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Accumulation of N reserves and vegetative storage protein (VSP) in taproots of non-nodulated alfalfa (*Medicago sativa* L.) are affected by mineral N availability

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Abstract

Our objective was to understand how mineral N availability alters accumulation of N reserves (nitrate, amino acids, soluble proteins and vegetative storage proteins known as VSP) in alfalfa (*Medicago sativa* L. cv Lodi) taproots. The effects of variation in NH_4NO_3 availability were followed by studying non-nodulated plants grown under hydroponic conditions during 21 days with (i) different N supplies which corresponded to N-replete plants (N100% = optimal N) and N-limited plants receiving only 50% (N50%) or 25% (N25%) of optimal N (Experiment I), or (ii) decreasing concentrations of NH_4NO_3 (1000, 250, 100 or 50 μM, Experiment II). Regardless of the N-limitation mode (Experiments I or II), and compared with higher N treatments (N100 or 1000 μM), there was a significant reduction of total shoot dry matter per plant for lowest N treatments (N25 or 50 μM). This was accentuated by the degree of N deficiency in Experiment I only. In Experiment II, taproot biomass significantly increased for low N treatments. In both experiments, total N, nitrate and amino acid concentrations in taproots increased for high N treatments, while the concentration of soluble proteins, and particularly VSP, increased for low N treatments. These results indicated that non-nodulated alfalfa was able to accumulate N reserves (mainly as VSPs), even under N-limited conditions, while under high mineral N availability, taproot amino acid concentrations (mainly asparagine) increased without a corresponding increase in soluble protein concentration. These results show that alfalfa was capable of optimizing N cycling and storage as a function of mineral N availability. These adaptive responses to low soil N environments also allow alfalfa to go dormant and perenniate, while awaiting more favorable conditions for shoot growth.

Keywords: *Medicago sativa* L.; N availability; N reserves; Amino acids; Nitrate; Vegetative storage protein; Roots

1. Introduction

Over the last decade, many studies on woody and herbaceous species have shown a key role of N reserves during crucial phases of plant development such as initial spring growth, post-defoliation herbage regrowth, and grain filling [1–6]. Although carbohydrates were the predominant storage compounds in terms of mass in underground storage organs, several results have shown that N reserve compounds (nitrate, amino acids, proteins) were more important than carbohydrate reserves in determining growth responses of herbaceous plants [7–10]. In forage species such as alfalfa (*Medicago sativa* L.), amino acids and water-soluble proteins represented the largest N fraction in taproots. Particular proteins were very abundant and were largely hydrolyzed during initiation of shoot growth in spring [11] or during shoot regrowth after cutting [12–14]. In addition, these proteins re-accumulated rapidly and to high concentrations during autumn or at the end of shoot regrowth

Abbreviations: HPLC, high performance liquid chromatography; PVDF, polyvinyldene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; VSP, vegetative storage protein.

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prior to alfalfa being cut for forage. Because of their abundance (about 40% of the total soluble protein) and their atypical patterns of extensive mobilization/accumulation (greater than the other soluble proteins), these proteins possess characteristics associated with vegetative storage protein (VSP), as previously described by Cyr and Bewley [15] and Staswick [4]. Three VSPs with molecular masses of 15, 19 and 32 kDa have been identified in alfalfa taproots [11]. These VSPs were specifically stored in vacuoles of parenchyma cells of wood rays in alfalfa taproots [14]. In addition, Gana et al. [16] have recently characterized another VSP of 57 kDa, which possess high amino acid sequence homology to ß-amylase.

Chapin et al. [3] divided the storage process into (i) reserve formation, which was in competition for nutrients with growth and (ii) reserve accumulation, which occurred when resource supply exceeded demand (without competition with growth). Moreover, it is now well established that N acquisition by uptake or symbiotic N_2 fixation, N partitioning within the plant and N reserve accumulation can vary as a function of resource availability. Several studies, mostly in poplar (*Populus deltoides*) and soybean (*Glycine max* L. Merr.), suggested that environmental factors (photoperiod, light intensity, temperature) and endogenous molecular signals (soluble sugars, jasmonic acid) that modified N source/sink relationships within the plant also regulated the N reserve accumulation, particularly as VSPs [17,4,18–21]. Soil or nutrient solution N availability and tissue N status also affected VSP accumulation. In soybean leaves [22] and poplar bark [21], VSP transcript accumulation increased in parallel with N supply. Removal of pods also modified the N partitioning between source and sink tissues during reproductive development of soybean and induced VSP gene expression in leaves [23]. However, Staswick [4] suggested that the available mineral N level may have an indirect effect on VSP gene induction of soybean leaves. Nevertheless, it appeared that VSP accumulation did not always increase with enhanced high mineral N supply. For example, altering N nutrition had no significant effect on VSP accumulation in chicory (*Cichorium intybus* L.) [24]. Similarly, increasing the N concentration in nutrient solution from 1 to 5 mM KNO_3 increased shoot growth in alfalfa, but did not change N partitioning in the different organs or alter taproot VSP concentrations [25]. Moreover, Kalengamaliro et al. [26] have shown that high N fertilization (10 mM NH_4NO_3) increased total plant growth, but did not influence root protein concentrations or the onset of VSP accumulation in young alfalfa seedlings. Ourry et al. [27] also reported that decreased mineral N supply to alfalfa (from 1 to 0.2 mM NH_4NO_3) over 15 days decreased shoot growth rates and the amount of total N in taproots. However, these authors did not investigate the impact of N deficiency on the partitioning of N reserves among taproot N pools.

Because alfalfa is a widely grown perennial legume adapted to low N environments, and because the role of the mineral N level on the accumulation of N-storage compounds has not been extensively evaluated in alfalfa, our objectives were to study the effects of different levels of mineral N availability on the accumulation and partitioning of N reserves (amino acids, total soluble proteins and VSP) in taproots of non-nodulated alfalfa. Our results indicate that alfalfa is able to accumulate N reserves (particularly VSPs) in taproots even if the mineral N nutrition is low.

2. Materials and methods

2.1. Plant material

Experiment I was initiated in January 1999 and alfalfa (*M. sativa* L. cv Lodi) seeds were germinated on sand. For Experiment II, alfalfa seeds (cv Lodi) were sown on a synthetic substrate (Oasis pinpot growing medium, Agrimedia, France) in March 2000. After 15 days, when the primary trifoliate leaves appeared, seedlings were transplanted to plastic pots filled with sand (four plants per pot). Each pot was irrigated three times per week with 300 ml of a nutrient solution containing 0.4 mM KH_2PO_4, 1 mM K_2SO_4, 3 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.15 mM K_2HPO_4, 0.2 mM Fe–Na EDTA, and 14 μM H_3BO_3, 5 μM $MnSO_4$, 3 μM $ZnSO_4$, 0.7 μM $CuSO_4$, 0.7 μM $(NH_4)_6Mo_7O_2$, and 0.1 μM $CoCl_2$ [28]. Nitrogen was added to the nutrient solution (1 mM NH_4NO_3) to repress nodule formation. Plants were grown under greenhouse conditions with a thermoperiod of 20 °C (day) and 18 °C (night) and a photoperiod of 16 h (day) and 8 h (night). After 3.5 months, plants were defoliated 6 cm above crown level, crowns and roots removed from pots, and transferred to 8 l plastic containers containing a continuously aerated nutrient solution. During the initial 15 days after transfer, this nutrient solution containing 1 mM of NH_4NO_3, was renewed every 3 days to avoid N deficiency. In addition, 25 ml of 1 mM $CaCO_3$ were added to maintain pH value close to 6.5.

Because previous results [29,12,14] have shown that the depletion of N storage occurs during the first 2 weeks following shoot removal, the N-limiting treatments (see Experiments I and II described below) were applied after 15 days of shoot regrowth (Day 0), when the re-accumulation of proteins begins. During the experiments, light was supplemented with high-pressure sodium lamps (400 W phytoclaude) supplying approximately 400 μmol of photons m^{-2} s^{-1}, 15 cm above crown level.

2.2. Experiment I: application of optimal and sub-optimal N feeding

This experiment started on the 15th day of regrowth after defoliation and continued for 21 days (Fig. 1). At Day 0 (first day of treatments), one-third of the plants received 1 mM of NH_4NO_3 in the nutrient solution and the depletion of NH_4NO_3 in the nutrient solution was measured daily in order to determine the daily N uptake. NH_4^+ and NO_3^- disappearance was determined from 1 ml of nutrient solution by high performance anionic (Ionpac AS9 analytical column) and cationic (Ionpac CS12 analytical column) chromatography (HPLC, DX100, Dionex Corp., Sunnyvale, CA, USA), respectively. Based on these results, NH_4NO_3 was directly added to the nutrient solution to compensate for daily N uptake by the plants (control plants corresponding to treatment N100%). The remaining plants were divided into two groups that received either 50% (treatment N50%) or 25% (treatment N25%) of the NH_4NO_3 taken up daily by the control plants (N100%). During the 21 days of treatments, the nutrient solution without N (see the composition above) was renewed every 3 days.

2.3. Experiment II: application of decreasing concentrations of NH_4NO_3

In this study, plants were grown during 21 days in nutrient solution containing four different concentrations of NH_4NO_3 (1000, 250, 100 or 50 μM), renewed every 3 days (Fig. 1). Moreover, in order to study the partitioning of N derived from uptake within the plant as a function of the reduction of mineral N availability, the N ($^{15}NH_4^{15}NO_3$) was labeled with 2.5 atom% ^{15}N excess.

2.4. Tissue sampling

In both experiments, plants were harvested at the end of the 21 days treatment period and were separated into lateral roots, taproots, leaves and stems. Taproot samples were immediately frozen in liquid N_2. After freeze drying, taproot samples were ground into a fine powder and kept at −80 °C for further N fraction analysis. Lateral roots and shoot tissues were dried at 70 °C for 72 h. The dry weight of each sample was determined.

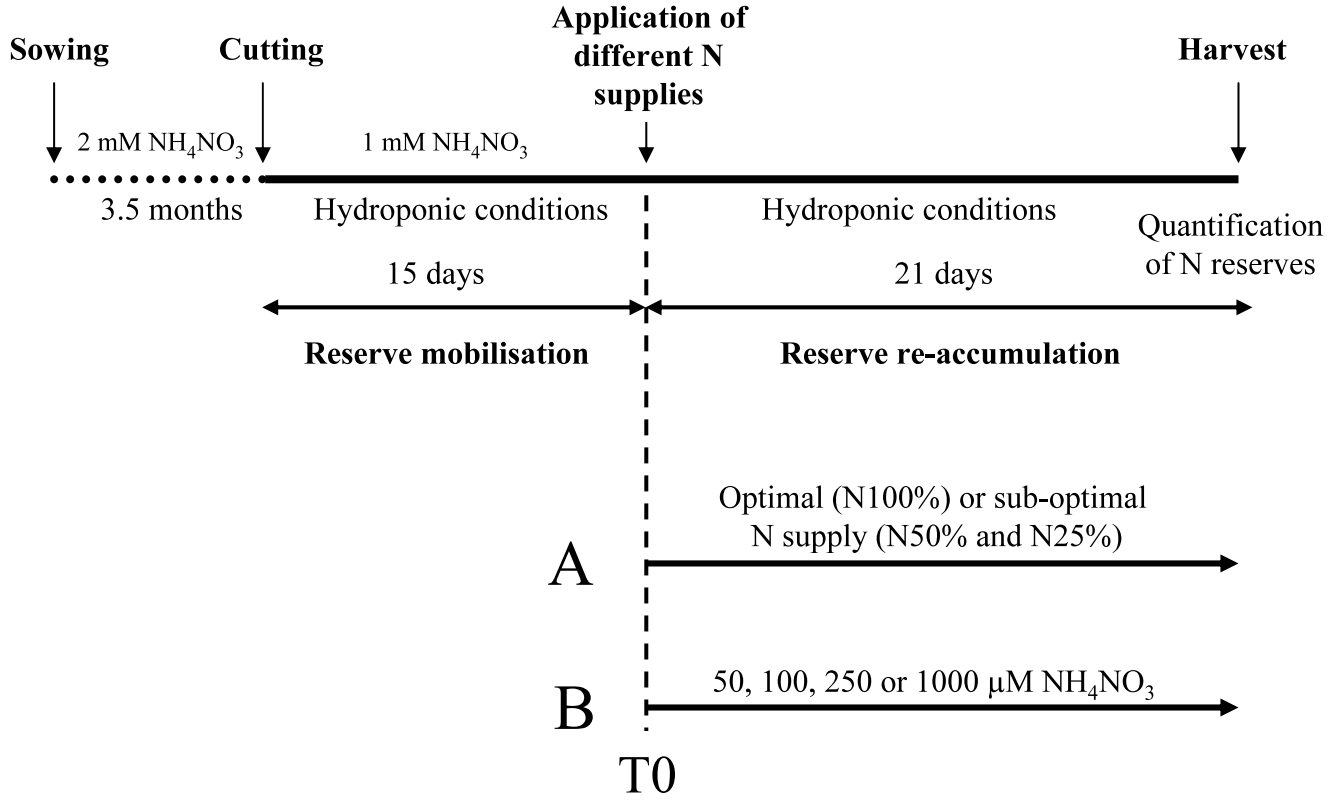

Fig. 1. Cultural conditions and experimental protocol used to provide different N concentrations to hydroponically grown alfalfa (*M. sativa* L., cv Lodi). (A) Experiment I: optimal (N100%) or sub-optimal (N50% or N25%) NH_4NO_3 feeding; (B) Experiment II: plants were provided with decreasing NH_4NO_3 concentrations (1000, 250, 100 or 50 μM) beginning 15 days after cutting and continuing for 21 days.

2.5. Analysis of N, nitrate, amino acids, soluble proteins and VSP concentrations in taproots

N concentrations in taproots were measured in continuous flow using a C/N analyzer linked to an isotope ratio mass spectrometer (IRMS, Roboprep CN and mass spectrometer, PDZ Europa Scientific Ltd., Crewe, UK).

Extraction of nitrate, amino acids and soluble proteins was adapted from Barber et al. [30] and Cunningham and Volenec [31]. Proteins were extracted at 4 °C by suspending 300 mg of ground freeze-dried taproot tissue in 5 ml of extraction buffer (pH 7) containing 100 mM sodium-phosphate and 10 μM β-mercaptoethanol. After centrifugation (3200 × *g*, 4 °C for 20 min), the pellet was re-extracted in 5 ml of extraction buffer, and after a second centrifugation, the supernatants were pooled. An aliquot of the resulting supernatants was used to determine nitrate and soluble protein concentrations. Nitrate concentration of the root extract was determined by the sulfanilamide method after reduction of nitrate to nitrite using a continuous-flow autoanalyzer (Bran+ Luebbe, Noderstedt, Germany). Soluble protein concentrations were quantified by protein dye-binding [32] and VSP quantification by the ELISA method as previously described by Noquet et al. [25]. One milliliter of protein extract was precipitated using the sodium deoxycholate-trichloroacetic acid protocol described by Peterson [33]. After centrifugation (10 000 × *g*, 4 °C for 10 min), the supernatant was used for amino acid analysis as described below. The resulting pellet was air-dried and resuspended in 50 mM Tris–HCl (pH 7.5), resuspended in Laemmli lysis buffer [34] and boiled for 5 min to denature proteins. SDS-PAGE was performed using a 150 g l^{-1} acrylamide separation gel. Separated proteins were blotted onto PVDF membrane for Western blotting analysis using the anti-32, -19 and -15 kDa VSP antibodies [35,25].

Amino acids were analyzed using HPLC on a Beckman Gold 8.0 system (Beckman, Roissy, France). The remaining supernatant obtained after protein precipitation was freeze-dried, resuspended in 1 ml of water and then filtered on 0.45 μm membrane. After dilution (1/5000), the extract was derivatized for 2 min with 50 mM *o*-phtaldialdehyde and separated on a C18 Bio-Rad column (150 × 4.5 mm). The amino acids were detected by using a Shimadzu R-F 551 fluorimeter. The internal standard was α-amino butyric acid (2.5 mM). The separation was performed using a gradient of pure methanol in Na-acetate buffer (50 mM, pH 5.9).

2.6. Calculation of N partitioning in plants

N content (Experiments I and II) and ^{15}N abundance (Experiment II) in all organs were measured in continuous flow using a C/N analyzer linked to an isotope ratio mass spectrometer (IRMS, Roboprep CN and mass spectrometer, PDZ Europa Scientific, Crewe, UK). Natural ^{15}N abundance (0.3663% ± 0.0004) of atmospheric N_2 was used as a reference for ^{15}N analysis.

In Experiment II, nitrogen derived from current N uptake (N_{Up}) between Day 0 and Day 21 in a given organ was calculated as follows:

$$N_{Up} = N_T \times (E\ (\%)/E_S\ (\%))$$

where N_T is the total nitrogen in the organ (mg per plant), E (%) is the atom% ^{15}N excess in a given organ and E_S is the nutrient solution atom% ^{15}N excess (2.5%).

2.7. Statistical analysis

The experiments were replicated four times (each replicate containing three or four plants). Results represented the mean ± S.E. for n = 4. The effects of the N supply were tested using *t*-tests (STATVIEW Student Software, Abacus Concepts, Berkeley, USA).

3. Results

3.1. Total biomass and shoot root ratio

In Experiment I, total biomass was significantly reduced by sub-optimal NH_4NO_3 supply. Compared with plants provided with optimal N (N100%), total dry matter was 44% (N50%) and 50% (N25%) less after 21 days of treatment (Fig. 2A). In Experiment II (Fig. 2B), irrespective of the NH_4NO_3 concentration used, the total biomass was not significantly modified and averaged 13 g per plant after 21 days. Total biomass at Day 0 in Experiment II was also higher than in Experiment I because of different greenhouse plant culture conditions (for details, see Section 2). Therefore, the biomass partitioning between shoots and roots was significantly altered by the level of N deficiency (Fig. 2, numerals above bars). The more N availability decreased in the nutrient solution, the greater the decline in shoot/root ratio. For example, in Experiment I, the shoot/root ratio reached 3.06 for N100% treatment, while this ratio markedly decreased for plants provided a sub-optimal N feeding (1.92 for N25%). N limitation had different effects on taproot biomass in both experiments. In Experiment II, taproot biomass was significantly increased as NH_4NO_3 concentration in nutrient solution decreased from 1 mM to 250 or 100 μM (Fig. 2B), while in Experiment I, taproot biomass was not affected by NH_4NO_3 concentration of the nutrient solution (Fig. 2A).

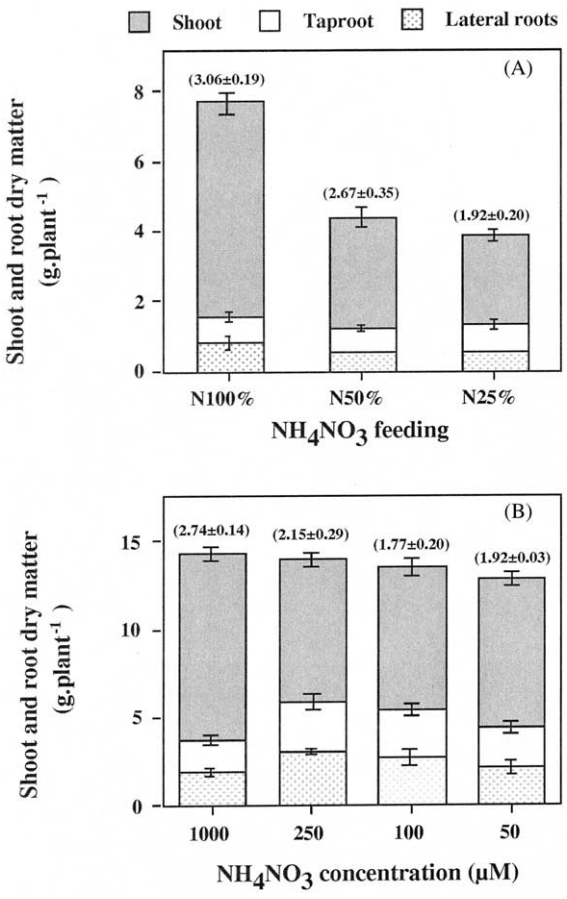

Fig. 2. Shoot, taproot and lateral root dry matter of alfalfa (*M. sativa* L., cv Lodi) after 21 days growth at different N concentrations. (A) Experiment I, and (B) Experiment II. Details about Experiments I and II are provided in Fig. 1. Vertical bars indicate ±S.E. of the mean for $n = 4$. Numbers in parentheses are the mean ±S.E. of the shoot to root dry matter ratio.

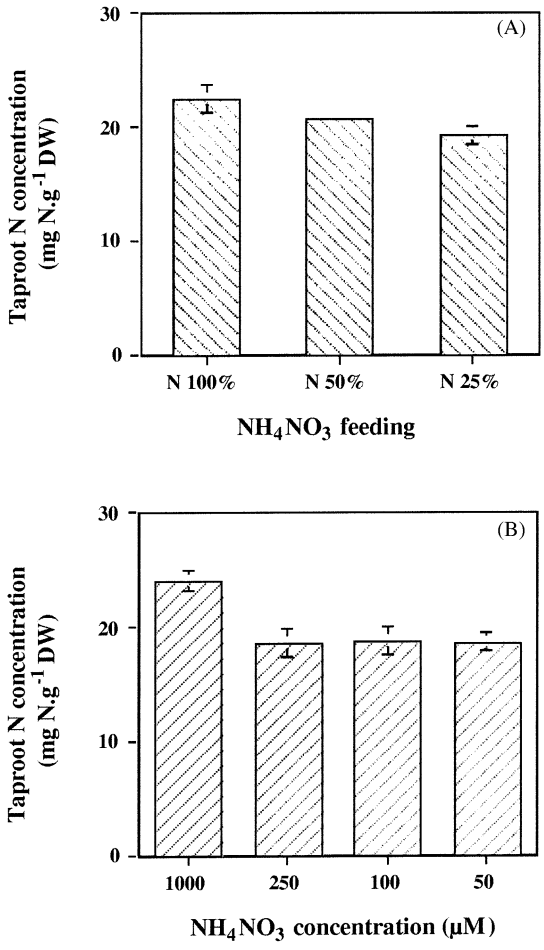

Fig. 3. Taproot N concentration of alfalfa (*M. sativa* L., cv Lodi) after 21 days growth at different N concentrations. (A) Experiment I, and (B) Experiment II. Details about Experiments I and II are provided in Fig. 1. Vertical bars indicate ±S.E. of the mean for $n = 4$.

3.2. Total N concentrations in taproot

In Experiment I, the taproot N concentration gradually decreased with the reduction of NH_4NO_3 availability (Fig. 3A). For example, when N supply was reduced by 75% (N25%) during 21 days, the taproot N concentration declined by 16% in comparison with control (N100%) plants (Fig. 3A). In Experiment II, a reduction of NH_4NO_3 concentration in the nutrient solution from 1000 to 250 µM (Fig. 3B) also severely decreased the taproot N concentration after 21 days of treatment. Taproot N concentrations of plants grown with 50–250 µM NH_4NO_3 concentrations were similar (Fig. 3B).

3.3. Exogenous N partitioning within the plant

The utilization of ^{15}N labeling methods during Experiment II showed that the relative partitioning of exogenous N derived from the nutrient solution was significantly altered by changes in NH_4NO_3 concentration of the nutrient solution (Fig. 4). The N taken up after 21 days was primarily found in regrowing shoots (up to 75% for 1000 µM plants, Fig. 4A) whereas the reduction in nutrient solution N supply decreased the allocation of exogenous N to shoots. Consequently, the reduction of N fertilization resulted in preferential N allocation to roots (taproots and lateral roots) at the expense of regrowing shoots. For example, when compared with plants provided with 1000 µM NH_4NO_3 (Fig. 4C), the allocation of exogenous N to taproots was doubled and reached approximately 10% of the total exogenous N for treatments supplied 50–250 µM NH_4NO_3 in the nutrient solution.

3.4. Amino acid and nitrate concentrations in taproots

In both experiments, the extent of N deficiency strongly affected the concentration of amino acids in taproots (Fig. 5A and B). Compared with control plants (N100% or 1 mM NH_4NO_3), the concentration in amino acids decreased by 29% after 21 days for plants provided

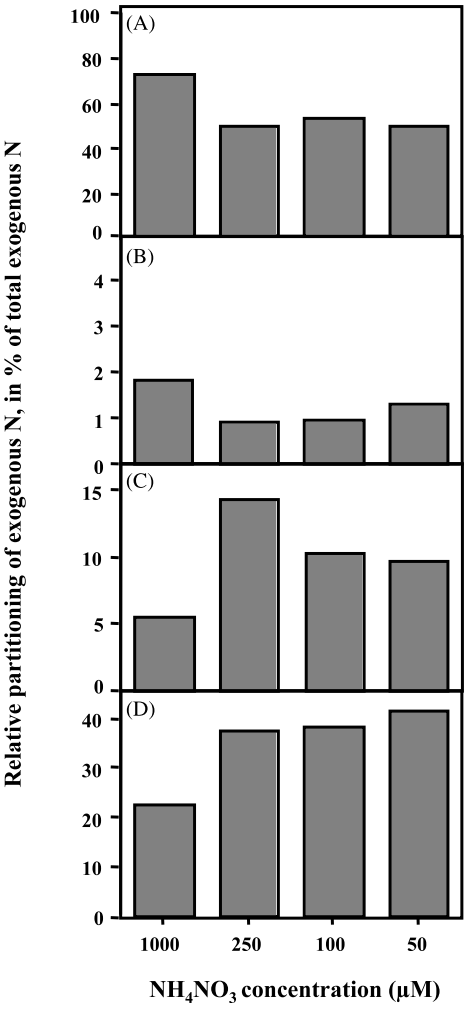

Fig. 4. Relative partitioning of N derived from the N uptake (as % of total N uptake) in different organs of alfalfa provided decreasing NH_4NO_3 concentrations (Experiment II: 1000, 250, 100 and 50 μM). (A) Regrowing shoots; (B) remaining shoots; (C) taproots and (D) lateral roots. Vertical bars indicate ±S.E. of the mean for $n = 4$.

low N (N25% or 250 μM NH_4NO_3, Fig. 5A and B). Asparagine was the most abundant amino acid and represented between 55 and 65% of the total amino acid pool in taproot irrespective of treatment (data not shown). Taproot nitrate concentration was also altered by N concentration of the nutrient solution (Fig. 5C and D). Compared with control plants, reducing NH_4NO_3 concentration of the nutrient solution from N100% to N25% (Fig. 5C) resulted in a slight decline in taproot nitrate concentration. In Experiment II (Fig. 5D) the taproot nitrate concentration was strongly reduced by decreasing mineral N from 1000 μM (3.39 mg N g^{-1} DW) to 250 μM (0.61 mg N g^{-1} DW) of NH_4NO_3.

3.5. Soluble protein and VSP concentrations in taproots

In comparison with total N, nitrate, and amino acid concentrations, the taproot protein concentration responded differently depending on the extent of N deprivation. For both experiments, the lowest soluble protein concentration was observed in taproots of control plants (Fig. 6A and B). The concentrations of soluble proteins increased with increasing N deprivation. The highest soluble protein concentrations were observed in taproots of plants grown at N50% and N25% (Experiment I, Fig. 6A) or when the nutrient solution only contained 50 μM NH_4NO_3 (Experiment II, Fig. 6B).

The effect of a restricted N supply on the pattern of VSP accumulation in taproots (Fig. 6C and D) was similar to that described for total soluble proteins (Fig. 6A and B). Compared with alfalfa grown with nutrient solution containing 1 mM of NH_4NO_3, the reduction of mineral N availability to 100 or 50 μM of NH_4NO_3 increased the soluble protein concentration by 22% (Fig. 6B) and the VSP concentration by 36% (Fig. 6D). These trends were confirmed using Western blot analysis of VSPs (Fig. 7A and B). For both experiments, Western blots showed that as N deprivation increased, that VSP increased markedly. In addition, accumulation of 32 kDa VSP was more extensive than the other VSPs as the intensity of N deprivation increased (Fig. 7).

4. Discussion

In both experiments, there was a significant reduction of shoot dry matter per plant and this was accentuated by the degree of N deficiency in the nutrient solution for the Experiment I only. This effect of N deprivation in reducing shoot biomass agrees with results obtained with field-grown alfalfa [36–38] and alfalfa grown under controlled conditions [39,40,27]. In Experiment II, the total dry matter was not significantly affected by the 250 or 100 μM of NH_4NO_3 treatments when compared with the plants receiving 1000 μM of NH_4NO_3 (Fig. 2B). These data suggest that the level of mineral N in nutrient solution containing 250 or 100 μM of NH_4NO_3 was not completely taken up by the plants between two renewals of nutrient solution (which occurred every 3 days in the Experiment II). This also indicates that, in our conditions of culture, the application of 250 or 100 μM NH_4NO_3 is not limitant for mineral N and does not result in a reduction of total growth. Moreover, in Experiment II our results showed that N deficiency reduced shoot/root dry weight ratio, and this effect was primarily due to an increase in root mass (taproot and lateral roots, Fig. 2B). This increase of root growth suggests that the plant is able to increase its capacity of N exploration when the availability of mineral N decreases. This change in biomass partitioning in favor of roots under N stress also agrees with previous results observed in alfalfa [39,41,26], other legumes such as

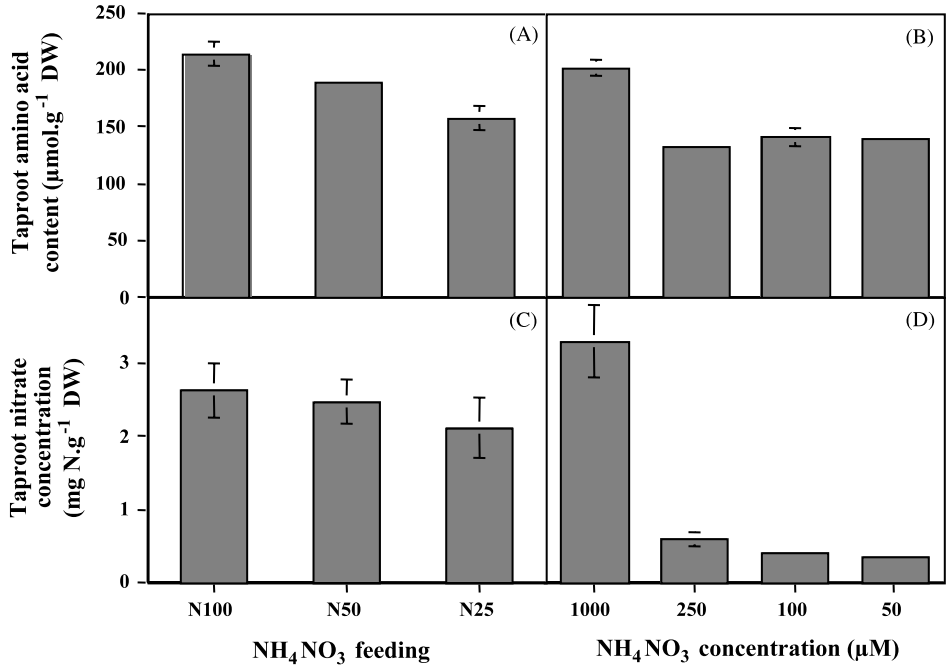

Fig. 5. Concentrations of amino acids (A, B) and nitrate (C, D) in taproots of alfalfa (*M. sativa* L., cv Lodi) after 21 days growth at different N concentrations. (A, C) Experiment I, and (B, D) Experiment II. Details of Experiments I and II are provided in Fig. 1. Vertical bars indicate ±S.E. of the mean for $n = 4$.

white clover (*Trifolium repens* L.) [42] or soybean [43], in chicory (*C. intybus* L.) [24], and in perennial grasses [44].

Results using $^{15}NH_4^{15}NO_3$ labeling gave us the opportunity to study the effect of N availability on the partitioning of N among different organs of alfalfa. These results showed that the relative partitioning of N to taproots increased at the expense of shoot tissues when N fertilization was reduced. Thus, N availability modified the partitioning of biomass and N, and led to changes in N partitioning within alfalfa taproots.

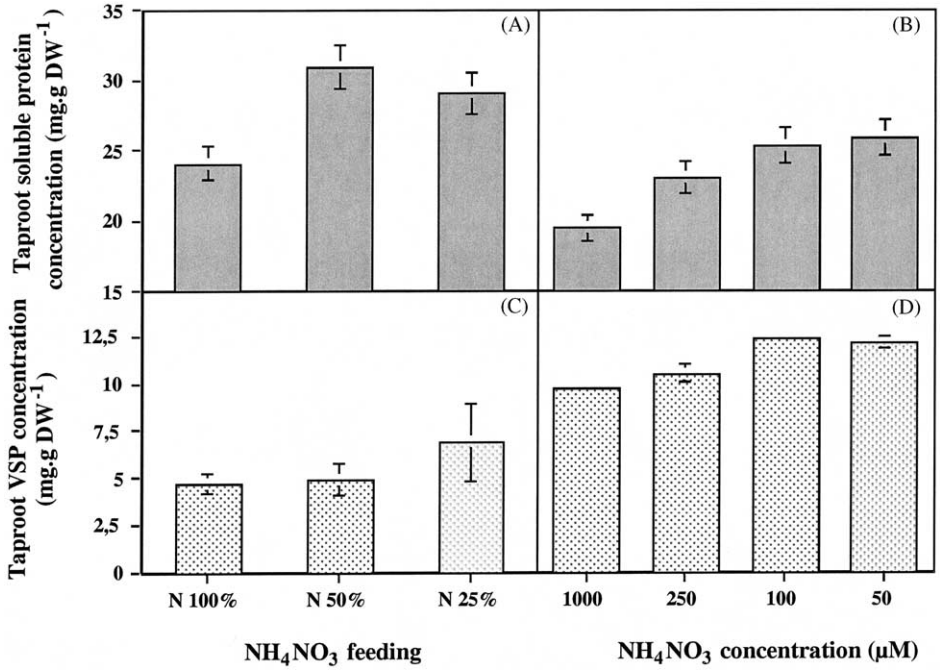

Fig. 6. Concentrations in total soluble protein (A, B) and VSP (C, D) in taproots of alfalfa (*M. sativa* L., cv Lodi) after 21 days growth at different N concentrations. (A, C) Experiment I, and (B, D) Experiment II. Details of Experiments I and II are provided in Fig. 1. Vertical bars indicate ±S.E. of the mean for $n = 4$.

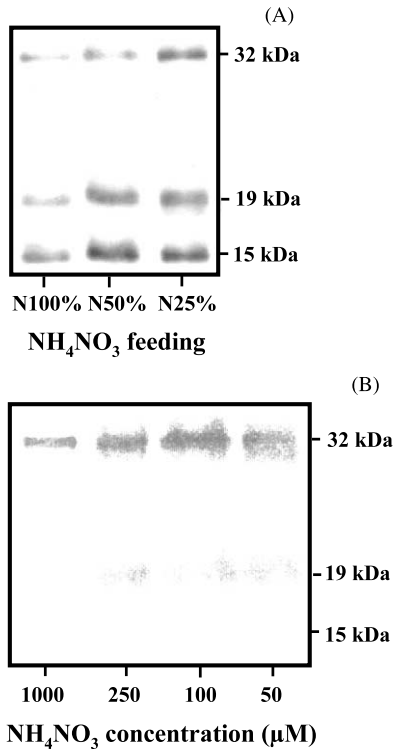

Fig. 7. Western blotting analysis of VSP (32, 19 and 15 kDa) accumulation in taproots of alfalfa (*M. sativa* L., cv Lodi) after 21 days growth at different N concentrations. (A) Experiment I, and (B) Experiment II. Details of Experiments I and II are provided in Fig. 1. Equal protein mass (30 μg) was loaded per lane. Values of molecular weight are indicated in kilodalton on the right side of the PVDF membrane.

However, total N, nitrate, and amino acid concentrations in taproots decreased as N concentration of the nutrient solution declined. Similar trends in taproot N concentration in response to N application have been reported previously by Trimble et al. [37] and Bélanger and Richards [38]. As previously reported by Girousse et al. [45] and Dhont et al. [46], asparagine represented the most abundant amino acid in taproots (up to 65% of the total amino acid pool) in both experiments (data not shown). This data confirmed that asparagine is an important form of stored N in alfalfa taproots. In contrast, we showed that the soluble protein concentrations in taproots (including VSP) increased with incremental increases in N deficiency. These results indicated that non-nodulated alfalfa was able to accumulate N reserves (mainly as VSPs) even when N concentration of nutrient solution was reduced. Similar results were obtained by Li et al. [47] who reported that N fertilizer did not change protein N concentration of alfalfa whereas root total N concentration increased. In the taproot of oilseed rape (*Brassica napus* L.), Rossato et al. [48] recently showed that increasing the supply of N from 1 to 50 mM KNO_3 in the nutrient solution did not increase the accumulation of a putative 23 kDa VSP. In chicory (*C. intybus* L.), Améziane et al. [24] reported that VSP accumulation was unaffected by the increase of N supply. During chicory senescence, the remobilization of previously absorbed N from the shoot to the tuberized root greatly increased in N-limited plants, whereas it increased only slightly in N-sufficient plants [24]. As a consequence, these authors reported the accumulation of the N reserves (mainly as VSP and arginine) also occurred in N-limited plants. The present study contradicts results obtained in soybean [22,49] and poplar [21] where accumulation of VSP polypeptide and transcript significantly increased with improved N availability. For example, Staswick [49] has showed that soybean leaf VSP and VSP mRNA levels correlated with the amount of N provided for non-nodulated plants. Under N deficiency, steady-state transcript levels for soybean leaf VSP was nearly undetectable whereas, at near toxic concentrations of N, soybean VSP transcript levels were greater than in N_2 fixing control plants [17]. These different responses to high N fertilization could be explained by the specific tissue localization of VSP, which are stored in the leaf of soybean (representing a strong sink for N) versus the taproot of alfalfa (representing a low sink for N). However, because there was less need to mobilize N from leaves to pods of soybean when mineral N was plentiful, Staswick [4] suggested that the effect of N availability during plant growth was related to altered source/sink relationships for N, rather than having a direct regulatory role. In alfalfa, our results suggested that VSP accumulation in taproots was not directly regulated by availability of N, but controlled indirectly via the changes of N partitioning and demand between source and sink organs.

Regardless of the mineral N limitation method used, both of our experiments showed that N deficiency led to increase taproot soluble protein (particularly VSP) concentrations. However, this positive effect of low N availability on VSP concentrations could be concomitant with an increase of taproot biomass (Experiment II) or not (Experiment I). Previous field experiments [50,51] also reported that root dry matter and concentrations of soluble proteins and VSP in roots may fluctuate together. For example, Justes et al. [51] have shown that N fertilization (100 kg ha^{-1}) at sowing did not improve root mass, total N, soluble protein, or VSP concentrations of roots when compared with alfalfa grown without mineral N supply, and that this resulted in similar shoot growth rates the following spring. However, under hydroponic conditions, our previous studies [25,52] showed that the increase of taproot dry matter did not parallel increases in taproot soluble protein or VSP concentrations. These studies showed that the increase in N concentration of the hydroponic solution from 1 to 5 mM KNO_3 [25] or 1–20 mM NH_4NO_3 [52] during 21 days strongly increased the total dry matter production (and root biomass) of 4-month-

old alfalfa plants, but did not significantly affect N partitioning between shoots and roots, and had no influence on root VSP concentration. Our results also showed that non-nodulated alfalfa was capable of adjusting its N storage as a function of the level of mineral N availability. With high mineral N availability, alfalfa could increase N reserves by increasing the amino acid and nitrate concentrations without changing in the concentration of soluble protein pools. In the case of N deficiency, alfalfa could increase the size of N reserves in taproots by increasing soluble protein (including VSP) concentrations and/or the size of this storage organ. As previously suggested by Millard [1], N storage as protein in N-deficient plants may have several advantages for plants. For example, the storage of N in the form of protein in vacuoles of taproot cells avoids the potential osmotic problems that accompany the accumulation of N as nitrate. This makes it possible to sequester N for extended periods without major consequences for cellular metabolism.

In conclusion, increasing exogenous N concentrations did not increase N reserve accumulation as VSP in alfalfa taproots. Protein N storage appeared to be controlled by the source/sink status and by the N demand of roots. Overall these results agree with the hypothesis of Chapin et al. [3] who suggested that, from an ecological point of view, optimized N cycling and storage within a plant were required in species adapted to low N environments. Moreover, because it was reported that alfalfa regrowth in spring or after defoliation was linearly correlated with taproot soluble protein concentrations [10,51], this adaptive response to low soil N environments also makes it possible for alfalfa to go dormant and perenniate, while awaiting more favorable conditions before resuming growth.

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