Influence of summer sowing dates, N fertilization and irrigation on autumn VSP accumulation and dynamics of spring regrowth in alfalfa (*Medicago sativa* L.)

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Abstract

Herbage yield of alfalfa (*Medicago sativa* L.) depends on forage management or environmental conditions that change C and N resource acquisition, and endogenous plants factors such as root organic reserves and number of active meristems. The aim of this work is to study the influence of two sowing dates in summer (12 July or 9 August), N fertilization (0 or 100 kg ha^{-1}) and/or irrigation applied during the first year of alfalfa establishment on (i) the accumulation of N organic reserves (soluble proteins and more specifically vegetative storage protein) in taproots during autumn, (ii) the number of crown axillary meristems present at the end of winter and (iii) the dynamics of spring shoot growth. Delaying the sowing date for one month reduced root growth and root N storage, especially vegetative storage proteins (VSP) during autumn. Irrespective of sowing dates, N fertilization did not affect root biomass, number of crown buds, total root N, root soluble protein or VSP concentrations. By contrast, water deficiency during alfalfa establishment in the early summer reduced both root growth and N reserve accumulation. When spring growth resumed, there is a significant linear relationship between leaf area development and soluble protein and VSP concentrations in taproots, and also the number of crown buds. The results showed that an early sowing date and adequate water status during the summer allowed alfalfa plants to accumulate N reserves by increasing taproot mass and soluble protein concentrations, especially VSPs. This resulted in rapid shoot regrowth rates the following spring.

Key words: Alfalfa, spring growth, sowing date, N fertilization, water, VSP.

Introduction

Herbage yield of perennial forage legumes such as alfalfa (*Medicago sativa* L.) depends on crop management (date of sowing, harvest frequency, N fertilization, irrigation, etc.) and environmental conditions (e.g. soil-N availability, water deficit, photoperiod, temperature, plant diseases). These factors induce large changes in resource acquisition and modify endogenous plant factors (root organic reserve levels, number of active meristems) that are required for rapid shoot growth in the spring or regrowth after defoliation. In the last decade numerous

studies have re-evaluated the broadly accepted view that total non-structural carbohydrates (TNC) in roots (mainly represented by starch) determined alfalfa regrowth potential in the spring or after defoliation (see Volenec *et al.*, 1996, for a review). Because large reductions in TNC content of roots occurs during regrowth in comparison to N reserves, TNC have long been considered as having a key role in shoot growth. Recent studies have shown, however, that C and N reserves may serve different purposes in shoot regrowth and plant survival after defoliation. The large decrease in root TNC reserves is primarily due to the use of carbohydrates in root and stubble respiration (Ta *et al.*, 1990; Avice *et al.*, 1996*a*). Using ^{13}C pulse-chase labelling of organic reserves in taproots, it was found that 61% of the stored ^{13}C was used for root respiration for 30 d of regrowth following defoliation, while only 5% was recovered in regrowing alfalfa shoots (Avice *et al.*, 1996*a*). In addition, a poor relationship between root TNC concentrations and shoot growth of perennial legumes has been reported in several studies. Using different cutting frequencies applied in November, Brink and Marten showed that the various root TNC levels did not explain the many differences in alfalfa yield in the spring (Brink and Marten, 1989). In addition, shoot regrowth rates after defoliation were not significantly different between alfalfa genotypes differing up to 4-fold in root starch concentrations (Fankhauser *et al.*, 1989; Habben and Volenec, 1990), and were not reduced in plants containing a very low level of starch (Boyce and Volenec, 1992; Ourry *et al.*, 1994).

Evidence from recent research on alfalfa has suggested that shoot regrowth after defoliation is more dependent on the availability of reserve N pools than C reserves (Kim *et al.*, 1993; Ourry *et al.*, 1994; Volenec *et al.*, 1996; Avice *et al.*, 1996*a*, 1997*b*). During a critical period of forage legume development such as spring growth or regrowth after defoliation, the acquisition of N by uptake (Kim *et al.*, 1993) or symbiotic fixation (Vance and Heichel, 1979) is very limited. Under these circumstances root N reserves became the major source of N mobilized for shoot regrowth. Several investigations using ^{15}N labelling methods during regrowth after cutting (Kim *et al.*, 1993; Ourry *et al.*, 1994; Barber *et al.*, 1996; Avice *et al.*, 1996*a*) indicated that taproot N reserves (mainly soluble proteins and amino acids) were mobilized and contributed significantly to the supply of N (and also C) needed to sustain early leaf regrowth. Studies on seasonal fluctuations of N and C reserves in taproots of alfalfa (Li *et al.*, 1996; Cunningham and Volenec, 1998) indicated that N concentrations increased markedly in autumn, and substantial reductions in root N occurred as shoot growth resumed in the early spring. Among the different pools of N, amino acids and soluble proteins significantly accumulated in autumn and were extensively mobilized

from taproots when meristem reactivation occurred in the spring (Hendershot and Volenec, 1993*a*; Li *et al.*, 1996). Similarly, shoot regrowth following forage harvest in the summer induced an extensive depletion of amino acids and soluble proteins pools from taproots (Hendershot and Volenec, 1993*b*).

Investigations on taproot soluble proteins initiated by Hendershot and Volenec (Hendershot and Volenec, 1993*a*, *b*) identified three specific polypeptides (32, 19, and 15 kDa) in alfalfa taproots that behaved in a manner consistent with criteria for vegetative storage proteins (VSP) (as defined by Cyr and Bewley, 1990). These polypeptides represented a large proportion of the water-soluble protein pool and exhibited a cyclic pattern of accumulation/mobilization which was induced by the spring regrowth (Hendershot and Volenec, 1993*a*, Li *et al.*, 1996) or by shoot removal (Hendershot and Volenec, 1993*b*; Avice *et al.*, 1996*b*) in summer. By studying alfalfa plants having different hierarchical positions for light within a dense canopy, Avice *et al.* showed that shoot regrowth was linearly related to taproot soluble protein and VSP contents on the day of defoliation (Avice *et al.*, 1997*b*). By contrast, no relationship was detected between shoot regrowth and initial starch or root total N concentrations. These results suggested that root protein, and more specifically VSP, are key organic nutrients for alfalfa shoot regrowth after harvest.

The importance of root N reserves (and more specifically VSP) in shoot regrowth in spring has been evaluated in field experiments conducted using established stands (Lemaire *et al.*, 1992, Li *et al.*, 1996; Avice *et al.* 1997*a*, *b*; Cunningham and Volenec, 1998). Little is known regarding the effects of varied management during alfalfa establishment on the accumulation of N pools and their consequences on the dynamics of spring shoot growth the following year (Bélanger and Richards, 2000). In the French region of Champagne, alfalfa is traditionally cultivated for 2 to 3 years in arable farming systems (in rotation with cereals and sugar beet) and dehydrated by factories to produce high-quality feed. As a consequence, all alfalfa fields in this region are sown after cereals harvested in summer.

The objective of this study was to determine the effects of two different dates of sowing in summer, the impact of N fertilization and irrigation applied during establishment on the shoot regrowth rates the following spring. The impact of these management factors on the accumulation of root organic reserves (total N, soluble protein and vegetative storage proteins), on the number of potential active meristems (crown axillary buds) present at the end of winter season, and their influence on shoot regrowth dynamics (leaf area index, shoot dry matter production and radiation use efficiency) in spring was investigated.

Materials and methods

Plant material, culture and treatments

Effects of variation of sowing dates, N fertilization and irrigation on *Medicago sativa* L. (cv. Alegro) were monitored in 1995. Experiments were conducted on a typical chalky soil (calcareous rendosol) at Châlons en Champagne in France (48°57' N and 2°25' E). The soil consisted of a rendzina 28 cm in thickness, overlying a horizon (28–60 cm) that has been altered by cryoturbation and was situated on a gelifracted chalk layer (60–110 cm). The Ap soil characteristics were: $CaCO_3$ 72.3%, clay 11.1%, silt 13.2%, sand 2.3%, organic C 1.75%, organic N 0.19%, and pH 8.3. Seeds were not inoculated with *Rhizobium* because the bacteria were present naturally in soil of this area. Seeds were sown at a density of 25 kg ha^{-1} (15.8 cm between rows) at two different sowing dates: on 12 July 1995 (treatment D1) or 9 August 1995 (treatment D2). For each sowing date, plants were (i) unfertilized with N (treatment N0), (ii) unfertilized but irrigated (treatment N0Irr) or (iii) fertilized with 100 kg N ha^{-1} (as ammonium nitrate) and irrigated (treatment N100Irr). Three replicates were arranged as a split-plot with irrigation as whole-plots and N treatments as subplots. The N fertilizer was applied at sowing in order to eliminate putative N deficiency during early development when symbiotic N_2 fixation may be insufficient to sustain growth. Pesticides were used to control insects and diseases. In order to avoid water stress for N0Irr and N100Irr treatments, potential evapotranspiration was calculated daily using the formulae of Penman as modified by Monteith to determine crop water balance and estimate crop irrigation needs (Penman, 1948; Monteith, 1965). The amount of irrigation in 1995 was 105 mm for D1 and 52 mm for D2. In addition, all six treatments were all fully irrigated in spring 1996 (84 mm of water for D1 and 88 mm for D2) in order to prevent water stress from masking the treatment effects imposed in autumn 1995.

Plant sampling and analysis

Throughout the experiment from emergence in summer 1995 until the first cutting of spring regrowth in May 1996, roots were regularly sampled by removing the uppermost 20 cm of soil along 0.5 m of two adjacent rows. Previous research indicated that this portion of the root represented more than 80% of the total root biomass (Khaïti and Lemaire, 1992). Root samples were freeze-dried for 48 h, weighed, and ground to a fine powder for chemical analysis. Root samples were also obtained on 29 February 1996 (end of winter) and 28 May 1996 (date of the first forage harvest in 1996), washed free of soil and immediately frozen in liquid N_2 and finally stored at -80 °C for analysis of soluble proteins and VSPs. Total root dry matter (RDM) was estimated by multiplying the dry mass of roots obtained from the uppermost 20 cm of soil by 1.23; this coefficient was determined from measurements carried out at the end of winter and in early spring for the six treatments where the total root dry matter was recovered from soil cores sampled to a depth of 100 cm in 20 cm layers.

Shoot dry matter was sampled every 7–14 d between 29 February to 28 May 1996, depending on the crop growth rate. Shoots were cut 6 cm above the crown to simulate the cutting height of mechanical harvesters (Gosse *et al.*, 1984; Khaïty and Lemaire, 1992). These shoot samples were termed harvestable dry matter (HDM), and were sampled on three subplots per block from two adjacent 1 m rows for each plot. The crown fraction also was sampled from these rows, but only 0.5 m of each row was used to determine crown dry masses. Shoot samples of HDM were dried at 80 °C for 48 h and weighed. To determine green leaf area index (LAI, in m^2 leaves m^{-2} soil surface), leaf area was measured on HDM plus crown parts (CDM) using a planimeter with a video camera (Delta-T Devices, UK). Total dry matter (TDM) was determined as HDM plus CDM and RDM.

Analysis of radiation use efficiency (RUE)

Under field conditions, a simple model was developed for alfalfa growth (Gosse *et al.*, 1984) assuming a linear relationship between harvestable dry matter production (HDM) and the cumulative intercepted photosynthetically active radiation ($\sum PAR_i$; MJ m^{-2} j^{-1}) by the crop as follows:

$$HDM = RUEh \times \sum PAR_i \qquad (1)$$

where the slope of the regression is the RUEh (radiation use efficiency for harvestable shoots) estimated to be 1.76 for spring and summer regrowths when not limited by water or nutrient supply, and the cumulative intercepted PAR ($\sum PAR_i$) was calculated using the relationship described previously (Gosse *et al.*, 1982; Varlet-Grancher and Bonhomme, 1982):

$$PAR_i = (0.48 \times Rg) \times 0.97 \times (1 - e^{-0.88 \times LAI}) \qquad (2)$$

Here, *Rg* is the daily global radiation measured at the experimental site; 0.48 corresponds to the proportion of PAR in global radiation; and LAI is the daily leaf area index measured or estimated between two sampling dates by linear regression between LAI and thermal time. Thermal time was calculated using a 5 °C base temperature (Christian, 1977; Fick, 1984, E Justes *et al.*, unpublished data) and accumulated from 1 January 1996. In addition, $\sum PAR_i$ was also accumulated from this date. This model was used to compare the radiation use efficiency (RUEh) of treatments during spring shoot growth. The RUE also was calculated for TDM and termed total RUE (RUEt).

Chemical analysis

Taproot total N concentration was determined by using an elemental analyser (Carlo Erba N autoanalyser 1500, Italy). Proteins were extracted by suspending 100 mg of ground, freeze-dried taproot tissue in 3 cm^3 of 50 mol m^{-3} imidazole-HCl buffer (pH 6.5) containing 2 mol m^{-3} phenylmethylsulphonyl-fluoride, 10 mol m^{-3} 2-mercaptoethanol, 100 mg of insoluble polyvinylpolypyrrolidone, and 10 cm^3 dm^{-3} Triton X-100 at 4 °C. Tubes were vortexed and centrifuged at 3200 *g*, at 4 °C for 20 min. The supernatant was recovered and centrifuged twice at 12 000 *g* at 4 °C for 10 min. An aliquot of the resulting supernatant was analysed for soluble proteins (Bradford, 1976) using BSA (bovine serum albumin) as standard protein. For SDS-PAGE analysis, protein in 1 cm^3 of supernatant was precipitated overnight using sodium deoxycholate-trichloroacetic acid (Peterson, 1983). After centrifugation (10 000 *g*, at 4 °C for 5 min), the pellet was air-dried and resuspended in 0.065 mol dm^{-3} TRIS-HCl, 200 cm^3 dm^{-3} glycerol, 23 g dm^{-3} sodium dodecyl sulphate, pH 6.8, containing 100 mol m^{-3} dithiothreitol and 0.4 g dm^{-3} bromophenol blue, and boiled for 5 min to denature the proteins. SDS-PAGE was performed as described by Laemmli (Laemmli, 1970), using a 55 g dm^{-3} acrylamide stacking gel and a 150 g dm^{-3} acrylamide separation gel and stained using Coomassie Brillant Blue R-250 (Merril, 1990). Electrophoretic transfer of polypeptides from SDS-PAGE gels to PVDF membrane (Immobilon-P, Proteigene, Saint-Marcel, France) was conducted using semi-dry

electroblotting (2.5 mA for 20 min, Milli Blot system, Proteigene), according to the protocol described previously (Towbin *et al.*, 1979). After blotting, PVDF membranes were treated with affinity-purified polyclonal anti-VSP (15, 19 and 32 kDa) primary antibodies as described earlier (Cunningham and Volenec, 1996). The antigen–antibody complex was visualized with alkaline phosphatase linked to goat anti-rabbit IgG as described previously (Blake *et al.*, 1984). Gels and immunoblots were quantitatively analysed by integrating the density of each band using an image analysis system (Bioimage, Proteigene). Relative concentration of VSP (mg g^{-1} RDM) was deduced from the soluble protein concentrations using the value of relative integrated density obtained for the 15, 19 and 32 kDa polypeptides in each lane.

Statistics

The experiment was replicated three times and results represent the mean ± standard error (SE). Variance and co-variance analyses (5% level of significance), and linear regression fittings were performed using the ANOVA, GLM and REG procedures of the SAS software, respectively (SAS Institute Inc., 1987).

Results

Deposition of root dry matter (RDM)

When alfalfa was sown in early summer (12 July, treatment D1) the root dry matter (RDM) increased markedly during late autumn (Fig. 1) compared to plants sown later in the summer (9 August, treatment D2). The enhanced root development occurred irrespective of the level of N fertilization or irrigation. However, when alfalfa was sown early in the summer (D1), plants without N fertilizer or irrigation (N0) had a significantly lower RDM than plants irrigated with or without N fertilization (Fig. 1). By contrast, there was no significant difference in RDM between all D2 treatments during the initial development in late summer, but RDM became lower for D2N0 when compared to the other D2 treatments in the autumn (Fig. 1). In the winter, RDM declined in all treatments (mean reduction of 30%). The RDM on 29 February was about 180 g m^{-2} for N100Irr and N0Irr, and 120 g m^{-2} for N0 seeded at D1, respectively, while root biomass reached only 60 g m^{-2} for all D2 treatments (Fig. 1).

When shoot growth resumed in March, RDM progressively declined in all treatments (Fig. 1). The depletion of RDM was higher for D1N0Irr and D1N100Irr than the other treatments. Thus, the RDM decreased by about 35 g m^{-2} between 20 February and 19 April. By comparison, plants subjected to the D1N0 and all the D2 treatments exhibited a RDM decline of approximately 25 g m^{-2} between February and April (Fig. 1). Thereafter, the RDM gradually increased in roots of plants, irrespective of treatment, until the end of spring. The RDM of D1N0 plants was similar to values observed in D1N0Irr or D1N100Irr treatments at the end
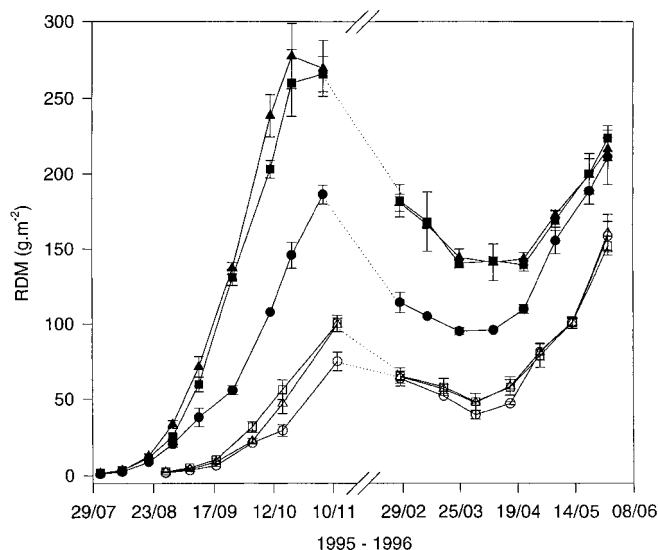

Fig. 1. Influence of six different crop management treatments during alfalfa establishment on the dynamics of root biomass (RDM) during the seeding year. D1: sowing on 12 July 1995; D2: sowing on 9 August 1995; Irr: plants irrigated; N100: fertilization with 100 kg N ha^{-1}; N0: no fertilization. Symbols of treatments: (●) D1N0, (■) D1N0Irr, (▲) D1N100Irr, (○) D2N0, (□) D2N0Irr, (△) D2N100Irr. Vertical bars indicate ± SE of the mean for $n = 3$.

of May (cutting date), while the RDM of all D2 treatments was always significantly lower than all D1 treatments (Fig. 1).

### Crown buds, LAI and N taproot reserve status at the end of winter

Treatments influenced the number of crown buds, LAI and root N reserves (N, soluble proteins, VSP) in alfalfa plants at the onset of meristem reactivation in spring (Table 1). The number of crown buds per plant observed at the end of winter period was always higher in plants sown early in summer (D1) than in D2. Crown buds per plant averaged about 3.9 versus 2.3 for the D1 and D2 treatments, respectively. Application of N fertilizer or irrigation during establishment at D1 did not significantly affect the number of buds observed on crowns at the end of winter. However, irrigation and N fertilizer application during establishment at D2 significantly affected the number of crown buds (Table 1). Leaf area index (LAI) on 29 February differed between the D1 and D2 treatments (Table 1). Maximum LAI values were obtained for D1N0Irr, while the lowest LAI values were observed for D2N0 plants. In D1 sowing treatment, LAI was *c.* 2- and 2.5-fold higher, respectively, in N0Irr and Nl00Irr than in the N0 treatment. Compared to plants from the D1 sowing treatments, LAI of D2 sowing treatments was very low.

The percentage N in roots at the end of winter was slightly higher in all D2 treatments when compared to D1 treatments, with no significant difference between the

Table 1. *The effects of management treatments on crown buds and taproot N reserves*

Number of buds per plant, number of buds m^{-2} of soil, LAI and N, soluble proteins (SP), vegetative storage proteins (VSP) concentrations in taproots. Results are given as $\pm$ SE of the mean for $n = 3$.

| Treatment	Buds per plant	Buds m^{-2} of soil	LAI (m^{-2} m^{-2})	N in roots (% RDM)	SP in roots (mg g^{-1} RDM)	VSP in roots (mg g^{-1} RDM)
D1N0Irr	3.95 (0.24)	3022 (209)	0.177 (0.007)	3.40 (0.10)	23.2 (1.3)	6.59 (0.71)
D1N100Irr	4.07 (0.23)	2991 (171)	0.134 (0.011)	3.30 (0.07)	17.5 (1.6)	6.83 (0.59)
D1N0	3.71 (0.20)	2259 (150)	0.069 (0.009)	3.41 (0.06)	14.9 (0.7)	2.29 (0.11)
D2N0Irr	2.32 (0.09)	1901 (119)	0.028 (0.003)	3.51 (0.01)	11.0 (0.6)	1.14 (0.15)
D2N100Irr	2.70 (0.14)	2020 (62)	0.052 (0.016)	3.45 (0.06)	11.2 (1.3)	1.13 (0.13)
D2N0	2.00 (0.12)	1610 (92)	0.010 (0.002)	3.57 (0.06)	11.1 (1.2)	1.11 (0.11)
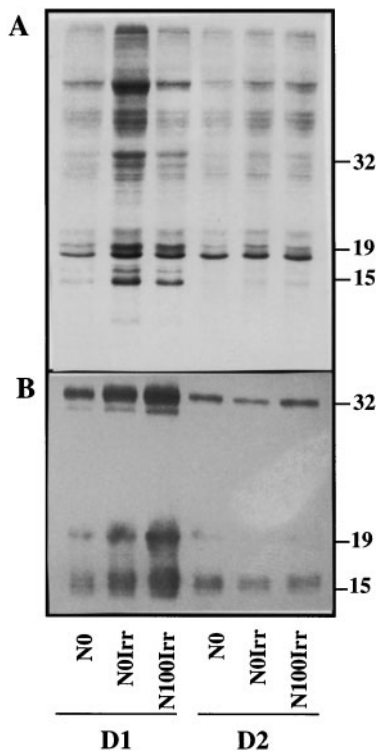
Fig. 2. Influence of six different crop management treatments during alfalfa establishment on soluble protein and VSP contents of taproots at the end of winter (29 February 1996). (A) SDS-PAGE of taproot soluble proteins after staining with Coomassie Brillant blue; (B) immuno-detection of VSP by Western blotting after separation by SDS-PAGE. Equal amounts of soluble proteins (100 μg for SDS-PAGE, 30 μg for Western blotting) were loaded per lane. Molecular mass (kDa) of different VSPs are given on the right side of the gel or the Western blot. Details about treatments are given in Fig. 1.

N100Irr, N0Irr, and N0 treatments within a sowing date (Table 1). By contrast with percentage N in roots, soluble protein concentrations in taproots was higher in plants sown early in the summer (mean value of all D1 treatments = 18.6 mg g^{-1} DM) when compared to plants sown later (mean value of all D2 treatments = 11 mg g^{-1} DM) (Table 1). The analysis of SDS-PAGE and Western-blotting given in Fig. 2 showed that the abundance of

taproot VSPs (32, 19 and 15 kDa) at the end of winter were higher in all D1 treatments than in D2 treatments. In addition, soluble protein and VSP concentrations were significantly higher in roots from the D1N0Irr and D1N100Irr treatments when compared to the D1N0 treatment, while VSP concentrations were similar among the D2 treatments. Highest soluble protein and VSP concentrations were observed in D1N0Irr and D1N100Irr treatments (Table 1). In contrast with D1, N fertilization and irrigation had no significant effect on VSP concentrations when plants were sown in August (D2 treatment).
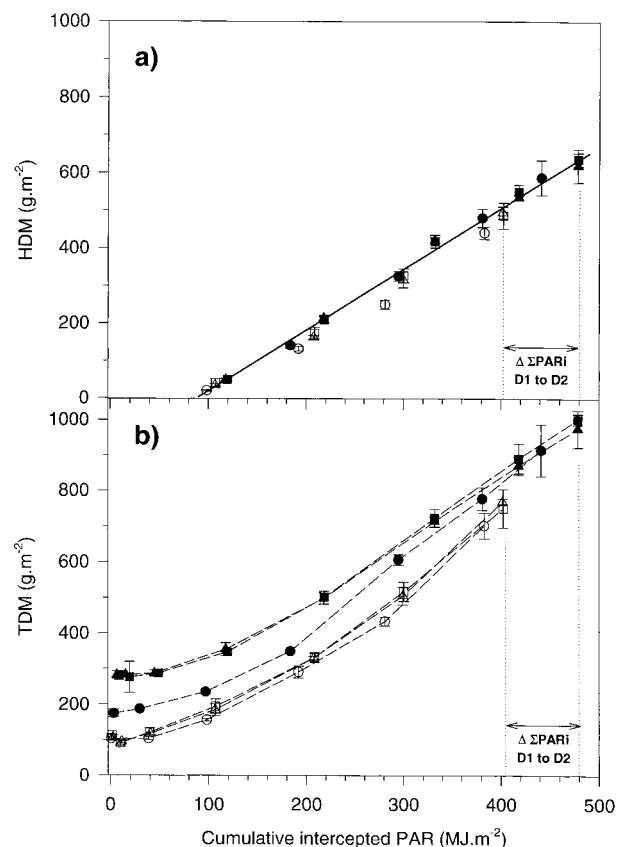
Dynamics of spring regrowth

Examination of LAI development versus thermal time (base temperature of 5 °C) when shoot growth resumed in March 1996 (Fig. 3) indicated that the plants sown in early summer (D1) exhibited a more rapid increase in LAI between 50 and 300 °C d^{-1} when compared to plants in the D2 treatment. For example, at 150 °C d^{-1}, LAI was *c*. 1.5-fold higher in plants sown on 12 July when compared to plant sown on 9 August. These significant differences of LAI between D1 and D2 were observed irrespective of forage management (N0, N0Irr, N100Irr) as indicated by the greater slopes from the regression of LAI versus thermal time, using LAI values of 4 or lower in order to avoid the effect of leaf senescence on LAI expansion rate (Fig. 3). After 200 °C d^{-1}, the LAI development subsided for D1N0Irr and D1N100Irr and LAI attained values of approximately 5 for these two treatments. The LAI of D1N0 plants continued to increase between 200 and 300 °C d^{-1} and attained the same maximum value of 5. Beyond 350 °C d^{-1}, the LAI remained unchanged in all D1 treatments because of competition for light in the dense alfalfa canopy and its effects on enhancing leaf senescence (Gosse *et al.*, 1988; Lemaire *et al.*, 1991). Irrespective of sowing dates, the slope of the relationship between LAI and thermal time was similar between N0Irr and Nl00Irr, but significantly lower for the N0 treatments (Fig. 3). In contrast with D1 treatments, the rapid LAI development of D2 treatments
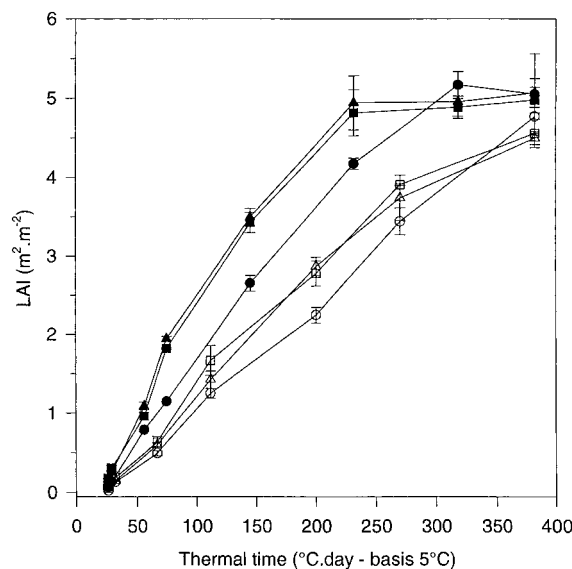
Fig. 3. Influence of six different crop management treatments during alfalfa establishment on LAI development during spring regrowth the following year. Symbols of treatments: (●) D1N0, (■) D1N0Irr, (▲) D1N100Irr, (○) D2N0, (□) D2N0Irr, (△) D2N100Irr. Details about treatments are given in Fig. 1. Vertical bars indicate $\pm$ SE of the mean for $n = 3$.


Fig. 4. Effects of six different crop management treatments during alfalfa establishment on production of (a) harvestable dry matter (HDM, harvestable shoot obtained 6 cm above crown level) or (b) total aerial dry matter (TDM, crowns plus harvestable shoots). Results are expressed as g m^{-2} against cumulative intercepted PAR (MJ m^{-2}). Symbols of treatments: (●) D1N0, (■) D1N0Irr, (▲) D1N100Irr, (○) D2N0, (□) D2N0Irr, (△) D2N100Irr. Details about treatments are given in Fig. 1. Vertical bars indicate $\pm$ SE of the mean for $n = 3$.

was maintained until the end of the regrowth period (Fig. 3). Plants from the D1N0 treatment had more rapid LAI development and higher LAI than any of the D2 treatments even though some of these latter plants were irrigated or fertilized with N (Fig. 3).

The influence of LAI development on shoot regrowth was measured in terms of total dry matter (TDM) and harvestable dry matter (HDM) as a function of cumulative PAR intercepted (Fig. 4). The total $\sum PAR_i$ accumulated at the end of spring regrowth was significantly lower for D2 than D1 treatments due to differences in LAI development. The HDM production averaged 23% lower for D2 treatments than D1 irrigated plants. The forage production of D1N0 was significantly reduced by 10% in comparison to the D1N100Irr treatment. However, irrespective of sowing dates (D1 or D2), the RUEh (which correspond to the slope of the linear regression between harvestable shoot dry matter and $\sum PAR_i$ shown in Fig. 4a) was not significantly different ($P > 0.05$) between treatments, with a mean value of 1.62 g of DM MJ^{-1}. As a result, differences in HDM could be attributed to differences in $\sum PAR_i$ that resulted from rapid LAI expansion. Significant differences in TDM between treatments were observed at the end of winter (Fig. 4b). At the end of spring regrowth, the TDM production was more than 1000 g m^{-2} for D1 irrigated treatments but only *c.* 750 g m^{-2} for D2 treatments. Up to 22 April, the RUEt (RUE for TDM) was very low and not significantly different ($P > 0.05$) between D1 and D2 treatments (mean value of 0.72 g of DM MJ^{-1}), but thereafter, the value of RUEt became largely higher and

was lower ($P > 0.10$) for D1N100Irr treatment (1.83) than for the other treatments (2.14 in average). This result was probably due to more rapid foliar senescence for D1Irr treatment where plants exhibited more rapid HDM production (Figs 3, 4).

Relationships between RDM, soluble proteins, VSP accumulated in taproots, or number of crown buds at the end of winter and LAI expansion

When slopes of the regressions of LAI versus thermal time for the different dates of sowing, N fertilization and irrigation treatments were regressed against RDM, root soluble protein concentrations, VSP concentrations or number of crown buds observed at the end of winter, significant linear relationships were obtained (Fig. 5). High coefficients of determination was observed for root dry mass ($R^2 = 0.99$***; Fig. 5a), taproot soluble protein concentrations ($R^2 = 0.82$; Fig. 5b), and taproot VSP concentrations ($R^2 > 0.96$**; Fig. 5c). This indicated that the rate of LAI increase in spring was closely associated
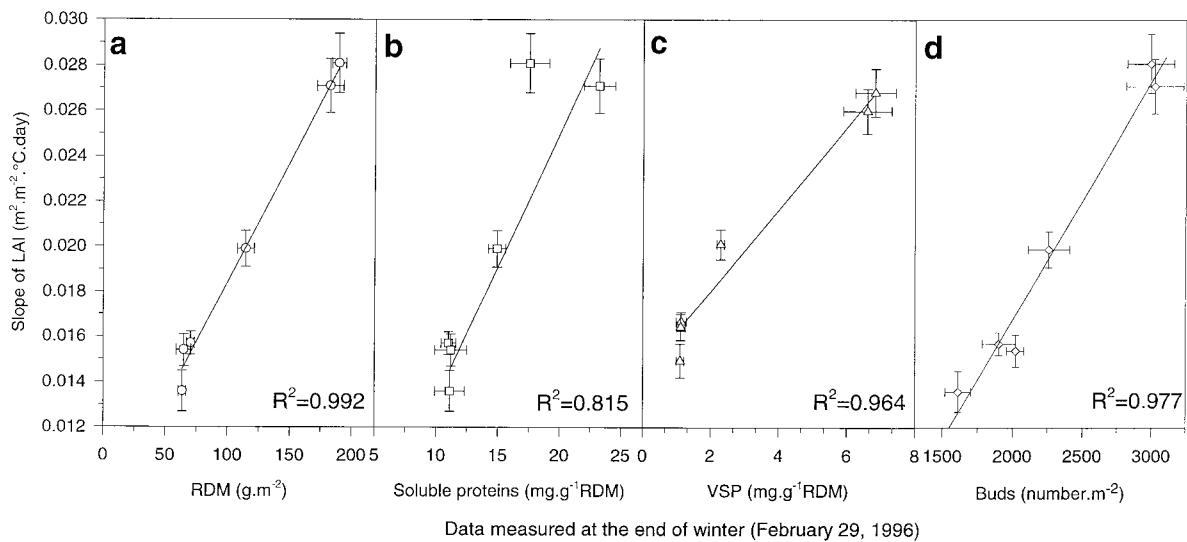
Fig. 5. Relationship between slope of the regression of LAI on thermal time (rate of leaf area development, Fig. 3) measured during the first spring shoot growth and (a) root dry matter (RDM), (b) taproot soluble protein concentration (SP), (c) taproot VSP concentration, and (d) number of buds m^{-2} of soil measured at the end of winter. Plants were established the preceding year using the six different crop management treatments described in Fig. 1. Vertical bars indicate ±SE of the mean for $n = 3$. The equations for the lines obtained using linear regression: (a) LAI-slope $= 1.06 \times 10^{-5}$RDM + 0.0078; (b) LAI-slope $= 0.0012$SP + 0.0028; (c) LAI-slope $= 0.0022$VSP + 0.0129, and (d) LAI-slope $= 1.06 \times 10^{-5}$Buds − 0.0044.

with RDM and root N reserves (soluble and vegetative storage proteins) accumulated in taproots during establishment the previous summer and autumn. Moreover, the number of crown buds m^{-2} present at the end of winter explained 98% of the variance of LAI expansion rate (Fig. 5d).

N reserves accumulated in RDM at the end of spring regrowth

At the end of spring regrowth (28 May 1996), the soluble protein concentration in taproots (Table 2) and the corresponding electrophoretic profiles (Fig. 6) were similar among the six treatments. By contrast, irrespective of N and irrigation treatments, the VSP concentration tended to be higher in roots of D1 plants (1.48 mg g^{-1} RDM) when compared to roots of plants planted on D2 (1.03 mg g^{-1} RDM) (Table 2; Fig. 6). However, root mass per plant and per soil area differed between D1 and D2 treatments. As a consequence, soluble protein and VSP contents in taproots were 50% and 100% higher, respectively, for the D1 than D2 treatments (Table 2). By the end of shoot regrowth on 28 May plants in the D2 treatments did not accumulate taproot N reserves to such high concentrations as the D1 plants. This significant carryover effect of planting date treatments influenced the establishment of the seeding year as well as subsequent shoot regrowth. Moreover, soluble proteins and VSP concentrations in taproots obtained at the end of spring regrowth (Table 2) were lower than those measured at the end of winter for each treatment (Table 1).

Discussion

Effect of sowing date, N fertilization and irrigation on taproot N reserve accumulation in autumn

The present study reveals that N storage in alfalfa taproots during the autumn of the first year of establishment depends strongly on crop management. Delaying the sowing date by one month reduced the mass of reserve organs (mainly represented by taproots) and with it, limited the capacity of alfalfa to store N in the taproot by altering the soluble proteins and, specifically, VSP concentrations at the end of the winter following establishment. From an agronomic point of view, the sowing date must be as early as possible in the summer (immediately after the harvest of the preceding winter crop) in order to maximize the accumulation of N reserves before the winter.

Irrespective of sowing date treatments, N fertilization at sowing did not improve root mass, total N, soluble protein, or VSP concentrations of roots. These results suggest that under this study's experimental conditions, the onset of symbiotic N$_2$ fixation was not a factor limiting growth and development during alfalfa establishment. By contrast, when alfalfa was sown early in summer (D1), water availability had greater importance than N availability on alfalfa establishment and growth. The primary effect of water deficiency during the emergence of alfalfa sown early in the summer was to reduce N acquisition by absorption or symbiotic fixation (Lemaire *et al.*, 1985). In this study, the lowest N concentration in HDM, which is an indicator of plant N status (Lemaire *et al.*, 1985), was

Table 2. *Means and standard errors (SE) of soluble proteins and VSP concentrations in RDM and contents m^{-2} of soil at the end of spring regrowth (28 May 1996) for the six treatments*

Numbers followed by different letters are significantly different (Newman–Keuls group $P > 0.05$).

Treatment	Soluble proteins		Vegetative storage proteins	
	Concentration (mg g^{-1} RDM)	Content (g m^{-2} of soil)	Concentration (mg g^{-1} RDM)	Content (mg m^{-2} of soil)
D1N0Irr	9.22 NS (0.41)	1.95	1.45 AB (0.043)	349
D1N100Irr	8.87 (0.99)	2.06	1.33 AB (0.248)	324
D1N0	9.24 (1.07)	1.92	1.65 A (0.146)	289
D2N0Irr	8.14 (0.33)	1.28	1.04 B (0.056)	164
D2N100Irr	9.01 (0.35)	1.24	1.02 AB (0.222)	158
D2N0	8.05 (1.02)	1.45	1.03 AB (0.224)	165
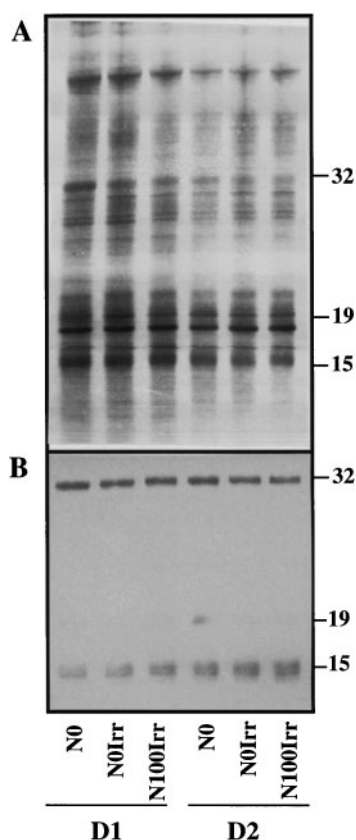
Fig. 6. Influence of six different crop management treatments during alfalfa establishment on soluble protein and VSP contents of taproots observed at the end of spring regrowth (28 May 1996). (A) SDS-PAGE of taproot soluble proteins after staining with Coomassie Brillant blue; (B) immunodetection of VSP by Western blotting after separation by SDS-PAGE. Equal amounts of soluble proteins (100 μg for SDS-PAGE, 30 μg for Western blotting) were loaded per lane. Molecular mass (kDa) of different VSP are given on the right side of the gel or the Western blot. Details about treatments are given in Fig. 1.

significantly reduced in the early spring in treatments without irrigation, i.e. D1N0 and D2N0 in comparison with irrigated treatments (data not shown). This indicates that the water deficit stress of non-irrigated treatments also resulted in temporary N deficiency. Ultimately this water deficit stress limited shoot and root growth in

the summer and autumn, and with it, reduced the accumulation of taproot N reserves.

Consequences of N reserves accumulated in taproots during the autumn on spring regrowth

Irrespective of sowing dates, a reduction in RDM occurred when the crown meristems reactivated in early spring; a pattern shown previously by others (Khaïty and Lemaire, 1992; Bélanger and Richards, 2000). This decline in RDM is caused by the mobilization of taproot TNC and N reserves (Bula *et al.*, 1956; Hodgkinson, 1973; Volenec *et al.*, 1991; Li *et al.*, 1996). When spring growth resumed, plants with large taproots with high concentrations of soluble proteins and VSPs exhibited rapid LAI expansion and shoot growth that resulted in significant differences in forage production (HDM). Nevertheless, RUEh was not affected by sowing date or crop management during establishment. The RUEh values were slightly lower (−8%) than those reported previously for spring growth of alfalfa (Gosse *et al.*, 1984; Khaïty and Lemaire, 1992). Nevertheless, RUEt values varied during spring regrowth: for all treatments, RUEt was lower than 0.9 in the first period of growth, and increased to values of 1.8 or greater during the second growth period. This lower efficiency in early spring can be interpreted as an effect of temperature on RUE, as has been shown for other species (Justes *et al.*, 2000). Mean air temperatures averaged only 6.4 °C for the first period, but increased to 11.4 °C for the second period. The average for the entire spring growth period (8.4 °C) could explain the lower RUEh and RUEt obtained in this experiment compared to values measured in a warmer location (Gosse *et al.*, 1984). The RUEt values were not significantly different between treatments during the first growth period when temperature may have limited RUEt. During early spring regrowth, the level of root N reserves had no effect on RUE, but N reserve levels enhanced LAI establishment. By contrast RUEt for D1N0Irr and D1N100Irr declined when compared to the other treatments during the second phase of growth. This could be explained by the earlier onset and more extensive leaf

senescence occurring at the end of regrowth as the light competition in the lower leaf layers of the canopy increased (Lemaire *et al.*, 1992). In the second phase of spring growth, the differences between RUEt and RUEh were, on average, higher for the D2 treatments (0.63 g DM MJ^{-1}) than the D1 treatments (0.25 or 0.44 g DM MJ^{-1} for D1Irr and D1N0, respectively), suggesting that the reaccumulation of reserves in roots and crowns was lower for D1 than D2 treatments at the end of spring growth. This assumption was verified by the concentrations of soluble proteins and VSPs in roots at the end of spring growth which were similar between D1 and D2 plants, although RDM and N reserves were much lower when winter was over.

The LAI development in spring was closely associated with the number of crown buds per plant or m^{-2} of soil observed at the beginning of spring, suggesting that this factor is a major determinant of alfalfa shoot growth in spring. Moreover, the number of crown buds per area was positively associated to N reserve levels as shown by the close relationship between RDM ($R^2 = 0.992$***), soluble proteins ($R^2 = 0.965$**) or VSP concentrations ($R^2 = 0.952$**) measured at the end of winter and the number of buds m^{-2} of soil in March (data not shown); the more N reserves, the more buds and the greater rate of LAI development.

The decrease of N concentration in HDM with the increase of HDM has been described previously (Lemaire *et al.*, 1992) and was also found in all treatments of this study. This N dilution process in regrowing alfalfa herbage may be related to the progressive shading of the leaves of the canopy and leaf senescence and a critical dilution curve was proposed for alfalfa as a tool to diagnose the crop N status (Lemaire *et al.*, 1985). In this experiment, crop management strategies that allowed a higher accumulation of root protein and VSP (D1N0Irr or D1N100Irr treatments) led to high N percentage in harvestable shoots despite the fact that the level of N in HDM was strongly affected in plants having low N reserves (data not shown) indicating that the crop N status was significantly lower for D2 treatments. This diagnosis of N deficiency in HDM could explain the lower LAI expansion rate in early spring.

Unfortunately, shoot regrowth after shoot removal on 28 May was not investigated in this experiment. However, considering that N reserve levels in taproots at the end of the spring were lower for D2 treatments, it can be hypothesized that shoot regrowth rate after defoliation on 28 May could be limited by N reserves when compared to plants in the D1 treatments. This hypothesis is based upon previous observations concerning the significant relationship between the level of N reserves in the taproot and the dynamics of the first spring shoot growth. Additionally, previous field experiments have shown that the second or third shoot regrowths following defoliation

was positively correlated to taproot soluble protein and VSP concentrations on the day of cutting (Avice *et al.*, 1997*b*). This suggests that the physiological consequences of alfalfa establishment may persist well into the succeeding year.

Was shoot growth rate determined by root soluble N reserve content or VSP concentration?

The correlations between N reserves and shoot growth reported in this study were confirmed by previous results obtained under field conditions. In a field experiment where variations in N and C reserve levels were induced by genetics or variation in crop management, Avice *et al.* reported that shoot regrowth was linearly related to both taproot soluble protein and VSP contents on the day of defoliation (Avice *et al.*, 1997*b*). By contrast, no relationship was detected between shoot regrowth and initial starch or N taproot contents. These authors suggested that root protein and VSP are key organic reserves for alfalfa shoot regrowth after cutting; nevertheless they did not determine the relative importance of soluble protein versus VSP reserves on shoot regrowth. In order to increase the soluble protein pool, alfalfa plants could increase root biomass, the concentration of root proteins (especially VSP) without changing RDM, or increase both factors. In this experiment and others (Avice *et al.*, 1997*a*, *b*), root mass (RDM) and the concentrations of soluble proteins and VSP in roots increase together, and does not offer the opportunity to know if root mass or VSP concentration is the principal determinant of shoot regrowth potential. However, under greenhouse conditions, the increase of RDM does not always parallel increases in root soluble protein or VSP concentration. For example, Noquet *et al.* reported that high N nutrition increased TDM without modifying taproot VSP concentration (Noquet *et al.*, 2001). These authors also showed that increasing the N concentration of the hydroponic solution from 1 to 5 mol m^{-3} KNO$_3$ doubled total dry matter production of alfalfa, but did not significantly affect the relative N partitioning between shoots and roots and had no influence on root VSP concentration. Similar results were also obtained for young alfalfa seedlings (Kalengamaliro *et al.*, 1997) when it was shown that the addition of N fertilizer (up to 10 mol m^{-3} NH$_4$NO$_3$) increased total plant growth, but did not influence root protein concentrations or the onset of VSP accumulation in taproots. Additionally, it has recently been found that when high fertilizer N rates are applied in the nutrient solution (20 mol m^{-3} NH$_4$NO$_3$) to 5-month-old alfalfa plants, VSP accumulation after 21 d of treatment is similar to that of plants provided 1 mol m^{-3} of NH$_4$NO$_3$ (data not shown). This suggests that VSP accumulation in alfalfa taproots was not regulated directly by availability of inorganic N. This also suggests

that alfalfa will increase the size of soluble reserve protein pool in roots as a first priority, and secondly, accumulate VSPs if the environment and stage of plant development permit. From this it appears that taproot mass and VSP reserve accumulation are the main factors determining the shoot regrowth potential of alfalfa. Future research will test this hypothesis as it needs verification in controlled conditions where RDM and VSP concentrations can be manipulated independently of each other (as shown by Noquet *et al.*, 2001).

Acknowledgements

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