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Seasonal changes of nitrogen storage compounds in a rhizomatous grass *Calamagrostis epigeios*

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Abstract

The seasonal dynamics in content and distribution of N-rich compounds between overwintering organs of *Calamagrostis epigeios* were examined. Samples were taken both from plants grown in natural conditions and in containers with controlled nutrient supply. There were significant changes in content of nitrate, free amino acids and soluble protein in all investigated plant parts during the course of a year. Amino acids showed both the highest maximum and seasonal fluctuation among the all N compounds observed and, therefore, appear to have a central role in N storage. Their content rises in the autumn, remains stable during winter and declines quickly at the beginning of spring. The most abundant amino acids in the end of winter storage period - asparagine, arginine and glutamine - constituted about 90 % of N in fraction of free amino acids. The portion of N stored in soluble proteins, however, was considerably smaller compare to both amino acids and nitrate. The amount of N stored in rhizomes of *C. epigeios* was smaller than in roots and stubble base before the onset of spring re-growth. This indicates that roots and stubble base are particularly important for winter N storage in this species.

Additional key words: free amino acids, nitrate, nitrogen mobilization, vegetative storage proteins.

Introduction

Perennial species often use their belowground structures not only for uptake of nutrients and water but also for storage of nutrients. Nutrient stores in roots and rhizomes allow perennial plants easily overcome fluctuations in actual nutrient availability. Significant role of storage compounds in plant survival and re-growth after cutting was extensively studied with forage species (see Volenec *et al.* 1996 for review), but information on wild species is very scarce. The role of saccharides, which are usually the prevailing storage compounds in plants, was originally considered as dominant for plant re-growth. However, several experiments showed only weak or no relationship between the amount of storage saccharides and the rate of re-growth (Volenec and Nelson 1984, Hogg and Lieffers 1991a,b). On the other hand, there was found close positive correlation between content of nitrogen compounds in remaining organs and the re-growth rate (Ourry *et al.* 1994, Volenec *et al.* 1996). The ability of plants to take up and assimilate inorganic nitrogen is lowered or stopped both in early spring and after defoliation (Clement *et al.* 1978, Ourry *et al.* 1990, Thornton and Millard 1993). The plant demand for nitrogen is met by the N uptake from soil after the restoring of positive carbon balance of the whole plant (Clement *et al.* 1978). Thus, the availability and mobilization of nitrogen reserves must be considered as an important factor in survival and competitive ability of perennial plants.

Nitrogen is primarily stored as storage proteins, free amino acids and nitrate ions (Millard 1988, Chapin *et al.* 1990). Nitrate is an important storage compound in ruderal and crop species (Rosnitschek-Schimmel 1985a, Yoneyama 1991). However, it is not a much suitable nitrogen source for growth when the rate of photosynthesis is limited because reduction and assimilation of nitrate have a high requirements for the energy and carbon skeletons. Many plant species accumulate amino acids and amides in their vegetative tissues in large quantities (Millard 1988). Several studies showed importance of free amino acids for N storage in

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below-ground structures (Rosnitschek-Schimmel 1985b, Sagisaka 1987, Lähdesmäki *et al.* 1990, Nordin and Näsholm 1997). Vegetative storage proteins may also serve as a mobilizeable nitrogen reserve. Convincing evidence for their importance has recently been summarised by Stepien *et al.* (1994, woody plants) and by Staswick (1994, herbaceous species). It is now clear, that the contribution of storage proteins in plant tissues to whole-plant N storage may be in many cases significant and should not be ignored.

The building of N stores and their effective use for fast re-growth in early spring could be one of the important traits supporting extraordinary successful growth and spreading of perennial grass *Calamagrostis epigeios* L. Roth. The extensive belowground organs (roots and rhizomes) of this species may serve not only for an efficient nutrient uptake and vegetative spreading but also for storage and translocation of stored compounds. Regular analyses of plant biomass from field plots were conducted to show changes in content of the N-storage compounds during annual vegetative cycle. However, the analysis of field samples does not show the real amount of N stored in plant, because the dry mass of belowground organs can not be precisely estimated in the field. Thus, a biomass of plants cultivated in containers with controlled supply of nutrients and water was also examined. The natural fluctuation of soil nitrogen availability as well as short-term fluctuation in plant N content were minimized in these plants. Known amounts of belowground biomass enabled quantification of nitrogen in storage compounds allocated in overwintering plant parts.

Materials and methods

Sampling and analysis of field-grown plants: The samples were taken from experimental plots established on one year old forest clearing 30 km west of Brno at an altitude of 400 m above the see level. The soil was classified as humic podzol with pH 4.2 (extracted with water). Average soil temperature in depth 10 cm during sampling (Fig. 1) varied from 1 °C (in February) to 16 °C (in July). Population of *C. epigeios* was in initial stage of colonization when sampling started. Selected plants of *C. epigeios* were collected monthly approximately 5 h after the sunrise. In each of the 7 experimental plots, 10 to 20 plants were collected to get sufficient amount of biomass for chemical analyses. Immediately after the sampling the plants were divided in roots, rhizomes, stubble bases (first 2 cm of the stubble above roots) and the rest of above-ground parts. Samples were then frozen at -80 °C, lyophilised for 48 h and ground. The extract of plant material in 0.05 M phosphate buffer (pH 7.5) was used for all chemical analyses. Nitrate was determined after reduction to nitrite (Cataldo *et al.* 1975) and the content of soluble proteins by the staining with the Coomassie Brilliant Blue (Bradford 1976) using bovine serum albumin as a standard. Content of total free amino acids was estimated with ninhydrin (Rosen 1957) using leucine as a standard. The proteins were removed from extract prior the analysis of amino acids by precipitation with 5 % (final concentration) sulfosalicylic acid followed by centrifugation (14 000 *g* for 20 min) because of the possible interference with assay.

The development of leaf area of plants was measured non-destructively on 21 selected plants (3 per plot) from April to July. The area of single leaf was estimated from its length multiplied by breadth and by a coefficient that describes a relationship between the result of multiplication leaf length times breadth and the real area of respective leaf. A reference set of ten excised leaves of various sizes was used for calculation of the coefficient and this determination was done for each set of field measurements separately. Leaf area of the whole plant was then calculated as a sum of area of all green leaves.

Analysis of cultivated plants: Plants were grown from June 1999 to July 2000 at the University Campus in Brno. Single plant seedlings were planted into 4 dm^3 plastic containers filled with the pure inorganic substrate (quartz sand and zeolite, 1:1). Plants were watered sufficiently with demineralised water and once weekly received modified Hoagland nutrient solution (100 cm^3 per container) containing 10 mol m^{-3} of N in form NH_4NO_3. Plants were harvested in regular intervals from September to July but only the sample sets with the highest and the lowest contents of N-rich compounds were chosen for this paper. Six plants per harvest were divided into leaves, stubbles, stubble bases, rhizomes and roots. Samples were then frozen in -80 °C, lyophilized for 48 h. Ground dry biomass from two plants was pooled and used as one replicate for chemical analysis. The content of nitrate and total soluble proteins was determined in the same way as in the samples from the field. Free amino acids were extracted with 0.01 M HCl for 1 h and then analysed as their 9-fluorenylmethyl formate (FMOC) derivates on HPLC (*Waters Alliance 2690XE*, *Waters 474* fluorescence detector, Milford, USA) as described in Näsholm *et al.* (1987) with modification according to Nordin and Näsholm (1997). Altogether 18 amino acids were routinely detected including asparagine, glutamine, aspartic and glutamic acids, serine, arginine, threonine, tyrosine, alanine, methionine, valine, phenylalanine, isoleucine, leucine, proline, gamma-aminobutyric acid, ornithine and lysine. The nitrogen content of soluble proteins was estimated as a sum of N contents in single amino acids resulted from hydrolysis of soluble protein fraction with 6 M HCl for 16 h. The total amount of nitrogen per organ and

N-compound (nitrate, free amino acids, soluble proteins) was calculated as nitrogen content in compound [mg N g^{-1}(d.m.)] multiplied by dry mass of the pertinent organ.

Statistics: The *SPSS v. 7.0* statistical package (*SPSS Inc.*, Chicago, USA) was used to evaluate the results. The analysis of variance was employed and the multiple comparison of means was based on the method of LSD contrasts. The homogeneity of variances was checked by Bartlet's and Cochran's tests, and heterogeneous sets of data were log-transformed before calculation.

Results

Field-grown plants: The leaf area of plants was rapidly increasing from April till the end of May but there was only minor change from June till the end of July (Fig. 1). The content of nitrate (Fig. 2*A*) was the highest in the end of vegetation season and during winter. Its minimum values were recorded at the beginning of vegetation period in roots an stubble bases but in rhizomes was the minimum in July. The content of free amino acids (Fig. 2*B*) in rhizomes declined dramatically in April by about 40 %. Subsequent decrease was slower but significant and lead to the minimal amino acid content 10 mg g^{-1}(d.m.) in July, which represents approximately 15 % of the content recorded in March. Similar changes were also found in stubble base. The content of amino acids in roots reached only 30 to 50 % of the content in rhizomes and its decline from March to June was slower. The content of soluble proteins (Fig. 2*C*) in roots and rhizomes was smaller than in case of amino acids but with similar annual changes. The soluble protein content dropped to its minimum in July in all analysed organs and started to increase again in autumn.

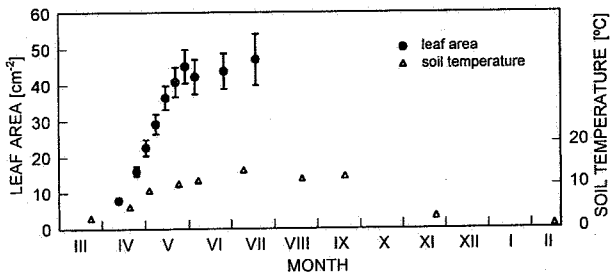

Fig. 1. Growth of *Calamagrostis epigeios* plants in the field as described by average leaf area of the whole plant. The average soil temperature in depth 10 cm on sampling days in experimental plots. *Vertical bars* indicate SE (if larger than symbols), $n = 21$ (leaf area) and 7 (temperature).

Cultivated plants: The amount of N that was found in nitrogen storage compounds in roots, rhizomes and stubble bases was several times higher at the beginning of March than in June (Table 1). In March there was the greatest portion of nitrogen present in amino acids (Table 2), namely in asparagine, glutamine and arginine (Fig. 3), whereas the smallest amount of N was in soluble protein fraction. In June there was only minor difference between contents of N present in form of nitrate, free amino acids and soluble proteins. Significantly lower content of N present as amino acids was found in roots compare to rhizomes and stubble bases in March as well

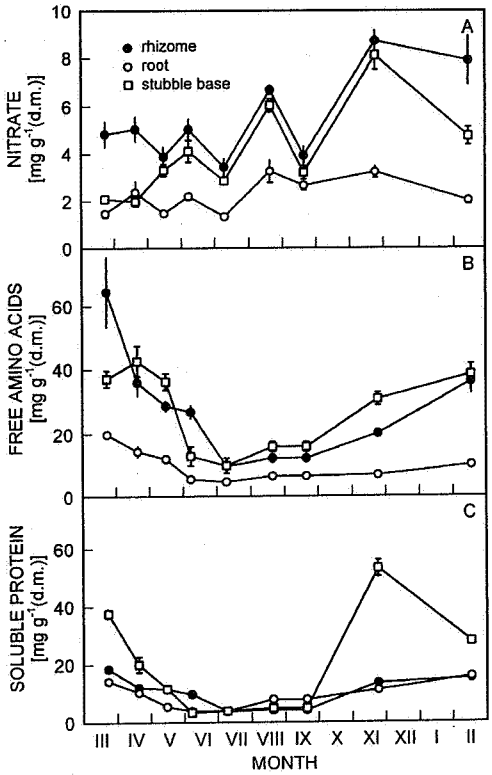

Fig. 2. Monthly variation of nitrate (*A*), free amino acids (*B*), and soluble proteins (*C*) in roots, rhizomes and stubble bases of *Calamagrostis epigeios* plants in the field. *Vertical bars* indicate SE (if larger than symbols), $n = 7$.

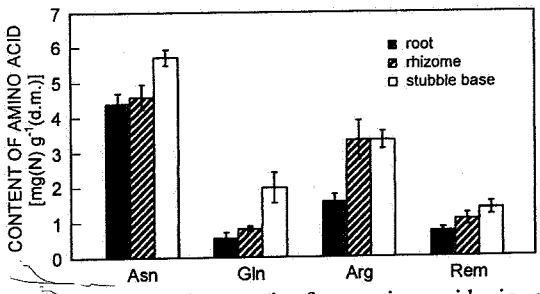

Fig. 3. Content of nitrogen in free amino acids in over-wintering organs of *Calamagrostis epigeios* on 1st March. Asn - asparagine, Gln - glutamine, Arg - arginine, Rem - sum of nitrogen in remainder pool of free amino acids analyzed. Means ± SE, $n = 3$.

as in June. The highest total amount of N allocated in all perennial organs in March (Table 2) was in form amino acids whereas amounts of N allocated in nitrate and soluble proteins did not differ. The size of N reserves in roots and stubble bases was similar but there was significantly less N allocated in rhizomes (Table 2).

Table 1. Nitrogen content [mg g^{-1}(d.m.)] as nitrate, free amino acids and buffer soluble protein in perennial organs of *C. epigeios*. Cultivated plants were harvested on 1 March and on 1 June. The letters indicate significance between types of N compounds, the asterisk indicates significantly different mean within N compounds and sampling season ($P < 0.05$). Mean ± SE ($n = 3$).

	Season	Nitrate N	Free amino acid N	Soluble protein N
Root	March	2.10 ± 0.07 b	7.24 ± 0.55 c*	1.39 ± 0.04 a
	June	0.45 ± 0.04 a*	0.37 ± 0.04 a*	0.68 ± 0.05 b*
Rhizome	March	2.32 ± 0.16 b	9.77 ± 0.93 c	1.54 ± 0.11 a
	June	0.95 ± 0.03 a	0.82 ± 0.06 a	0.85 ± 0.03 a
Stubble base	March	1.99 ± 0.26 b	12.35 ± 0.51 c	1.32 ± 0.17 a
	June	0.73 ± 0.05 a	0.93 ± 0.17 a	0.94 ± 0.06 a

Table 2. The amount of N [mg] present in organs of cultivated *C. epigeios* plants on 1 March. The amount of N was summarized separately for nitrate, free amino acids and buffer soluble protein fractions. Sum N represents the amount of N allocated in all three groups of compounds in particular organ. The letters indicate significant difference between types of N compounds, asterisk indicates significantly different means between plant organs ($P < 0.05$). Mean ± SE ($n = 4$ - 6).

	Nitrate N	Free amino acid N	Soluble protein N	Sum N
Root	4.19 ± 0.85 a	14.82 ± 3.31 b	2.78 ± 0.57 a	22.14 ± 3.34
Rhizome	2.36 ± 0.45 a	10.21 ± 2.33 b	1.56 ± 0.30 a	12.33 ± 1.84*
Stubble base	3.01 ± 0.51 a	18.94 ± 2.91 b	2.00 ± 0.34 a	22.83 ± 0.94

Discussion

All types of N storage compounds often vary seasonally in their concentrations (Millard 1988) and this was also the case in *C. epigeios* plants. The content of nitrate in plants in the field was fluctuating during the vegetation season but there was a distinct maximum found in December. Similar results were found also with other perennial species (Cyr and Bewley 1989, Cyr *et al.* 1990). This pattern probably reflects the relationship between the good availability of nitrate in soil and very limited potential of plants for nitrate reduction. The rate of nitrate reduction declines together with its transport rates to senescing shoot parts in the autumn. At the same time the content of nitrate in below-ground parts rises, because the capacity of roots of *C. epigeios* for NO_3^- reduction is very low compare to shoots (Seidlová 1998). An increase in nitrate content during winter dormancy when soil temperature was above the freezing point has been previously observed (Rosnitschek-Schimmel 1985a and references therein).

Of all investigated nitrogen storage compounds, the free amino acids showed both the highest maximum and seasonal fluctuation in all organs of *C. epigeios*. The accumulation of large quantities of amino acids in vegetative tissues was shown in many species (Cyr *et al.* 1990, Sagisaka 1987, Nordin and Näsholm 1997). Their content in belowground organs usually rises in autumn, remains stable during winter and declines quickly at the beginning of the new vegetation season (Cyr and Bewley 1989, Cyr *et al.* 1990, Rosnitschek-Schimmel 1985b). The same basic pattern was observed in *C. epigeios*. The marked increase in amino acid content in autumn is closely connected with remobilization of N from senescing plant parts (Millard 1988) but may also reflect the temporary higher availability of N in the soil.

The composition of free amino acid pool varies considerably during the growing season. High levels of glutamine, asparagine, arginine and alanine usually accumulate in belowground organs before the winter dormancy (Rosnitschek-Schimmel 1985b, Nordin and Näsholm 1997). These compounds possess low C to N ratio and thus minimise the requirement of carbon skeletons for N storage. The most abundant free amino acids from roots, rhizomes and stubble bases of *C. epigeios* plants grown in controlled conditions were in March asparagine, arginine and glutamine. The predominant accumulation of asparagine and glutamine is a common trait also for other grass species (Sagisaka 1987, Nordin and Näsholm 1997).

High variability in amino acid content during vegetation in comparison to proteins and close correlation between their depletion rate and plant re-growth indicate their role as the main N storage compounds (see also in Sagisaka 1987, Rosnitschek-Schimmel 1985b, Lähdesmäki *et al.* 1990). In comparison to storage proteins, amino acids are readily accessible for growth processes (no enzymatic decomposition is necessary), and their good solubility facilitate rapid mobilization of internal N resources in spring as well as after defoliation. The maximum values and the amplitude of changes in the content of amino acids during vegetation suggest that also in *C. epigeios* amino acids are the most important N-storage compounds.

The role of soluble proteins in nitrogen storage was assumed to be minor (Sagisaka and Araki 1983, Rosnitschek-Schimmel 1985b). However, the experiments with perennial weeds (Cyr and Bewley 1989, Cyr *et al.* 1990) showed similarly significant annual changes in soluble protein as those in the amino acid fraction. Recently published two reviews (Staswick 1994, Stepien *et al.* 1994) summarized experimental evidence for the importance of proteins for N storage in vegetative organs of some species (namely dicots and woody species). In contrast to findings of Nordin and Näsholm (1997) there were found significant changes in the content of soluble proteins in *C. epigeios* organs. Therefore, the contribution of proteins to N storage in this species was carefully evaluated.

The seasonal changes in content of N-rich compounds in plant biomass are only a raw measure of the importance of particular N store. Better evaluation of the importance of each compound in N-storage is the portion of N available to the growing tissues. However, the precise calculation of size of N reserves in mg(N) g^{-1}(d.m.) is less frequent (Rosnitschek-Schimmel 1985a, Nordin and Näsholm 1997). Plants of *C. epigeios* cultivated in containers were used to clarify the importance various types of N-reserves. In March, most of the N-reserves was present as free amino acids in all perennial organs supporting hypothesis that amino acids play a key role in N storage (Sagisaka 1987, Nordin and Näsholm 1997). Conversely, the amount of N in to the soluble proteins was in March several times smaller than N stored in amino acids and also significantly lower than N present in nitrate. This was probably due to both lower total content of soluble proteins in the tissues (when compared with amino acids) and relatively low portion N in protein molecules (when compared with nitrate). Therefore, soluble proteins probably play in N storage only marginal role in *C. epigeios*.

Roots and rhizomes are the main storage organs in perennial dicots (Heilmeier and Monson 1991, Volenec *et al.* 1996). On the other hand the stubble base is an important storage compartment for grass species since it contains basal leaf meristems. Roots and stubble base in *C. epigeios* plants are equally important N-stores during winter whereas rhizomes contain less N-reserves. This finding fits well with previous results showing that content of storage compounds in rhizomes of *Calamagrostis canadensis* is only poorly related to re-growth capacity of plants (Hogg and Lieffers 1991a). The relationship between the size of N reserves and spring re-growth capacity of *C. epigeios* plants in controlled conditions will be described in a following paper (Gloser *et al.*, in preparation).

In summary, there were found significant changes in content of nitrate, free amino acids and soluble proteins in overwintering parts of *C. epigeios*. Amino acids are probably the most important compounds for N-storage in this species because the highest portion of nitrogen was stored in this form. The highest level of N reserves for spring re-growth was found in roots and stubble bases of *C. epigeios* plants.

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