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The consequences of lower nitrogen availability in autumn for internal nitrogen reserves and spring growth of *Calamagrostis epigejos*

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Abstract

The building and use of internal N stores in the grass *Calamagrostis epigejos* was investigated in context of complex ecological study focused on mechanisms underlying competitive ability of this highly successful invasive species. Induced changes in nitrogen availability in the course of two subsequent vegetation seasons were used as a tool for finding (i) to what extent high N availability in substrate is important for building N reserves in autumn that support spring regrowth and, (ii) if contrasting contents of N storage compounds may result in differences in growth in the next season. Plants were grown in solely inorganic substrate and received a nutrient solution containing 5 mol m^{-3} of NH_4NO_3. The nitrogen supply was reduced in a low nitrogen (LN) treatment to 0.25 mol m^{-3} in August whereas in high nitrogen (HN) treatment remained high till December. During the following growing season were plants from both treatments grown at the low N supply (0.25 mol m^{-3}). An increase in the content of N storage compounds was observed from September to December in both treatments. Plants in the LN treatment showed significantly lower total N content and also N allocated to mobilizable reserves (20–50% of HN plants), namely due to a smaller accumulation of amino acids and soluble protein in autumn. External nitrogen availability in autumn is hence highly important for building N reserves in this species. A major portion of the nitrogen stored in HN plants during winter was taken up from growth medium in late autumn, whereas translocation from senescing shoots dominated in LN treatment. During the winter about 50% of N in plants was permanently present in shoots bearing several frost resistant green leaves. Spring regrowth was accompanied by a fast decrease of both total N and the content of N storage compounds in both treatments. Amino acids were identified as the most prominent source of mobilizable N during spring regrowth. Development of leaf area in LN plants was significantly slower in March and April than in HN plants namely due to smaller number of tillers and green leaves per plant. Low N availability in autumn, therefore, may result in restrictions of plant growth and development in the following season.

Introduction

The storage of nutrients in belowground organs allows perennial species to overcome periods when the demand of growing tissues for mineral nutrients is greater than the external supply. The significant role of nitrogen storage compounds in the support of plant regrowth has been illustrated in several reviews (Heilmeier and Monson 1994; Stepien et al. 1994; Volenec et al. 1996). Amino

acids, soluble protein or nitrate were recognized as the main compounds used in plants for storage of nitrogen that is subsequently used for building new shoot biomass (Ourry et al. 1988; Hendershot and Volenec 1993; Thornton and Millard 1993).

The size of nitrogen reserves can fluctuate any time during the whole vegetation season (e.g. after defoliation) but its greatest increase takes place at the end of the growing season (Cyr and Bewley 1989; Cyr et al. 1990; Gloser 2002). The content of nitrogen storage compounds in overwintering organs usually rises at the end of the vegetation season and is often positively related with plant winter survival and shoot regrowth in spring (Chapin et al. 1990; Volenec et al. 1996). The content of nitrogen storage compounds may rise in overwintering organs in response to several processes: (a) increased nitrogen availability in the soil, (b) the uptake of nitrogen being less limited than its assimilation and use for growth, c) the translocation of nitrogenous compounds from senescing shoot parts and (d) the synthesizing and accumulating of compounds with a cryoprotective function (i.e. proline). The actual increase in content of nitrogen reserves usually depends on a combination of several of these processes. Little is known, however, about relative importance of these individual processes to final amount of N stored in plants.

Nitrogen reserves support the regrowth of perennial plants primarily in early spring, when the soil temperature is low and, consequently, the mineralization and uptake of nitrogen are reduced or stopped (Clarkson et al. 1992; Menyailo and Huwe 1999). Rapid decrease of nitrogen storage compounds in spring was shown in several experiments (Rosnitschek-Schimmel 1985; Cyr et al. 1990; Hendershot and Volenec 1993; Gloser 2002). In contrast to numerous studies focused on use of N storage compounds in plants after defoliation, very little is known about the relationship between the size of N reserves in perennial plants during winter dormancy and plant growth and development in the new vegetation season.

In this study the importance of internal nitrogen stores was evaluated in a perennial, invasive grass *Calamagrostis epigejos*, as a part of more complex research projects focused on mechanisms underlying competitive ability of this species (Gloser and Gloser 1996; Gloser et al. 1996). *C. epigejos* showed not only strong growth response to nitrogen fertilization (Rebele and Lehmann 2001) but also significant seasonal dynamics of nitrogen compounds in overwintering organs in the field (Gloser 2002). In experiments described in the present paper induced changes in nitrogen supply to plants grown in sand culture were used to test: (i) to what extent high N availability during the last few month before dormancy can modify total amount and composition of N reserves in overwintering organs of *C. epigejos*, and (ii) if induced changes in status N storage compounds in overwintering organs *C. epigejos* may result in significant differences in growth and development of the plants in the next vegetation season.

Methods

Cultivation of plants

Calamagrostis epigejos (L.) Roth. plants were grown from June 1999 to July 2000 at the university campus in Brno. Single plant seedlings were planted into plastic containers filled with purely inorganic substrate (quartz sand and zeolite, 1:1). The plants were exposed to natural precipitation and watered sufficiently with demineralized water when necessary. Once a week they received modified Hoagland nutrient solution (100 ml per container) containing 5 mol m^{-3} of NH_4NO_3. At the end of August the growth medium in each container was washed with demineralized water and plants were randomly divided into two groups. Once a week one group received 100 ml of the same nutrient solution as before (= high nitrogen treatment, HN) while the second group received the nutrient solution with a concentration of NH_4NO_3 only 0.25 mol m^{-3} (= low nitrogen treatment, LN) with the concentrations of other nutrients kept unchanged. Plants were supplied with these nutrient solutions until mid December when the substrate became frozen. From February 2000, all the remaining plants received weekly 100 ml of the nutrient solution with 0.25 mol m^{-3} of NH_4NO_3. Plants were harvested in September and December 1999 and from March to July 2000 once a month. Altogether seven harvests were done. Six plants from each treatment were washed with tap water and divided into roots, rhizomes, stubble bases (basal 20 mm of the shoot), stubbles (the rest of the shoot above stubble base) and

leaves. Leaf area was measured using a computer scanner and simple program for image analysis. Number of tillers included all true stubbles found on examined mother plant and its ramets longer than 20 mm. The samples of biomass were then frozen at −80 °C, and then lyophilized for 48 h (Heto Maxi Dry Lyo, Denmark). Dry samples were weighed and biomass of each organ was pooled from two plants, ground and used as one replicate for chemical analysis. Calculation of root to shoot ratio involved root and rhizome biomass as a root part and stubble base, stubble and green leaves as a shoot part. Dry leaves were not included in comparisons of total plant biomass because the degree of their decomposition was highly variable between harvests.

Chemical analyses

Lyophilized biomass samples were extracted in phosphate buffer (0.05 M, pH 7.5) and the extract was used for determining the nitrate content (Cataldo et al. 1975) and total soluble protein (Bradford 1976). Free amino acids were extracted with 0.01 M HCl for 1 h. This extraction method was optimized for high yield of amino acids namely with low C:N ration in molecule (Nordin 1998). Extracts were analyzed as their 9-fluorenylmethyl formate (FMOC) derivates on HPLC (Waters Alliance 2690XE, Waters 474 fluorescence detector, Milford, USA) as described in 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 in fraction of free amino acids was estimated as a sum of nitrogen from all quantified amino acids in sample. Total nitrogen concentration in plant biomass was determined by combustion gas chromatography (Elemental Analyser 1106, Carlo Erba, Italy). Calculation of N content in organs was based on organ dry mass and corresponding N concentration. Amount of N present in amino acid was calculated as a sum of N present in all amino acids analyzed. Nitrogen content in soluble proteins was estimated in a similar way, i.e., in amino acids present in protein hydrolysate.

Statistics

The effects of experimental factors were tested by the multifactorial analysis of variance. STATISTICA v. 5 (StatSoft Inc., Tulsa, USA) was used to evaluate the results. 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

Plant growth and development

Plants in both treatments increased their dry mass similarly from September to December by about 50% (Figure 1). Also regrowth of plants in spring did not result in significant differences in total dry mass between treatments (Table 1, Figure 1). The root to shoot dry mass ratio (RSR) was almost doubled in the LN treatment in December compared to HN plants and then declined (Figure 2) but remained significantly higher in the LN treatment until the end of the experiment (Table 1, Figure 2).

The LN treatment had a negative effect on the plant leaf area during the whole cultivation period (Table 1, Figure 2). The leaf area of the HN plants was particularly higher than that of the LN plants from December to April (Figure 2). Both the higher amount of leaf dry mass and higher leaf area per unit of leaf dry mass (SLA) in HN plants were responsible for the observed difference (data not shown). Tillers were more numerous in HN plants in early spring (March and April, Figure 2) but their number decreased later due to high mortality, and, eventually, in May did not differ from LN plants.

Plant chemical composition

Nitrogen content in whole plants (including shoots) increased substantially in HN plants from September to December, but minimal change occurred in LN plants (Figure 3). When changes in total N content were examined separately in root, rhizome and stubble base an increase was

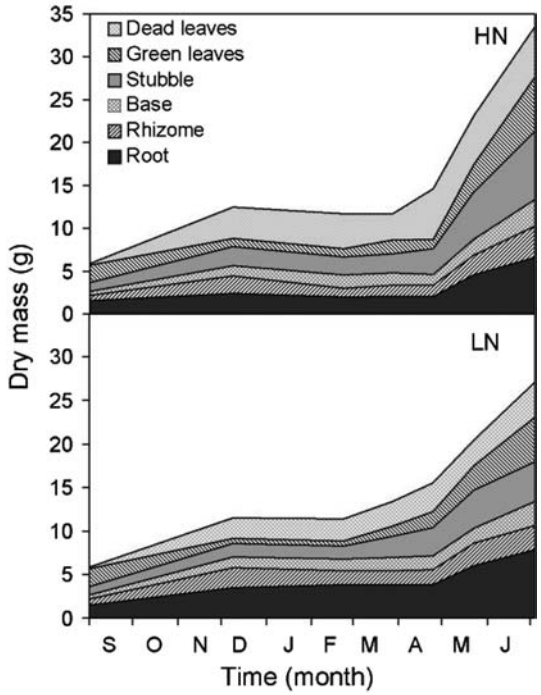

Figure 1. Seasonal changes in contribution of plant organs and dead shoot biomass to total dry mass of *C. epigejos*. Shown are cumulative data of dry mass per plant for each sampling date. Plants were exposed to high (HN) or low (LN) nitrogen availability from September to December and to low N availability thereafter. Mean values from six plants.

Table 1. Results of multifactorial ANOVA of parameters describing growth and development of plants.

	DM	LA	TN	RSR
Sampl. date	**0.000**	**0.000**	**0.000**	**0.000**
Nitrogen	0.388	**0.000**	**0.000**	**0.000**
S × N	**0.025**	0.053	0.075	**0.000**

p-Values indicate significance of the effects of sampling date, availability of nitrogen and their interaction. DM – plant dry mass (data in Figure 1), LA – leaf area (Figure 2), TN – number of tillers per plant (Figure 2), RSR – root to shoot dry mass ratio (Figure 2).

found in both HN and LN plants during this period. The ANOVA of N content data showed highly significant effects of sample date, nitrogen availability and interaction these two factors for all examined organs. The content of nitrogen in storage compounds in root, rhizome and stubble base in autumn followed the pattern found for total N (Figure 4). The content of amino acid N and protein N was, however, significantly lower in

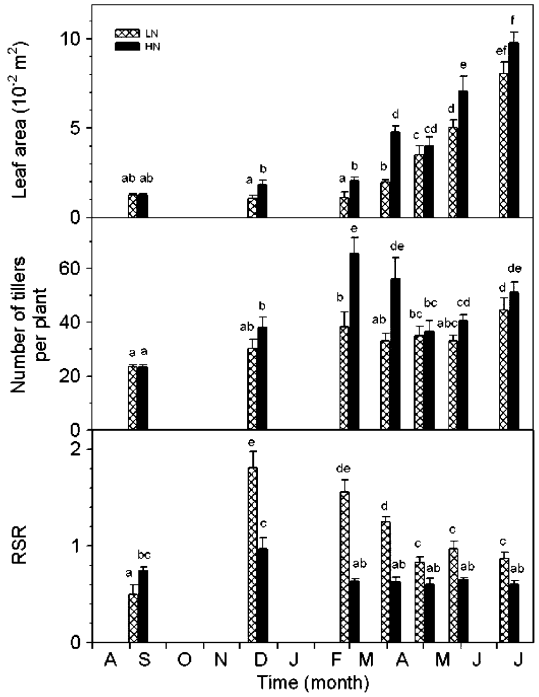

Figure 2. Seasonal changes in leaf area, number of tillers per plant and root to shoot dry mass ratio (RSR) of *C. epigejos*. Plants were exposed to high (HN) or low (LN) nitrogen availability from September to December and to low N availability thereafter. Vertical bars indicate mean ± SE, $n = 6$. Dissimilar letters above columns denote significant difference ($p > 0.05$).

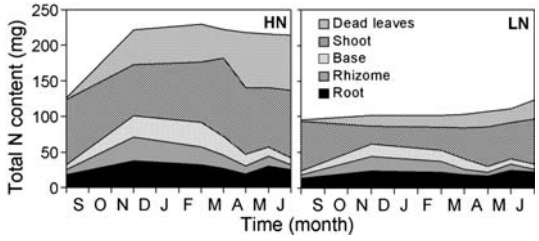

Figure 3. Seasonal changes in contribution of roots, rhizomes, stubble base, the rest of shoot and dead leaves to total nitrogen content of *C. epigejos* plants. Shown are cumulative data of N mass per plant for each sampling date. Plants were exposed to high (HN treatment) or low (LN treatment) nitrogen availability from September to December and to low N availability thereafter. Mean values from six plants.

LN than in HN treatment. The portion of insoluble N remaining after subtraction of N allocated in storage compounds from total N was similar in both treatments and comprised, on average, 37% of total N content.

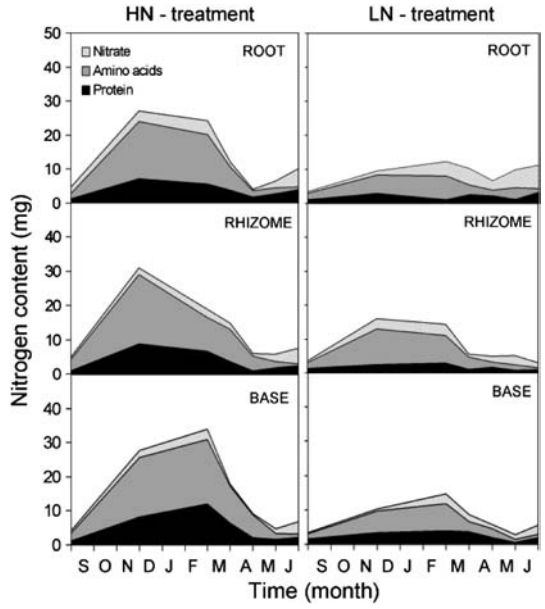

Figure 4. Seasonal changes in contribution of nitrate ions, amino acids and soluble protein to total N content in roots, rhizomes, stubble base of *C. epigejos* plants. Shown are cumulative data of N mass per plant for each sampling date. Plants were exposed to high (HN) or low (LN) nitrogen availability from September to December to low N availability thereafter. Average portion of non-extractable N in examined organs was 37% of total N content. Mean values from six plants.

Significant reduction in both total N content and N allocated to storage compounds was observed from March to May (Figures 3, 4). Decreased N content in below-ground parts of LN plants in the spring was inversely related to increased N content in shoots. Plants in HN treatment experienced net loss of N in the spring due to increased portion of N lost in dead leaf biomass (Figure 3) whereas no such trend was observed in LN treatment. Changes in the content per organ of individual N storage compounds in spring were also highly significant (Figure 4, Table 2). Generally, changes in the concentration of N in storage compounds and the N content in storage compounds per organ were parallel until May. Then nitrogen concentrations reached its minimum but N content was increasing due to accelerated growth of organs. Nitrate N contributed relatively more to the N budget in LN plants due to their smaller content of amino acid and protein N. Nevertheless, the amino acids in all examined organs were the most plentiful nitrogen storage compound in both treatments. The maximum content of amino acids per organ was 2–3 times higher in HN plants than in LN plants (Figure 4) and the same was found for amino acid concentration (data not shown). The pattern of decrease in amino acid content was similar in plants from both treatments, and all plants reached the same minimum in July irrespective of treatment. Arginine, asparagine and glutamine were the most abundant free amino acids. Asparagine dominated in the amino acid pool in the HN treatment (Table 3). The pattern of mobilization of N from soluble protein in spring was similar for both treatments but LN plants had significantly lower minimum content in June (Figure 4).

Discussion

Changes in the pool of nitrogen reserves

It has been known that nitrogen availability can affect the timing of deposition of N in storage organs, but not necessarily the total amount of stored N. The deposition of storage compounds in overwintering organs of a biennial herb *Cirsium vulgare* occurred simultaneously with enlargement of storage organs. Under low N availability, N

Table 2. Results of multifactorial ANOVA of the content of N storage compounds in root (R), rhizome (Rhi) and stubble base (Base).

	Nitrate			Amino acids			Protein		
	R	Rhi	Base	R	Rhi	Base	R	Rhi	Base
Sample date	*	***	ns	***	***	***	***	***	***
Nitrogen	***	***	***	***	***	***	***	***	***
SxN	***	***	***	***	***	*	***	***	***

Shown is the significance of effects of sampling date, availability of nitrogen and their interaction. Level of significance is indicated by asterisks: $*p < 0.05$, $***p < 0.001$, ns – non-significant.

Table 3. Content of nitrogen in free amino acids in root, rhizome and stubble base (mg g^{-1} DM) of *Calamagrostis epigejos* plants on 1st March before the onset of growth after overwintering.

	LN			HN		
	Root	Rhizome	Stubble base	Root	Rhizome	Stubble base
Asn	0.52 ± 0.05	1.98 ± 0.31	2.25 ± 0.10	4.40 ± 0.30	4.59 ± 0.35	5.70 ± 0.22
Gln	0.18 ± 0.02	0.34 ± 0.04	0.62 ± 0.04	0.55 ± 0.16	0.80 ± 0.07	1.97 ± 0.44
Arg	0.59 ± 0.04	2.10 ± 0.32	2.12 ± 0.13	1.56 ± 0.21	3.32 ± 0.57	3.33 ± 0.25
Rem	0.51 ± 0.13	0.78 ± 0.07	0.86 ± 0.06	0.73 ± 0.10	1.06 ± 0.16	1.36 ± 0.19

Plants were exposed to either low (LN) or high (HN) nutrient availability in substrate in preceding vegetative season from August till December. Asn – asparagine, Gln – glutamine, Arg – arginine, Rem – sum of nitrogen in remainder pool of free amino acids analyzed. Means ± SE, $n = 3$.

reserves build up closer to winter dormancy with the major part of stored N recycled from the shoot (Monson et al. 1994). The total size of N reserves present in *C. vulgare* during winter was, however, similar in both treatments. In the experiment with *C. epigejos* the major increase in the content of N storage compounds was found during the final part of the vegetation period (Figure 4). The observed changes in nitrogen content in plant organs (Figure 3) suggest that a major portion of nitrogen (about 70%) stored in roots, rhizomes and stubble bases of HN-grown plants during winter was not retranslocated from shoot, but taken up from the substrate and assimilated in late autumn.

Low N availability in autumn seriously impaired filling of storage organs. Plants in the LN treatment had in their overwintering organs only 50% of total nitrogen present in HN plants and the N content of soluble nitrogen storage compounds was also similarly lower (Figures 3, 4). In contrast to HN treatment, mobilization from senescing shoot parts was a major source of stored N in plants in LN treatment and lower content of N in dead leaves indicates that this mobilization was probably more efficient than in HN plants. Therefore, the soil nitrogen availability in autumn has, in *C. epigejos*, a crucial importance for the size of nitrogen reserves which could be used for enhancement of spring growth.

A spring mobilization of nitrogen stored in below-ground organs has been documented in several experiments using ^{15}N tracer (Tagliavini et al. 2000; Bausenwein et al. 2001a, b). Remobilized nitrogen contributed 30–80% to the total nitrogen in newly regrowing tissue, depending on the duration of regrowth and the species. The significant decrease in nitrogen content in roots, rhizomes and stubble base of examined *C. epigejos* plants in March and April (40–50%) also suggests massive translocation of N from these organs to the shoot.

Nitrogen in regrowing shoot parts of the grasses *Agrostis capillaris* and *Festuca rubra* was preferably derived from senescing leaves present on overwintering tillers but not translocated from roots (Bausenwein et al. 2001a). This is in accord with finding for *C. epigejos* where changes in root N content were also quite small. Importance of green leaves present on *C. epigejos* during winter for N storage N was not of a primary focus in the present experiment but it was included in the final analysis of N budget of plants (Figure 3). Overwintering shoot parts of *C. epigejos* (with predominant share of green leaves) comprised 50 and 40% of N in living plant parts in HN and LN treatment, respectively, and, thus, should be considered as an important source of mobilizable N in this species.

The composition of the pool of nitrogen compounds in plant organs may vary both seasonally and according to examined species (Nordin and Näsholm 1997). Changes in nitrate content usually reflect temporary fluctuations of nitrate availability in soil and in plant capacity for its reduction during a vegetation season (Millard 1988). In *C. epigejos* nitrate was not a dominant storage compound. The high amplitude of changes in amino acid N (Figure 4), on the other hand, suggests that amino acids were the most important nitrogen storage compound in this species irrespective of nitrogen availability. Pattern of annual changes in the content of amino acids was in both treatments similar to that previously observed in the field (Gloser 2002). The nitrogen availability, however, affected the total amino acid content and also the composition of the amino acid pool. Plants in the

HN treatment contained great amounts of asparagine (Table 3). Preferential accumulation of asparagine and glutamine has previously been observed in several other grass species, particularly at high nitrogen availability (Sagisaka 1987; Ohlson et al. 1995; Nordin and Näsholm 1997).

The role of proteins in nitrogen storage and overwintering of grasses was recently discussed (Louahlia et al. 1999; Bausenwein et al. 2001a). Significant changes of the protein N content in *C. epigejos* were found both in this experiment and in the field (Gloser 2002). Although there is so far no evidence supporting existence of vegetative storage proteins in grasses, massive decomposition of proteins in plant organs is undoubtedly an important mechanism for nitrogen redistribution during regrowth of *C. epigejos* in spring. Storage of nitrogen in proteins directly usable in the newly initiated growth processes of leaf meristems in stubble base, in apical meristems of rhizomes or in overwintering green leaves may potentially compensate for higher energy costs connected with this way of N storage (Millard 1988). The cost-benefit analysis of N storage in leaf proteins in grasses would be particularly important goal for future studies since it may bring more insight into this complex problem.

Regrowth of plants in spring

Plants grown in LN treatment developed significantly lower leaf area in spring than HN plants. The regrowing leaf tissue of grasses has been identified as the most prominent sink for the nitrogen remobilized from reserves in several previous studies (Ourry et al. 1988, 1990; Thornton et al. 1993). The processes leading to altered plant leaf area development may be of different character. For instance, in the present experiment the number of tillers as well as the number of green leaves per plant (data not shown) was lower in the LN treatment (Figure 2). The delay in new organ initiation and development coincided with a slower growth of leaf area in March and April. The positive relationship between an increased external supply of nitrogen on the formation of tillers has already been demonstrated (Harris et al. 1996) and the results of the present experiment suggest that a similar effect may also be connected with a content of nitrogen reserves. It is, however, difficult to differentiate between the effect of internal N reserve size and external N availability since the morphogenetic development of new tillers may have started in autumn along with filling of plant storage organs.

The ratio between below- and above-ground biomass (RSR) in HN plants remained significantly lower than in LN plants even during period of low N availability in spring (Figure 3). It has been shown in several species that RSR increases with decreased nitrogen availability (Reynolds and D'Antonio 1996). In *C. epigejos* high content of N reserves and/or previous morphogenetic development of plants under high N availability may have been responsible for maintaining low RSR of HN plants in spring when plants of both treatments were already cultivated at the same (low) concentration of N in substrate. This in turn probably contributed to deceleration of growth in HN treatment later in the second vegetation season.

A rapid decline in nitrogen content in overwintering organs in spring, was not accompanied by a proportional increase in whole plant dry mass, namely in HN treatment Figure 1). It is very likely, that decreased level of internal nitrogen reserves together with low external nitrogen availability lead to establishing a new balance between carbon and nitrogen acquisition. Putative regulatory mechanisms induced higher mortality rate of tillers in May and, subsequently, decreased leaf area, which was unfavourable for fast growth. Therefore, the changes in morphology observed in *C. epigejos* plants in the HN treatment, namely the enhanced initiation of new organs could become a potential advantage only when the nitrogen availability during the new vegetation season is sufficiently high for proper growth of all these organs. The results also indicate that remarkable phenotypic plasticity in *C. epigejos*, controlled by internal nitrogen status can act as a buffer against the effect of external fluctuations in nitrogen availability during the vegetation cycle on final production of plant biomass.

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