The accumulation of CI transcripts in October was significantly correlated with rapidly declining temperatures during that period (Figs 1, 4F; r=0.95; P <0.001; *msa*CIG). Except for the *msa*CID gene, all CI transcripts had returned to baseline or low levels in plants sampled in mid-March. The pattern of accumulation of the *msa*CID transcripts noticeably differed from that of the other CI genes. At the time of the second summer defoliation on 7 August, measurable amounts of *msa*CID transcripts were present in taproots of alfalfa and were significantly higher in extracts of Europe than in AC Caribou (Fig. 4C). In late summer (21 August to 29 September), *msa*CID transcripts followed a pattern very similar to that of the VSP-encoding genes (Fig. 4A, B). Later on, variations in *msa*CID transcipts departed significantly from those of the chitinase and β-amylase genes showing a response typical of CI genes with a significant increase in the period from 29 September to 4 November (Fig. 4A–C).

There were significant differences in the accumulation of the various CI transcripts between cultivars and in response to defoliation treatments. In plants defoliated only twice in the summer, the maximum level of *msa*CIA, *msa*CID, and *msa*CIG transcripts was significantly higher in AC Caribou than in Europe while transcripts of *msa*CIB were significantly higher in Europe than in AC Caribou (Fig. 4C–F). Compared with plants subjected to only two defoliations in the summer, an additional defoliation in the autumn markedly reduced the levels of the *msa*CIA transcripts in AC Caribou with a 75% reduction in the level of its transcripts on 10 February (Fig. 4D). Similarly, autumn defoliation significantly reduced the transcript levels of *msa*CIB in roots of Europe, but did not affect its levels in AC Caribou (Fig. 4E). In roots of both cultivars, the autumn accumulation of transcripts of the dehydrin-homologue *msa*CIG was not significantly affected by a third defoliation (Fig. 4F).

Northern blot analysis of chitinase gene and chitinase activity

Northern blot analysis confirmed that, at the time of defoliation, the level of chitinase transcripts was higher in roots of Europe than in those of AC Caribou (Fig. 5A). On 29 September, chitinase transcripts were no longer detectable in roots of both cultivars defoliated in the autumn compared with alfalfa defoliated only twice (Fig. 5A). In

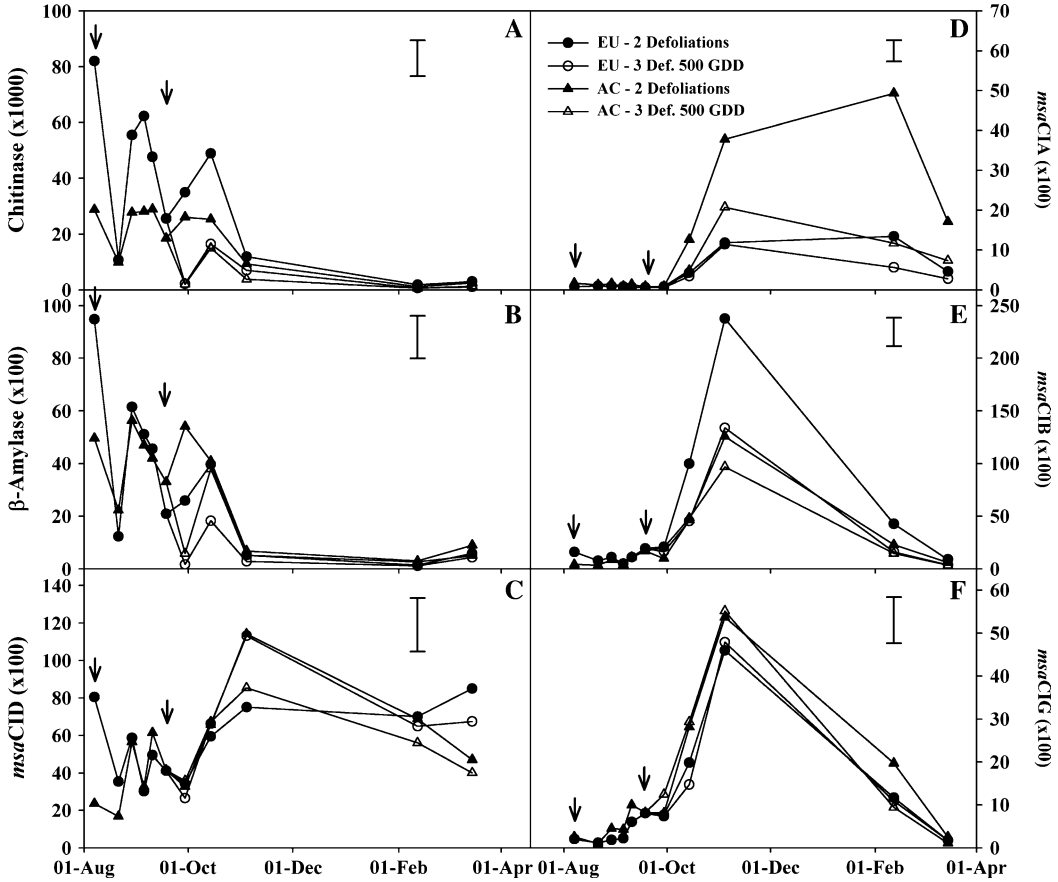

Fig. 4. Time-course analysis from August 2003 to March 2004 of the relative expression of the chitinase (A), β-amylase (B), *msa*CID (C), *msa*CIA (D), *msa*CIB (E), and *msa*CIG (F) genes in roots of two alfalfa cultivars, Europe (circles) and AC Caribou (triangles), defoliated either twice during the summer (filled symbols), or three times with the third defoliation taken in the autumn at 500 GDD after the second summer defoliation (open symbols). Vertical arrows indicate the second and third defoliations, i.e. 7 August and 18 September 2003, respectively. Values are means of three independent replicates from dot blot analysis and vertical bar indicates LSD.

both cultivars, the encoded chitinase polypeptide of 32 kDa in alfalfa showed significant chitinolytic activity *in vitro* on a glycol-chitin gel (Fig. 5C). The chitinolytic activity of 32 kDa VSP did not appear to be substantially reduced by a third defoliation at 500 GDD (Fig. 5C).

Discussion

This study, performed under hardening conditions in the field followed by the overwintering of plants exposed to natural variations of air temperatures in an unheated greenhouse during winter, provided detailed information on the evolution and regulation of soluble protein (TSPs and VSPs) accumulation in taproots of perennial alfalfa. Variation in the levels of these N reserve components were assessed in two cultivars of contrasting origins (North America versus Europe) and in response to autumn defoliation, in an effort to highlight the adaptive value of proteins that accumulate in overwintering taproots with regard to the productivity and persistence of alfalfa.

Cultivars responses to autumn hardening and autumn cutting

Autumn dormancy of alfalfa is a trait closely related to adaptation to harsh winter conditions in northern climates (Cunningham *et al*., 1998, 2001). Autumn dormancy ranking is based on the evaluation of shoot growth in autumn with autumn dormant cultivars typically exhibiting reduced shoot elongation and superior winterhardiness than non-dormant cultivars (Schwab *et al*., 1996; Haagenson *et al*., 2003*b*). The difference observed in autumn shoot height between AC Caribou and Europe (Table 2) clearly indicates that these cultivars differed in their autumn dormancy response. The lesser autumn dormancy of Europe was associated with a slight but significant reduction in the winter survival of plants that were defoliated in autumn. This is in agreement with Haagenson *et al*. (2003*a*) who reported that shoot removal in the autumn increases winter injury and reduces regrowth vigour of field-grown plants in mid-west USA. The absence of a defoliation stress could help to explain the observed lack of winter damage for plants of both AC Caribou and Europe defoliated only

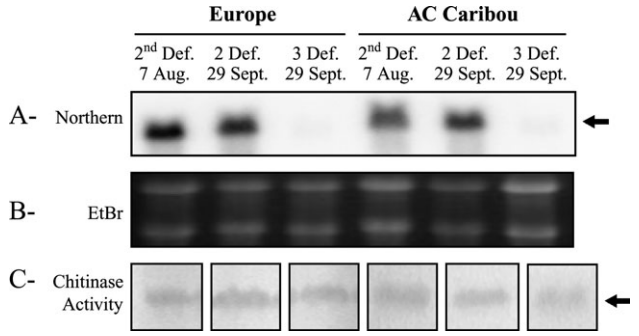

Fig. 5. Northern blot analysis of a class III chitinase gene homologue (A), ethidium bromide-stained gel (B), and chitinase activity on glycol-chitin gel (C), on 7 August (2nd defoliation) and 29 September 2003, in roots of two alfalfa cultivars, Europe and AC Caribou, defoliated either twice during the summer (2 Def.), or three times with the third defoliation taken in the autumn at 500 GDD after the second summer defoliation (3 Def.). The second (2nd Def.) and third defoliations occurred on 7 August and 18 September 2003, respectively.

twice. Maximum winter survival was also likely to be attributable to the optimal overwintering conditions to which plants were exposed. Factors like taproot exposure to extreme subfreezing temperatures ($\leqslant -15$ °C), high soil moisture and ice sheeting that are known to be conducive to winter damages in alfalfa (Bélanger *et al*., 2006) did not prevail in the current study. These results suggest that cultivars with less autumn dormancy could theoretically survive under harsh winter climates if no untimely defoliation is applied in autumn and adequate snow insulation prevents exposure to killing temperatures. Although spring regrowth was, in accordance with previous reports (Dhont *et al*., 2002, 2004; Haagenson *et al*., 2003*a*), significantly reduced by autumn defoliation, the response did not differ between the two cultivars. Moreover, the significantly higher pools of TSPs and VSPs observed in taproots of the cultivar AC Caribou compared with the cultivar Europe are in agreement with reports by Cunningham *et al*. (1998, 2001) and Haagenson *et al*. (2003*a*) that TSP concentrations are higher in populations of alfalfa having superior autumn dormancy. It is noteworthy that Europe defoliated in the autumn at 500 GDD presented both the lowest VSP pools values and the only significant winter damage.

Contrasting time-course of TSP and VSP accumulation in overwintering alfalfa

The adaptive value of soluble proteins, including VSPs, with regard to winter survival and the vigour of spring regrowth has often been proposed, based on their marked accumulation during autumn hardening and their induction by environmental changes associated with the cold acclimation process and autumn dormancy (reviewed by Avice *et al*., 2003). Major changes in the pool of translatable RNAs in poplar (*Populus* sp.) with concomitant accumulation of a 32 kDa VSP (Langheinrich and Tichner, 1991; Coleman *et al*., 1992; Van Cleve and Apel, 1993) was shown to occur in plants exposed to short photoperiods and low temperatures. A 17.3 kDa VSP from white clover (*Trifolium repens*) possesses characteristics common to stress-responsive proteins and its accumulation in roots and stolons was enhanced by exposure to low temperatures (Bouchart *et al*., 1998; Goulas *et al*., 2001). This study indicates that the marked accumulation of alfalfa VSPs in late summer was attributable to increases in both VSP concentrations and total root dry weight. This occurred at a time when daylength is rapidly changing in the context of relatively stable temperatures. The accumulation of VSPs stopped early in the autumn concomitant with a drop of the temperatures below the 10 °C threshold. These observations made with plants exposed to natural hardening conditions are in agreement with the results of Noquet *et al*. (2001) obtained under environmentally-controlled conditions. These authors showed that the levels of β-amylase transcripts (57 kDa VSP in alfalfa) and the accumulation of the encoded protein in roots of alfalfa was increased under short days and repressed by low temperatures (5 °C). The rapid disappearance of the chitinase (32 kDa VSP) and β-amylase transcripts in November, observed in the current study, brings further evidence that the expression of VSP-encoding genes in taproots of alfalfa is repressed by low temperatures with the consequential arrest of their synthesis. It was concluded that VSPs that are present in taproots of overwintering alfalfa are being deposited in late summer in response to the reduction in the photoperiod before air temperature had started to decline to values known to trigger the cold-hardening process (5–10 °C).

This study highlighted a marked and continuous increase in TSPs during the winter hardening period. A large accumulation of TSPs ($\approx$100 mg plant^{-1}) between the end of September and mid-February occurred after VSP synthesis had stopped. This confirms Noquet *et al*.'s (2001) observations that TSP concentrations increase without concomitant changes in VSPs in roots of growth chamber-grown alfalfa maintained at 5 °C. As a result, the proportion of VSPs relative to TSPs (Fig. 2C; %VSP/TSP) markedly declined from a maximum ratio near 90% in AC Caribou and 70% in Europe at the end of the summer, to a minimum that was, in some cases, below 40% in mid-winter. The marked decrease in the %VSP/TSP ratio during autumn hardening could be related to the combined repression of VSP-encoding genes in early autumn (Fig. 4A, B) and the later induction of CI genes by low temperature (Fig. 4C, D, E, F). Castonguay *et al*. (1993, 1997*b*) observed a marked accumulation of CI translation products in crowns of alfalfa cold acclimated under both environmentally-controlled and simulated winter conditions in an unheated greenhouse.

Alfalfa VSPs were shown to exhibit a typical cycle of mobilization/accumulation when shoot growth resumes after summer defoliation and to act as a key N store to sustain vegetative growth in periods when N acquisition is

limited (Hendershot and Volenec, 1993*b*; Kim *et al*., 1993*a*, *b*; Avice *et al*., 1997). The analysis of the seasonal fluctuation in the concentrations of soluble proteins in taproots of alfalfa corroborates previous observations by Hendershot and Volenec (1993*a*) of a continuous increase in TSPs throughout the winter-hardening period followed by full mobilization during spring regrowth. The rapidly declining VSP/TSP ratio in autumn (Fig. 2C) at the time of active deposition of TSPs in taproots strongly suggest that soluble proteins, other than known VSPs constitute an important source of N reserves and could contribute to an extent even larger than VSPs to the vigour of spring regrowth. In that perspective, it can be hypothesized that CI gene products that accumulate in the autumn and winter could play, in addition to their postulated protective roles against winter stresses, an important role in the productivity of alfalfa. Interestingly, Rinne *et al*. (1999) recently proposed that cold-induced dehydrins that accumulate in cold-acclimated birch (*Betula pubescens*), could play a significant role in rescuing metabolic processes especially during the early mobilization of stored proteins.

Differential expression of VSP-encoding and cold-inducible genes during the hardening period

The levels of expression of VSP-encoding and CI genes were characterized in taproots during the cold acclimation of alfalfa under natural outdoor conditions. Distinct responses to changing environmental conditions in autumn were observed between VSP-encoding and CI genes. RNA analysis confirms that the accumulation of known VSPs in alfalfa occurs mainly in late summer at the time when their transcripts are abundant while their synthesis rapidly declines when air temperatures drop in October and their transcripts disappear. These observations confirm previous results obtained under environmentally-controlled conditions by Noquet *et al*. (2003) and Avice *et al.* (2003). These authors reported that the accumulation of transcripts encoding a 32 kDa VSP in alfalfa was promoted under short-day conditions, but was markedly repressed at low temperatures. The regulation of VSP accumulation in overwintering alfalfa differs from the observation by Bouchart *et al*. (1998) and Goulas *et al*. (2001) that a VSP that accumulates in stolons of white clover is induced at low temperature. This illustrates the variety in the types and the modes of regulation of VSPs found in herbaceous perennials and it underscores the difficulty in defining VSPs (Bewley, 2002).

As expected, the accumulation of CI transcripts rose markedly when temperatures drop from daily averages of *c*. 10 °C at the end of September to values as low as –2 °C in early November (Fig. 4A–D). These changes in transcript levels are compatible with what is known of the acquisition of cold tolerance in alfalfa that begins in the autumn when the mean air temperature drops below 10 °C and accelerates as it approaches 5 °C (Mckenzie *et al*., 1988). Accordingly, Castonguay *et al*. (1993) previously reported that acclimation of alfalfa for 2 weeks at 2 °C followed by 2 weeks at −2 °C was associated with the appearance of new translatable mRNAs in crowns that were not present in plants only acclimated at 2 °C. Significant CI genes induction in roots of both cultivars were observed, with similar levels of transcripts for the *msa*CID and *msa*CIG genes in AC Caribou and Europe (Fig. 4C, F). There were, however, clear differences in the levels of the *msa*CIA and *msa*CIB transcripts between the cultivars (Fig. 4D, E). Higher levels of *msa*CIA transcripts accumulated in the winter-hardy cultivar AC Caribou than in the cultivar Europe (Fig. 4D). Two glycine-rich peptides homologous to MSACIA isolated from roots of shepherd's purse (*Capsella bursa-pastoris*) exhibited strong *in vitro* antimicrobial and antifungal activities (Park *et al*., 2000). If the peptide encoded by *msa*CIA has a similar function in alfalfa, this could confer meaningful protection against pathogen aggression during the winter. The polypeptide encoded by *msa*CID also shares a high identity with ABR17 (Iturriaga *et al*., 1994), a pathogenesis-related (PR) protein belonging to the PR10 family of proteins (Castonguay *et al*., 1997*a*). It is noteworthy that the cultivar AC Caribou was exposed during its development, to a selection pressure of low temperature pathogens typically encountered in eastern Canada (Dr R Michaud, personal communication). Richard *et al. (*1982) noted that *Fusarium*-infected plants did not tolerate a freezing stress as well as healthy plants. In that perspective, the striking decline in the expression of *msa*CIA in plants of alfalfa defoliated in the autumn, previously noted by Dhont *et al*. (2006) and corroborated in the current study, as well as the significant reduction of *msa*CID transcripts in plants of AC Caribou defoliated in the autumn, could have important consequences with regard to the overwintering potential of these plants. Couture *et al*. (2002) recently reported that defoliation in autumn increased the severity of *Fusarium* root rot in two cultivars of alfalfa including AC Caribou. Transcripts of root *car*1, a homologue to *msa*CIB, were shown to be associated with alfalfa winter survival (Cunningham *et al*., 2001), and to accumulate to higher levels in roots of alfalfa selected for higher autumn dormancy (Haagenson *et al*., 2003*b*). Contrary to expectations the levels of *msa*CIB transcripts were higher in the less autumn dormant Europe than in the winter-hardy AC Caribou (Fig. 4E). This underscores our limited knowledge of the functional roles of many COR genes (Pearce, 1999, 2004) and the importance of pursuing physiological and genetic studies to unravel their contribution to long-term persistence of perennial plants.

Chitinolytic activity of the alfalfa 32 kDa VSP during cold acclimation

The alfalfa 32 kDa VSP shares 75% and 68% homology with class III chitinases of *Sesbania rostrata* and *Glycine*

max, respectively, and enriched extracts of this 32 kDa VSP was shown to possess chitinolytic activity *in vitro* (Meuriot *et al.*, 2004). The present study confirmed that the 32 kDa alfalfa VSP from crude protein extracts exhibits chitinolytic activity *in vitro*, in roots in both cultivars (Fig. 5C). Staswick *et al*. (2001) suggested that the primary role assigned to VSPs as determinant factors of plant productivity might not be their sole contribution. In some species, VSPs were found to retain enzymatic activities (De Wald *et al*., 1992) or to possess significant homologies with PR proteins (Goulas *et al*., 2001; Peumans *et al*., 2002; Richard-Molard *et al*., 2004). Interestingly, antimicrobial and antifungal activities have recently been demonstrated for ocatin, a 18 kDa VSP from the Andean tuber oca, which belongs to PR10 family (Flores *et al*., 2002). A similar PR role for the 32 kDa VSP of alfalfa would provide an intrinsic protection against pathogens to this perennial legume.

In winter rye (*Secale cereale*), the translation products of two genes encoding for class I and class II chitinases which accumulate late during cold acclimation were characterized as antifreeze proteins (Yeh *et al*., 2000; Yu and Griffith, 2001; Griffith and Yaish, 2004). Antifreeze proteins isolated from plants act to initiate and regulate the growth of ice crystals in the extracellular spaces (reviewed by Hoshino *et al*., 1999). In the present study, the expression of the VSP-chitinase gene in alfalfa roots was repressed at low temperatures (Fig. 4E), which does not meet the current knowledge of the cold induction of antifreeze proteins in plants (Hoshino *et al*., 1999). However, Yeh *et al*. (2000) proposed that winter rye chitinases could acquire their antifreeze activity via chemical changes at low temperatures promoting the binding of existing chitinases to ice crystals. The putative roles of the 32 kDa alfalfa VSP in conferring potential protection against pathogens and freezing stress certainly warrant further investigation.

Conclusions

Autumn hardening of alfalfa begins early in August with the accumulation of N reserves in the form of VSPs. This is followed by a significant increase in other soluble proteins that occurs later in November at the time when CI transcript levels increased. The hypothesis that CI proteins constitute, in addition to their putative protective roles against winter stresses, an important reservoir of N reserves for spring regrowth warrants further investigation. The significant impact of autumn defoliation on the accumulation of VSPs, TSPs and changes in gene expression that occur during autumn hardening are likely to have significant consequences on alfalfa productivity and its long-term persistence.

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References

Avice JC, Le Dily F, Goulas E *et al.* 2003. Vegetative storage proteins in overwintering storage organs of forage legumes, roles and regulation. *Canadian Journal of Botany* **81,** 1198–1212.

Avice JC, Ourry A, Lemaire G, Volenec JJ, Boucaud J. 1997. Root protein and vegetative storage protein are key organic nutrients for alfalfa shoot regrowth. *Crop Science* **37,** 1187–1193.

Bélanger G, Castonguay Y, Bertrand A, Dhont C, Rochette P, Couture L, Drapeau R, Mongrain D, Chalifour FP, Michaud R. 2006. Winter damage to perennial forage crops in Eastern Canada: causes, mitigation and prediction. *Canadian Journal of Plant Science* **86,** 33–47.

Bélanger G, Richards JE, McQueen RE. 1992. Effects of harvesting systems on yield, persistence, and nutritive value of alfalfa. *Canadian Journal of Plant Science* **72,** 793–799.

Bewley JD. 2002. Root storage proteins, with particular reference to taproots. *Canadian Journal of Botany* **80,** 321–329.

Bouchart V, Macduff JH, Ourry A, Svenning MM, Gay AP, Simon JC, Boucaud J. 1998. Seasonal pattern of accumulation and effects of low temperatures on storage compounds in *Trifolium repens. Physiologia Plantarum* **104,** 65–74.

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

Castonguay Y, Nadeau P, Lechaseur P, Chouinard L. 1995. Differential accumulation of carbohydrates in alfalfa cultivars of contrasting winterhardiness. *Crop Science* **35,** 509–516.

Castonguay Y, Nadeau P, Laberge S, Vézina LP. 1997*a.* Temperature and drought stress. In: McKersie BD, Brown DCW, eds. *Biotechnology and the improvement of forage legumes.* New York: CAB International, 175–202.

Castonguay Y, Nadeau P, Laberge S, Vézina LP. 1997*b.* Changes in gene expression in six alfalfa cultivars acclimated under winter hardening conditions. *Crop Science* **37,** 332–342.

Coleman GD, Chen THH, Fuchigami LH. 1992. Complementary DNA cloning of poplar bark storage protein and control of its expression by photoperiod. *Plant Physiology* **98,** 687–693.

Couture L, Dhont C, Chalifour FP, Drapeau R, Tremblay G, Castonguay Y, Bélanger G, Nadeau P. 2002. *Fusarium* root and crown rot in alfalfa subjected to autumn harvests. *Canadian Journal of Plant Science* **82,** 621–624.

Cunningham SM, Gana JA, Volenec JJ, Teuber LR. 2001. Winter hardiness, root physiology, and gene expression in successive fall dormancy selections from 'Mesilla' and 'CUF 101' alfalfa. *Crop Science* **41,** 1091–1098.

Cunningham SM, Volenec JJ. 1996. Purification and characterization of vegetative storage proteins from alfalfa (*Medicago sativa* L.) taproots. *Plant Physiology* **147,** 625–632.

Cunningham SM, Volenec JJ, Teuber LR. 1998. Plant survival and root and bud composition of alfalfa populations selected for contrasting fall dormancy. *Crop Science* **38,** 962–969.

De Wald DB, Mason HS, Mullet JE. 1992. The soybean vegetative storage proteins VSPα and VSPβ are acid phosphatases active on polyphosphates. *Journal of Biological Chemistry* **267,** 15958–15964.

De Vries S, Hoge H, Bisseling T. 1988. Isolation of total and polysomal RNA from plant tissues. *Plant Molecular Biology Manual* **B6,** 1–13.

Dhont C, Castonguay Y, Nadeau P, Bélanger G, Chalifour FP. 2002. Alfalfa root carbohydrates and regrowth potential in response to fall harvests. *Crop Science* **42,** 754–765.

Dhont C, Castonguay Y, Nadeau P, Bélanger G, Chalifour FP. 2003. Alfalfa root nitrogen reserves and regrowth potential in response to fall harvests. *Crop Science* **43,** 181–194.

Dhont C, Castonguay Y, Nadeau P, Bélanger G, Drapeau R, Chalifour FP. 2004. Untimely fall harvest affects dry matter yield and root organic reserves in field-grown alfalfa. *Crop Science* **44,** 144–157.

Dhont C, Castonguay Y, Nadeau P, Bélanger G, Drapeau R, Laberge S, Avice JC, Chalifour FP. 2006. Nitrogen reserves, spring regrowth and winter survival of field-grown alfalfa (*Medicago sativa*) defoliated in the autumn. *Annals of Botany* **97,** 109–120.

Ferullo JM, Vézina LP, Rail J, Laberge S, Nadeau P, Castonguay Y. 1997. Differential accumulation of two glycine-rich proteins during cold-acclimation alfalfa. *Plant Molecular Biology* **33,** 625–633.

Flores T, Alape-Giron A, Flores-Diaz M, Flores HE. 2002. Ocatin. A novel tuber storage protein from the Andean tuber crop Oca with antibacterial and antifungal activities. *Plant Physiology* **128,** 1291–1302.

Gana JA, Kalengamaliro NE, Cunningham SM, Volenec JJ. 1998. Expression of β-amylase from alfalfa taproots. *Plant Physiology* **118,** 1495–1505.

Gervais P, Girard M. 1987. Effects of height and frequency of cutting on the yield, persistence, chemical composition and food reserves of alfalfa. *Canadian Journal of Plant Science* **67,** 735–746.

Goulas E, Le Dily F, Teissedre L, Corbel G, Robin C, Ourry A. 2001. Vegetative storage proteins in white clover (*Trifolium repens*): quantitative and qualitative features. *Annals of Botany* **88,** 789–795.

Griffith M, Yaish MWF. 2004. Antifreeze proteins in overwintering plants, a tale of two activities. *Trends in Plant Science* **9,** 399–405.

Haagenson DM, Cunningham SM, Joern BC, Volenec JJ. 2003*a.* Autumn defoliation effects on alfalfa winter survival, root physiology, and gene expression. *Crop Science* **43,** 1340–1348.

Haagenson DM, Cunningham SM, Volenec JJ. 2003*b.* Root physiology of less dormant, winter hardy alfalfa selections. *Crop Science* **43,** 1441–1447.

Hendershot KL, Volenec JJ. 1993*a.* Nitrogen pools in taproots of *Medicago sativa* L. after defoliation. *Journal of Plant Physiology* **141,** 129–135.

Hendershot KL, Volenec JJ. 1993*b.* Taproot nitrogen accumulation and use in overwintering alfalfa. *Journal of Plant Physiology* **141,** 68–74.

Hoshino T, Odaira M, Yoshida M, Tsuda S. 1999. Physiological and biochemical significance of antifreeze substances in plants. *Journal of Plant Research* **112,** 255–261.

Iturriaga EA, Leech MJ, Barratt DHP, Wand TL. 1994. Two ABA-responsive proteins from pea (*Pisum sativum* L.) are closely related to intracellular pathogenesis-related proteins. *Plant Molecular Biology* **24,** 235–240.

Justes E, Thiébau P, Avice JC, Ourry A, Lemaire G, Volenec JJ. 2002. Influence of summer sowing dates, N fertilization and irrigation on autumn VSP accumulation and dynamics of spring regrowth in alfalfa (*Medicago sativa* L.). *Journal of Experimental Botany* **53,** 111–121.

Kim TH, Bigot J, Ourry A, Boucaud J. 1993*a.* Amino acid content in xylem sap of regrowing alfalfa (*Medicago sativa* L.): relations with N uptake, N_2 fixation and N remobilization. *Plant and Soil* **149,** 167–174.

Kim TH, Ourry A, Boucaud J, Lemaire G. 1993*b.* Partitioning of nitrogen derived from N_2 fixation and reserves in nodulated *Medicago sativa* L. during regrowth. *Journal of Experimental Botany* **44,** 555–562.

Laberge S, Castonguay Y, Vézina LP. 1993. New cold- and drought-regulated genes from *Medicago sativa. Plant Physiology* **101,** 1411–1412.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Langheinrich U, Tischner R. 1991. Vegetative storage proteins in poplar. Induction and characterization of a 32- and a 36-kilodalton polypeptide. *Plant Physiology* **97,** 1017–1025.

Li R, Volenec JJ, Joern BC, Cunningham SM. 1996. Seasonal changes in nonstructural carbohydrates, protein, and macronutrients in roots of alfalfa, red clover, sweetclover, and birdsfoot trefoil. *Crop Science* **36,** 617–623.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193,** 265–275.

McKenzie JS, Paquin R, Duke SH. 1988. Cold and heat tolerance. In: Hanson AA *et al.*, eds. *Alfalfa and alfalfa improvement.* ASA, CSSA and SSSA, Madison, WI., Agronomy Monographs **29,** 259–302.

Meuriot F, Noquet C, Avice JC, Volenec JJ, Cunningham SM, Sors TG, Caillot S, Ourry A. 2004. Methyl jasmonate alters N partitioning, N reserves accumulation and induces gene expression of 32 kDa vegetative storage protein that possesses chitinase activity in *Medicago sativa* taproots. *Physiologia Plantarum* **120,** 113–123.

Monroy AF, Castonguay Y, Laberge S, Sarhan F, Vézina LP, Dhinsda RS. 1993. A new cold-induced alfalfa gene is associated with enhanced hardening at subzero temperature. *Plant Physiology* **102,** 873–879.

Noquet C, Avice JC, Ourry A, Volenec JJ, Cunningham SM, Boucaud J. 2001. Effects of environmental factors and endogenous signals on N uptake, N partitioning, and taproot vegetative storage protein accumulation in *Medicago sativa. Australian Journal of Plant Physiology* **28,** 279–287.

Noquet C, Meuriot F, Caillot S, Avice JC, Ourry A, Cunningham SM, Volenec JJ. 2003. Short-day photoperiod induces changes in N uptake, N partitioning and accumulation of vegetative storage proteins in two *Medicago sativa* L. cultivars. *Functional Plant Biology* **30,** 853–863.

Ourry A, Kim TH, Boucaud J. 1994. Nitrogen reserve mobilization during regrowth of *Medicago sativa* L. (relationships between availability and regrowth yield). *Plant Physiology* **105,** 831–837.

Park CJ, Park CB, Hong SS, Lee HS, Lee SY, Kim SC. 2000. Characterization and cloning of two glycine- and histidine-rich antimicrobial peptides from the roots of shepherd's purse, *Capsella bursa-pastoris. Plant Molecular Biology* **44,** 187–197.

Pearce RS. 1999. Molecular analysis of cold acclimation. *Plant Growth Regulation* **29,** 47–76.

Pearce RS. 2004. Adaptation of higher plants to freezing. In: Fuller BJ, Lane N, Benson EE, eds. *Life in the frozen state.* Boca Raton, USA: CRC Press, 171–203.

Peterson GL. 1983. Determination of total protein. *Methods in Enzymology* **91,** 95–119.

Peumans WJ, Proost P, Swennen RL, Van Damme EJM. 2002. The abundant class III chitinase homolog in young developing banana fruits behaves as a transient vegetative storage protein and most probably serves as an important supply of amino acids for the synthesis of ripening-associated proteins. *Plant Physiology* **130,** 1063–1072.

Prévost D, Bordeleau LM, Antoun H. 1987. Symbiotic effectiveness of indigenous arctic rhizobia on a temperatre forage legume: sainfoin (*Onobrychis viciifolia*). *Plant and Soil* **104,** 63–69.

Richard C, Willemot C, Michaud R, Bernier-Cardou M, Gagnon C. 1982. Low-temperature interactions in *fusarium* wilt and root rot of alfalfa. *Phytopathology* **72,** 293–297.

Richard-Molard C, Brugière N, Moille M, Carrayol E, Limami A. 2004. Molecular characterization of a gene encoding a vegetative storage protein (CiVSP) from *Cichorium intybus* and its expression in the root and shoot in relation to nitrogen status and pathogen resistance. *Physiologia Plantarum* **121,** 568–577.

Rinne PLH, Kaikuranta PLM, van der Plas LHW, van der Schoot C. 1999. Dehydrins in cold –acclimated apices of birch (*Betula pubescens* Ehrh): production, localization and potential role in rescuing enzyme function during dehydration. *Planta* **209,** 377–388.

SAS Institute. 1999–2001. *The SAS system for Windows, Software release version 8.2.* Cary, NC, USA: SAS Institute.

Schwab PM, Barnes DK, Sheaffer CC. 1996. The relationship between field winter injury and fall growth score for 251 alfalfa cultivars. *Crop Science* **36,** 418–426.

Staswick JE, Zhanyuan Z, Clemente TE, Specht JE. 2001. Efficient down-regulation of the major vegetative storage protein genes in transgenic soybean does not compromise plant productivity. *Plant Physiology* **127,** 1819–1826.

Trudel J, Asselin A. 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. *Analytical Biochemistry* **178,** 362–366.

Van Cleve B, Apel K. 1993. Induction by nitrogen and low temperatures of storage-protein synthesis in poplar trees exposed to long days. *Planta* **189,** 157–160.

Vincent JM. 1970. *A manual for the practical study of root nodule bacteria.* In: Oxford, England: IBP Hanbook no 15. Blackwell Scientific Publications.

Volenec JJ, Cunningham SM, Haagenson DM, Berg WK, Joern BC, Wiersma DW. 2002. Physiological genetics of alfalfa improvement: past failures, future prospects. *Field and Crop Research* **75,** 97–110.

Volenec JJ, Ourry A, Joern BC. 1996. A role for nitrogen reserves in forage regrowth and stress tolerance. *Physiologia Plantarum* **97,** 185–193.

Wolfraim LA, Dhindsa RS. 1993. Cloning and sequencing of the cDNA for cas17, a cold acclimation-specific gene of alfalfa. *Plant Physiology* **103,** 667–668.

Yeh S, Moffat BA, Griffith M, *et al.* 2000. Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. *Plant Physiology* **124,** 1251–1263.

Yu XM, Griffith M. 2001. Winter rye antifreeze activity increases in response to cold and drought, but not abscisic acid. *Physiologia Plantarum* **112,** 78–86.