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The nitrogen and carbon supply system of leaf growth in perennial ryegrass – Characterization by dynamic ^{15}N and ^{13}C labeling and compartmental analysis of tracer influx into the leaf growth zone

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“The secret of happiness is freedom
and the secret of freedom courage”

Thucydides

ABSTRACT

Aims: Subject of the present study was the characterization of the nitrogen and carbon supply system of leaf growth in perennial ryegrass (*Lolium perenne L.*) in terms of the numbers, half lives, size and importances of kinetically distinct pools supplying nitrogen and carbon substrates to leaf growth. In the first part, the structure of the supply system was assessed under high external nitrogen supply by an approach consisting of dynamic labeling with ^{15}N and ^{13}C of newly assimilated nitrogen and carbon and compartmental modeling. In the second part, the effect of nitrogen deficiency on i) the validity of the model structure evaluated under high nitrogen supply, ii) the half lives of the pools and iii) the role of current assimilates *versus* stores was studied.

Materials and Methods: Individual plants of *L. perenne* were grown in continuous light at 20°C at the level of the leaf growth zone with a relative humidity near 85 % and with a steady supply of nutrients. Half of the plants was supplied with 7.5 mM (high N), the other half with 1.0 mM (low N) nitrogen in the nutrient solution. To analyze the kinetics of tracer incorporation into the fluxes of nitrogen and carbon imported into the leaf growth zone, plants were dynamically labeled with $^{15}NO_3^-/^{14}NO_3^-$ and $^{13}CO_2/^{12}CO_2$ for 2 to 576 h (high N) or for 2 to 975 h (low N). Resulting tracer kinetics in the import flux into the leaf growth zone were analyzed by compartmental models.

Results and Discussion: That kinetics revealed that under ample external nitrogen supply both nitrogen and carbon were supplied by current assimilates and long-term stores, while carbon additionally was supplied by short-term stored material. Current assimilation provided most of the nitrogen and as well of the carbon supply (60% and 70%, respectively), but storage mobilization contributed also a substantial part (40% and 30%). Results of plants grown under nitrogen deficiency showed that a similar number of pools supplied nitrogen and carbon to leaf growth as under high nitrogen supply. But the importance of short and long-term storage pools slightly increased under low nitrogen supply (50% and 40%).

Conclusions: The presented study provides evidence that leaf growth of grass plants grown under nitrogen deficiency draws on the same substrate pools as in plants grown under ample nitrogen supply. Only contributions of single pools to the supply of leaf growth changed. Results further indicate that, within a plant, stores were synthesized and mobilized simultaneously, even under growth conditions that allowed for continuous photosynthesis or nitrogen uptake. This contradics to a common view of stores as fixed reserves.

ZUSAMMENFASSUNG

Zielsetzung: Die vorliegende Arbeit befasst sich mit der Stickstoff- und Kohlenstoffversorgung des Blattwachstums von Deutsch Weidelgras (*L. perenne*). Ziel war es, das Versorgungssystem der Blattwachstumszone mit Stickstoff- und Kohlenstoffsubstraten hinsichtlich der Anzahl der unterschiedlichen ‚Pools', deren Halbwertszeit, Größe und Bedeutung zu charakterisieren. Im ersten Teil der Arbeit wurde die Struktur des Versorgungssystems unter guter externer Stickstoffversorgung untersucht. Hierzu wurde ein Ansatz bestehend aus dynamischer Markierung des neu aufgenommenen Stickstoffs und Kohlenstoffs mit ^{15}N und ^{13}C und Analyse der Tracer-Kinetiken mit kompartimentellen Modellen verwendet. Im zweiten Teil der Arbeit wurde der Effekt von Stickstoffmangel auf i) die Gültigkeit der ermittelten Modellstruktur, ii) die Halbwertszeiten der verschiedenen ‚Pools' und iii) die Rolle von neu aufgenommenen Assimilaten gegenüber der der Speichermobilisierung ermittelt.

Material und Methoden: *L. perenne* Pflanzen wurden einzeln im Dauerlicht, bei 20°C auf Höhe der Wachstumszone, mit einer relativen Luftfeuchte von 85% und mit einer kontinuierlichen Nährstoffversorgung angezogen. Die Hälfte der Pflanzen wurde mit 7.5 mM (‚high N'), die andere Hälfte mit 1.0 mM (‚low N') Stickstoff in der Nährstofflösung versorgt. Um den Zeitverlauf der Beimischung der Tracer in die Stickstoff- und Kohlenstoffflüsse in die Blattwachstumszone zu analysieren, wurden die Pflanzen dynamisch mit $^{15}NO_3^-/^{14}NO_3^-$ und $^{13}CO_2/^{12}CO_2$ für 2 bis 576 Stunden (high N) oder für 2 bis 975 Stunden (low N) markiert. Die hieraus resultierenden Tracer-Kinetiken im Importfluß in die Wachstumszone wurden mittels kompartimentellen Modellen analysiert.

Ergebnisse und Diskussion: Diese Kinetiken zeigten dass, unter guter externer Stickstoffversorgung, sowohl Stickstoff als auch Kohlenstoff von neu aufgenommenen Assimilaten und von Langzeitspeichern bereitgestellt wurden, während Kohlenstoff zusätzlich von kurzzeitig gespeichertem Material stammte. Neu aufgenommene Assimilate stellten den Großteil der Stickstoff- sowie der Kohlenstoffversorgung dar (60% bzw. 70%). Die Mobilisierung von gespeichertem Material trug ebenso zu einem substanziellen Teil bei (40% bzw. 30%). Ergebnisse von Pflanzen, die unter Stickstoffmangel aufwuchsen, zeigten, dass dieselbe Anzahl an Pools zur Versorgung des Blattwachstums mit Stickstoff und Kohlenstoff beitrug wie unter guten Wachstumsbedingungen. Allerdings stieg die Bedeutung der Kurz- und Langzeitspeicher unter Stickstoffmangelernährung tendenziell an (50% bzw. 40%).

Schlussfolgerungen: Die vorliegenden Untersuchungen belegen, dass das Blattwachstum von Graspflanzen bei Stickstoffmangel von denselben Substratpools gespeist wird wie bei guter

Stickstoffversorgung. Nur die Beiträge der einzelnen Pools zur Versorgung des Blattwachstums verändern sich. Die Ergebnisse zeigen außerdem, dass die Anlage von Substratspeichern sowie deren Mobilisierung innerhalb von Pflanzen zeitgleich geschehen, und das obwohl die gewählten Wachstumsbedingungen eine ständige Photosynthese oder Stickstoffaufnahme erlauben würden. Diese Ergebnisse widersprechen der bisherigen Ansicht von Speichern als unangetastete Reserven.

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1 GENERAL INTRODUCTION

This thesis was conducted within the frame of the research program of the joint project SFB 607[1], whose special interest is the trade-off concerning substrate supply of conflicting demands within plants. To elucidate this complex field of allocation patterns to different metabolic processes like growth, defence against parasites or competitiveness against neighbouring plants, it is necessary to understand the patterns of resource allocation to growth processes. Thus the aim of this thesis was to characterize the supply system for leaf growth in terms of its structure and properties and to contribute to the question how plants allocate carbon and nitrogen to leaf growth under differing external nitrogen supply. As grasses have unidirectional linear leaf growth, they are a perfect object for the study of growth processes. In this study we focused on perennial ryegrass (*Lolium perenne*), because it is the most common forage grass in humid temperate grassland and is thus also of interest from an agronomical point of view. Grass leaves produce assimilates needed for the plants´ growth and maintenance and provide the food for heterotrophic organisms like cattle. Grassland utilization essentially consists in the periodic defoliation of grasses, *i.e.* in removal of the leaf laminae. Hence, the ability to maintain leaf production is essential for both survival of the grass plant and sustained grassland production. Therefore, it is necessary to understand the fundamental processes that are directly involved in the growth of a grass leaf and how leaf growth is controlled by resource availability within and to the plant (Schnyder et al. 2000).

1.1 THE LEAF GROWTH ZONE

The so called 'leaf growth zone' of a grass leaf is located at the base of the growing leaf (Davidson and Milthorpe 1966; Durand et al. 1999; Kemp 1980; Volenec and Nelson 1983) and is completely enclosed within the sheaths of older leaves. The leaf growth zone can be sub-divided into 'cell division' and 'elongation-only' zone. In the basal cell division zone, which is in the order of a 10^{th} or less of the length of the leaf growth zone (Durand et al. 1999), cells are produced by division of meristematic cells accompanied by mitotic growth. In the adjacent elongation-only zone, cells undergo a phase of post-mitotic expansion during which they attain

[1] SFB 607 'Growth and Parasite Defense – Competition of Resources in Economic Plants from Forestry and Agronomy' (http://sfb607.de) supported by the Deutsche Forschungsgemeinschaft

their final length (Kavanova et al. 2008). In the subsequent maturation zone they gain their photosynthetic activity by the ongoing synthesis of RubisCo (Gastal and Nelson 1994).

Any cell produced by the meristem is displaced away from the leaf base as a result of continuous production and expansion of cells at more basal positions in the leaf (Durand et al. 1995). It is evident that the distance between position and time is not linear in the growth zone: displacement is slow near the base and increases as the number of expanding elements located between the origin (meristem) and the cell increases with time (Schnyder et al. 1990). After a cell has reached the distal limit of the growth zone the rate of its displacement equals the rate of leaf elongation (Durand et al. 1995). Thus, in steady-state, the rate of cell production equals cell efflux from the leaf growth zone.

Initial leaf expansion is confined to the lamina part, the sheath only starts to expand actively when lamina expansion slows down (Schnyder et al. 1990; Skinner and Nelson 1995). Transition from lamina to leaf expansion can be easily recognized by the displacement of the ligule through the growth zone and away from the leaf base.

1.2 SUBSTRATE AND TISSUE FLUXES INTO AND OUT OF THE LEAF GROWTH ZONE

As it is completely enclosed by surrounding sheaths of older leaves, cells of the growth zone tissue are heterotrophic and thus form one of the main sink for substrates (especially for nitrogen and carbon) next to respiration. Continuous production of cells at basal positions and their expansion give rise to a flux of tissue and tissue-bound mass efflux out of the growth zone. This export is counterbalanced by import of carbon and nitrogen assimilates (Lattanzi et al. 2004) from mature parts of the grass plant (*i.e.* source tissue). Carbon and nitrogen assimilates enter the growth zone *via* phloem at the leaf base, where cell division takes place (Allard and Nelson 1991). They are mainly deposited within the leaf growth zone, thus by the end of the growth zone most of the C and N deposition has already taken place (Gastal and Nelson 1994; Maurice et al. 1997; Schnyder et al. 1988; Schnyder and Nelson 1988). The growth zone thus may be regarded as a place where substrates are imported, transformed and exported as structural and functionally differentiated tissue (Lattanzi et al. 2004). In steady-state, substrate influx of carbon and nitrogen compounds equals the substrate efflux in the form of tissue-bound carbon and nitrogen plus respiratory carbon losses.

1.3 SOURCES SUPPLYING LEAF GROWTH

Together with oxygen and hydrogen, carbon and nitrogen constitute the most important elements for plant growth (Lea and Morot-Gaudry J.-F 2001; Taiz L. and Zeiger E. 2000). In heterotrophic growing leaves nitrogen and carbon are supplied *via* the phloem from mature leaf tissues in the form of amino acids and sucrose (Amiard et al. 2004; Gastal and Nelson 1994; Lalonde et al. 2003; Schnyder and Nelson 1988; Volenec and Nelson 1984). As amino acids contain nitrogen and carbon, both sucrose and amino acids contribute to the carbon supply of leaf growth.

These substrates derive either from current photosynthesis or nitrogen uptake ('assimilation-derived') or from mobilization of stored compounds like vacuolar sucrose, fructans or proteins ('storage-derived'). While allocation of carbon and nitrogen towards, and mobilization from storage pools has been described in many species, and effects of changes in growth conditions on such patterns are relatively well known (Chapin F.S.3 et al. 1990; Chatterton et al. 1989; Millard 1988; Volenec et al. 1996), the role of storage mobilization and current assimilation in supplying leaf growth has been little explored. Tracer studies with pulse-chase labeling of ^{13}C showed commonly that carbohydrates in mature leaf and sheath tissue are located in a cytosolic sucrose pool, which turns over quickly and is readily available for export, and a vacuolar pool consisting of sucrose and fructans, which turns over more slowly (Baxter and Farrar 1999; Borland and Farrar 1988; Farrar and Farrar 1986; Geiger et al. 1983). Schnyder and de Visser (1999) showed that only 10 % of carbon accumulating in regrowing leaves after defoliation derived from assimilates older than 3 d, supporting the view that mainly current photosynthesis supplied leaf growth.

^{15}N pulse-chase labeling studies of leaves and roots found indications for a rapidly cycling cytoplasmic and a slowly turning over vacuolar nitrogen pool (Devienne et al. 1994; Hayashi et al. 1997), which probably consisted of amino acids (Caputo and Barneix 1999). Due to a limited labeling time leaf proteins were not considered in these studies. For nitrogen supply of leaf growth it seemed that recent nitrogen uptake was of lesser importance (Bausenwein et al. 2001; Louahlia et al. 2000).

Current views on the contribution of storage mobilization and current assimilation to growth are mainly based on observations of the disappearance or export of substrates from source or storage organs (Borland and Farrar 1988; Geiger et al. 1983; Prosser and Farrar 1981). However, such observations do not provide direct evidence of the fate of those materials, and more particularly, they provide no proof for the actual use and importance of current assimilation-derived *versus* storage-derived substances for leaf growth.

The study of Lattanzi et al. 2004) presented a method for estimating the flux of nitrogen and carbon within the growth zone. Furthermore, it evaluated the supply system for nitrogen and carbon substrates and particularly the kinetic properties of the pools supplying leaf growth in a C3 (*L. perenne*) and a C4 (*Paspalum dilatatum*) grass in different hierarchical positions in mixed stands (Lattanzi et al. 2005). They considered substrate supply for leaf growth, focusing on the role of stores, from the sink's perspective and thus integrated the whole plant to a supply system using a comprehensive approach of dynamic labeling with ^{15}N and ^{13}C and compartmental modeling (Moorby and Jarman 1975). These approaches were previously developed in biology and medicine (Jacquez 1972). In *L. perenne* they found that nitrogen as well as carbon was supplied by two distinct pools: a rapidly turned over transport and a slowly turned over storage pool. As suggested from the source perspective, for nitrogen supply of the growth zone storage mobilization was much more important than for carbon supply (Lattanzi et al. 2005). Due to a time resolution of 24 h, which did not allow to resolve pools with a half live shorter than 5 h, the transport pool likely consisted of a mixture of quickly turned over substrates (ready to be transported to the growth zone) and of short-term stored compounds. Thus, to characterize the contribution of 'current assimilation-' *versus* 'storage-' derived substrates a shorter time resolution is necessary to separate kinetics of transport and short-term stored compounds.

1.4 EFFECT OF NITROGEN ON LEAF GROWTH AND ON POTENTIAL SOURCES

The process of leaf growth is sensitive to the nutrient status of a plant (Evans 1996). Especially nitrogen deficiency severely inhibits leaf growth. Gastal and Nelson (1994) and Volenec and Nelson (1983) attributed the decrease of leaf elongation rate to a reduction of cell production rate. Kavanova et al. (2008), who analysed the effect of nitrogen deficiency on the cellular level of *L. perenne*, found that nitrogen deficiency decreased cell division, mitotic elongation and post-mitotic elongation rates leading to a reduced cell production rate and final cell lengths. Thus nitrogen seemed to have a direct impact on meristematic processes and on the cell-cycle.

Further, nitrogen and carbon status of the whole leaf growth zone also revealed an explicit reaction to external nitrogen supply. When external nitrogen supply was high the growth zone also showed a high content of total nitrogen and *vice versa* (Gastal and Nelson 1994). In contrast, the content of soluble carbohydrates -especially fructans- in the leaf growth zone increased under low nitrogen nutrition (Gastal and Nelson 1994; Volenec and Nelson 1984). This observation led to the suggestion that under low nitrogen nutrition leaf growth was inhibited by nitrogen rather than by carbohydrate availability. Total nitrogen, soluble nitrogen and nitrate nitrogen in leaves, whole shoots and whole plant tissue also increased with increasing external

nitrogen (Caputo and Barneix 1997; Evans 1996; Lea and Morot-Gaudry J.-F 2001; Nowakowski 1962). Similar to the reaction in the leaf growth zone, the content of water-soluble carbohydrates in the shoot increased with decreasing nitrogen nutrition, which was mainly ascribed to the increase in fructan content (Jones et al. 1965; Nowakowski 1962). The increases in nitrogen or carbon can be ascribed to storage processes in forms of accumulation and/or reserve formation when nitrogen or carbon are in excess (Chapin F.S.3 et al. 1990).

A study of the effect of nitrogen on the composition of phloem exudates in wheat showed that the amount of amino acids in the phloem was positively correlated to external nitrogen supply, while phloem water-soluble carbohydrate content seemed to be unchanged (Caputo and Barneix 1997). Leading to an increased ratio of phloem sugars: phloem amino acids under nitrogen deficiency. So far, effects of external nitrogen nutrition on phloem sap composition, on which the leaf growth zone draws, is clear. But as also respiration and other sinks draw on the supply of phloem solutes, the effect on substrates supplying leaf growth is still unclear. It further is unclear how nitrogen deficiency affects the contribution of current assimilation- *versus* storage-derived nitrogen and carbon.

1.5 AIMS

The first aim of this research was to characterize the kinetic properties of the substrate supply system for leaf growth as described by Lattanzi et al. (2005), in terms of the number of pools, their half lives and contributions to leaf growth, with focus on the contribution of current assimilates *versus* stores. To do so, plants under steady growth conditions and ample nitrogen supply were labeled dynamically with $^{15}NO_3^-$ and $^{13}CO_2$ and the tracer time-course of labeled and unlabeled nitrogen and carbon in the import flux into the leaf growth zone was evaluated by compartmental modeling (Chapter 2).

The second aim was to evaluate the effect of nitrogen deprivation on the substrate supply system and on the contributions of assimilation-derived *versus* storage-derived substrates. Thus plants grown under nitrogen deprivation were labeled and analyzed in a similar way. Results of the compartmental modeling were compared to the previously evaluated structure of the carbon and nitrogen supply system for leaf growth under ample nitrogen nutrition (Chapter 3).

To describe the nitrogen and carbon supply system, which delivers substrates to the leaf growth zone, we constructed a hypothetical model by adopting a previously evaluated 2-pool model describing the substrate supply system of leaf growth (Lattanzi et al. 2005). This was based on knowledge about substrate compartmentation in source tissues, and extended the model with basic biological knowledge about the kind of substrates supplying leaf growth (Amiard et al.

2004; Lea and Morot-Gaudry J.-F 2001). This resulted in a hypothetical comprehensive carbon and nitrogen model, which represented short-and long-term substrate pools within the whole plant (see Chapter 2).

We are aware that summarizing the plants metabolic complexity is a simplification, but exactly this is our goal. All pools described here need to be considered as defined by Jacquez and Simon (1993) – as an amount of some material that is kinetically and isotopically homogenous at all times. This implements that substrates represented by one pool i) may have a potential heterogeneous biochemical identity and ii) may be located in several tissues - but show similar kinetical properties.

The term ‘stores or storage pools’ as used here implements three different classes of storage: i) accumulation of compounds due to an excess, ii) formation of reserves, to buffer times of substrate shortage and iii) recycling of compounds, which primarily have a physiological function and later are degraded and remobilized (like leaf proteins) (*sensu* Chapin F.S.3 et al. 1990).

2 CHARACTERIZATION OF THE CARBON AND NITROGEN SUPPLY SYSTEM OF LEAF GROWTH IN PERENNIAL RYEGRASS UNDER FAVOURABLE GROWTH CONDITIONS

2.1 ABSTRACT

We characterized the nitrogen and carbon assimilate supply system for leaf growth in perennial ryegrass (*Lolium perenne L.*). Specifically, we assessed the half life ($t_{0.5}$), size and importance of kinetically distinct pools supplying nitrogen and carbon substrates to growth. Individual plants, growing in continuous light and with a high and steady supply of nutrients, were labeled with $^{15}NO_3^-/^{14}NO_3^-$ and $^{13}CO_2/^{12}CO_2$ for 2 to 575 h. Then, we used compartmental models to analyze the kinetics of tracer incorporation into the fluxes of nitrogen and carbon imported into the leaf growth zone –the place where substrates are converted into leaf tissue. Stores and current uptake/photosynthesis both supplied nitrogen and carbon for leaf growth. A transport pool ($t_{0.5}$ ~50 min), consisting presumably of cytosolic-apoplastic amino acids and sucrose, routed current assimilates directly to the leaf growth zone. These accounted for 60% of total nitrogen and 70% of total carbon import. Hence, 40% of nitrogen and 30% of carbon substrates used for leaf growth first cycled through stores. Nitrogen and carbon showed one long-term store ($t_{0.5}$ ~ 5d) related to the turnover of leaf proteins. Carbon further was supplied by a vacuolar short- term store ($t_{0.5}$ ~ 11 min). Results indicate that, within a plant, stores were synthesized and mobilized simultaneously, even under growth conditions that allowed for continuous photosynthesis or nitrogen uptake. This attests to a supply system requiring constant investments in storage, and with constant use of mobilized material, even in undisturbed plants.

2.2 INTRODUCTION

This work is concerned with the assimilate supply system of leaf growth in a perennial cool-season C3 grass. In grasses, the supply of nitrogen and carbon assimilates to leaf growth zones occurs in the form of amino acids and sucrose (Amiard et al. 2004; Hayashi et al. 1997). The leaf growth zone is the place where these substrates are converted into structurally and functionally differentiated tissue. Leaf growth zones contain no nitrate reductase and nitrate import is minor compared to that of organic nitrogen (Gastal and Nelson 1994). As the leaf growth zone of grasses is completely heterotrophic, being fully enclosed by sheaths of older leaves (Fig. 1) theses substrates all originate from external sources. Ultimately, all amino acids and sucrose derive from primary assimilation, *i.e.* nitrogen uptake and photosynthesis, but the link between assimilation and actual use in sink tissues is intricate: some may be delivered directly and some may first visit other places, such as stores dispersed throughout the plant, before being transferred to the leaf growth zone.

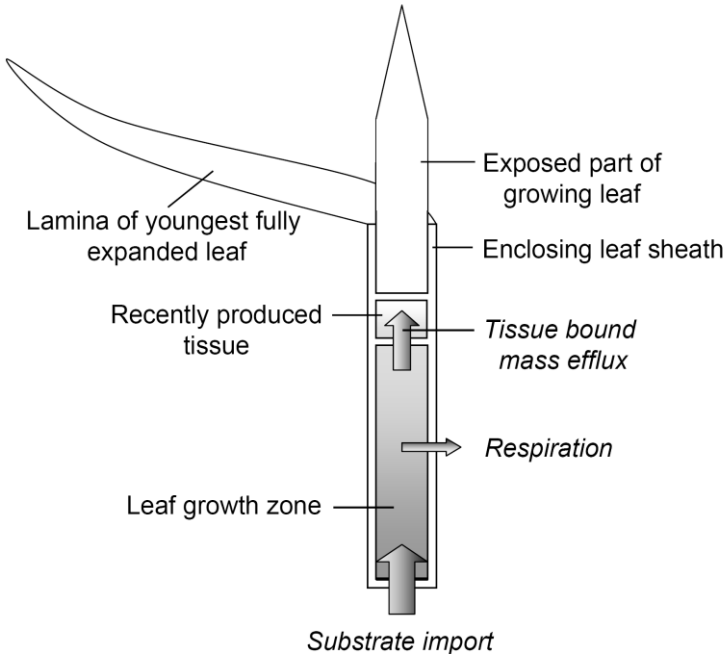

Fig. 1: Schematic of a grass tiller

The growing leaf and its growth zone is enclosed by the sheath of the surrounding youngest fully expanded leaf. The growth zone can be conceived as a place in which substrates (*e.g.* sucrose and amino acids) are imported, and subsequently converted into structural material, and exported as new -fully functional- leaf tissue by tissue-bound mass efflux. The conversion of carbon substrates involves respiration, thus in a steady-state the rate of import equals the export through tissue-bound mass efflux plus respiration (Lattanzi et al. 2004; Lattanzi et al. 2005). For other substances including nitrogen, the relationship reduces to ‘import equals export’. The rate of export can be assessed from measurements of

leaf elongation rate (mm h^{-1}) and the lineal density of recently produced leaf tissue, which is adjacent to the leaf growth zone of the substance of interest (Lattanzi et al. 2004).

Plant storage of nitrogen and carbon can be diverse, consisting of a complex system of biochemically distinct compounds with subcellular-, tissue- and organ-defined spatial locations. The physiology and biochemistry of storage and mobilization of some proteins and non-structural reserve carbohydrates has been studied extensively: in perennial forage grasses, such as *L. perenne*, they have an important role for the survival of stressful periods and re-growth following defoliation (reviews by Cairns et al. 2000, Chapin F.S.3 et al. 1990, Millard 1988 and Volenec et al. 1996). Substrate supply to leaf growth of grasses also continues at night (Schnyder et al. 1988), indicating that stores are also an important factor in the continuous supply of leaf growth, buffering diurnal cycles (and day-to-day) variations in nitrogen uptake and photosynthesis.

Carbohydrate storage in most cool season C3 grasses occurs in the form of vacuolar short-term stored sucrose and fructans in mesophyll and bundle-sheath cells of photosynthetically active leaf laminae (Farrar and Farrar 1986; Koroleva et al. 2000) and long-term fructan reserves in the base of the sheath of mature leaves (Cairns et al. 2000; Morvan-Bertrand et al. 2001; Volenec 1986). Nitrogen storage occurs in even more varied forms: nitrate and free amino acids can be stored in vacuoles in both roots and leaves (Devienne et al. 1994; Martinoia et al. 1981), while the xylem-phloem cycling pool of amino acids has also been suggested to act as a nitrogen store in grasses (Cooper and Clarkson 1989). Some proteins, particularly RubisCo, are widely thought to form a nitrogen store (Mae et al. 1983; Millard 1988; Peterson et al. 1973). Vegetative storage proteins are of little quantitative importance in grasses (Louahlia et al. 2000).

Little is known about the importance of stores during undisturbed vegetative growth, because direct evidence of the allocation of mobilized material (*e.g.* towards structural biomass synthesis or maintenance respiration) is scarce. Thus, the idea that reserves are laid down only to be mobilized following a perturbation such as grazing or mowing, or during spring regrowth is widespread, and led to the view that, whenever possible, plants would use current assimilation instead of stores (Chapin F.S.3 et al. 1990). But stores may nonetheless also have a constitutive function in supplying substrates for sinks. For instance, amino acids are continuously cycling between cytoplasm, chloroplasts and vacuoles (Winter et al. 1993), as well as in the xylem/phloem system (Cooper and Clarkson 1989). Likewise, vacuolar fructans – unlike starch – show continuous turn over in the light (Borland and Farrar 1988; Farrar and Farrar 1986), meaning that these stores simultaneously receive and release carbohydrates.

Storage and mobilization of reserve carbohydrates may partly be under developmental control (Smith and Stitt 2007). Ultimate mobilization of fructans stored in leaf sheaths seems to occur during the senescence phase of leaves (Volenec 1986). Similarly, proteins of mature tissue are mobilized during leaf senescence and acclimation to shade (Evans and Poorter 2001; Feller and Fischer 1994) recycling amino acids for new leaf growth. These relationships underline the role of stores as part of the assimilate supply system for leaf growth, also under conditions of no apparent shortage of current assimilate production.

The aim of this work was to provide a comparative, quantitative characterization of the nitrogen and carbon supply systems for leaf growth of *L. perenne* plants – from the sink's perspective. Assimilate supply is here viewed as a multi-compartment system consisting, as a 'minimum hypothesis', of transport and storage pools for amino compounds and carbohydrates (Fig. 2). Once in the transport compartment, products of current uptake or photosynthesis can be transferred directly to the leaf growth zone (current assimilation-derived sucrose or amino acids), or first visit one or several stores (storage-derived substrate). When nitrogen uptake and photosynthesis are labeled, the time lapse between tracer entering the plant (*via* photosynthesis or nitrogen uptake) and arriving to the leaf growth zone is shorter for directly transferred assimilates than for storage-derived material. So, the time-delay between fixation and arrival of carbon and nitrogen in the growth zone is a function of the kinetic properties of the different physical and metabolical compartments visited along their way to the growth zone. Our goal is to summarize such complexity into a few pools –defined as isotopically similar spaces (Jacquez and Simon 1993; Rescigno 2001) and therefore with a potentially heterogeneous biochemical identity and spatial distribution in various tissues / plant parts – to reveal the salient properties of the system supplying substrates for leaf growth.

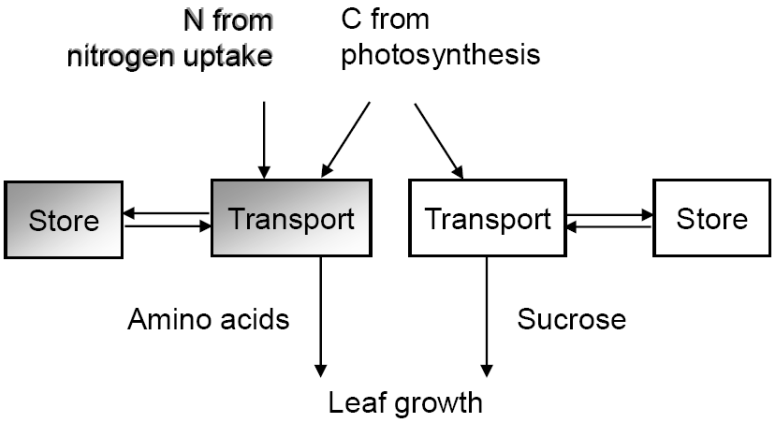

Fig. 2: Hypothetical minimum model
Hypothetical minimum four-pool model of the assimilate supply system feeding leaf growth in a grass. The leaf growth zone of grasses is completely heterotrophic and thus fully dependent on carbon and nitrogen assimilate supply from external sources. Assimilate supply occurs in two forms: amino acids and sucrose. Those in turn can derive either from current assimilation (photosynthesis or nitrogen up take) feeding the growth zone directly *via* a 'transport pool' or can derive from mobilization from stores, which exchange assimilates with the transport pool.

We used compartmental modeling of the time course of tracer incorporation into carbon and nitrogen substrates being imported into leaf growth zone (Moorby and Jarman 1975; Prosser and Farrar 1981) under quantitative dynamic labeling (de Visser et al. 1997) to infer the (i) number and (ii) size and half life of pools which make up the supply system, and (iii) to provide quantitative estimates of the contribution of individual pools to leaf growth (Lattanzi et al. 2005). To minimize the occurrence of diurnal cycles in carbon and nitrogen fluxes induced by day-night-cycles, plants were grown in continuous light and irrigated frequently.

2.3 RESULTS

Lattanzi et al. (2004) and Lattanzi et al. (2005) presented the rationale and procedure for the estimation of the amount of tracer imported into the leaf growth zone of grasses at different labeling times. This rests on the sequential measurements of leaf elongation rate, lineal density of carbon and nitrogen in recently produced leaf tissue, and carbon and nitrogen mass of the leaf growth zone itself, as well as ^{15}N and ^{13}C tracer content of these two tissues (eq 3b Materials and Methods; for abbreviations see Tab. 7). Accordingly, we present evidence showing that leaf growth and associated properties were in an approximate steady-state, and then we report the kinetics of tracer incorporation into the leaf growth zone and (adjacent) recently produced tissue, and in the import flux.

TILLER AND LEAF GROWTH

Analysis of tracer kinetics becomes most straightforward if a (near-) steady-state of carbon and nitrogen fluxes can be assumed. For this reason, a continuous light regime coupled with a frequent supply of water and nutrients was chosen to minimize diurnal cycles in carbon and nitrogen metabolism. These conditions, certainly affecting plant growth and development, resulted nonetheless in typical values of morphological and physiological parameters: (i) carbon use efficiency [growth / (growth + respiration)] was 0.68 (Lehmeier et al. 2008), (ii) the shoot to root ratio (2.8), was similar to that observed in the same species growing with a more typical 16 h-light photoperiod (Grimoldi et al. 2005) and (iii) nitrogen concentrations were 1.8 % for shoots and 1.3 % for roots (in a dry weight. basis) and thus were far from the high values usually associated with luxury consumption (Marschner 1995), and there was little nitrate accumulation in leaf growth zones (< 4 mg NO_3^- (g dry wt.)$^{-1}$, M. Kavanová, unpublished). This shows that results gained in this study are indeed comparable to plants grown under natural conditions.

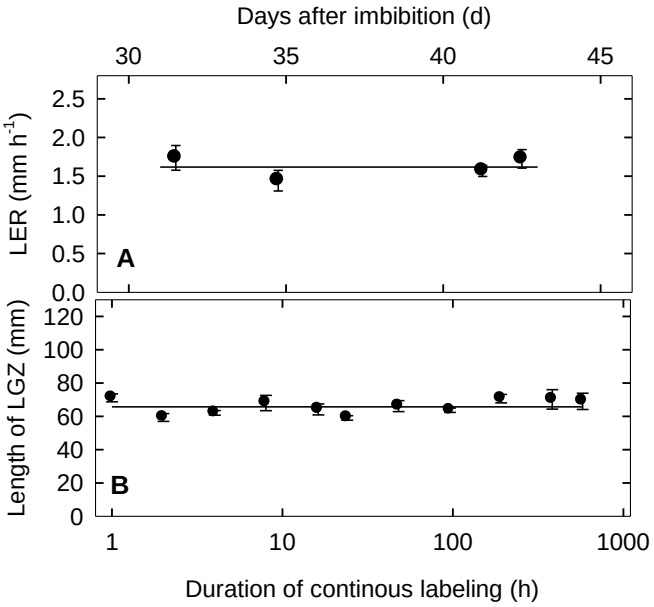

Fig. 3: Process variables – LER and length of the LGZ
Leaf elongation rate (LER) (A) and length of leaf growth zones (B; semi-log scale) in plants of *L. perenne* during the experimental phase of dynamic labeling with ^{15}N and ^{13}C.

As tillers harvested were all in the same developmental stage (length of growing leaf was 40 to 60% of its final length) and due to a nested labeling pattern (see Fig. 10), process variables only changed marginally among plants, even though they were labeled for different time intervals.

Leaves elongated rapidly (leaf elongation rate (LER) of 1.6 mm h^{-1}) and constantly during time of labeling (Fig. 3A). Also the length of the leaf growth zone was constant in this time interval (Fig. 3B). Mass of nitrogen and carbon in the growth zone and in recently produced (fully-expanded) leaf tissue (Fig. 4) did not change with duration of labeling. These relationships indicated a biological system in near steady-state, in which the fluxes of nitrogen and carbon into and out of the growth zone were stable and only changed marginally during the experimental period (Fig. 5).

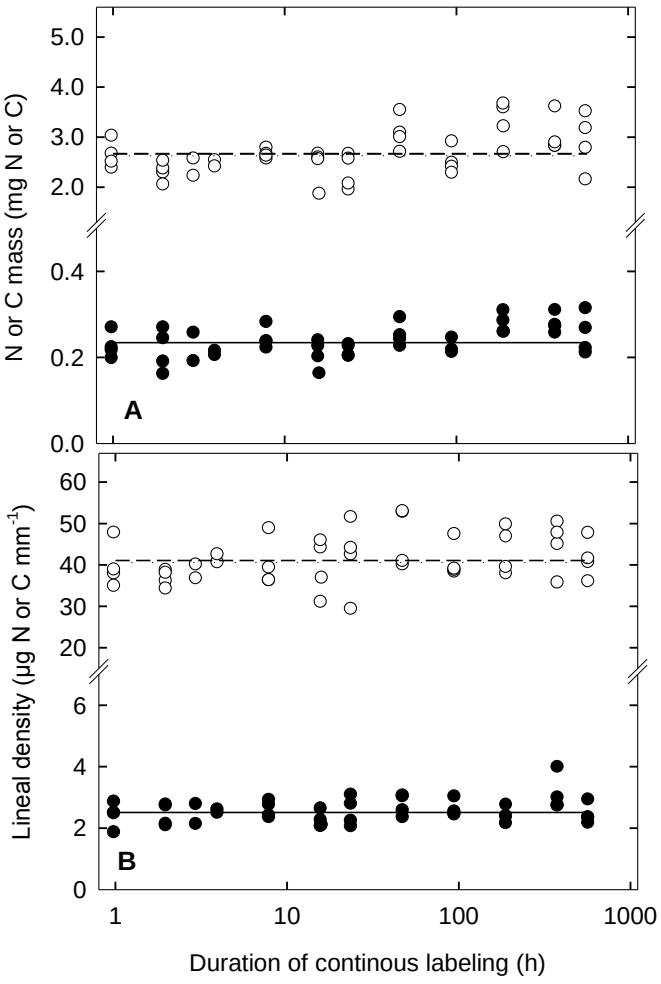

Fig. 4: Process variables – N & C mass and Lineal N and C density
Nitrogen (closed circles) and carbon (open circles) mass in the leaf growth zone (A) and lineal nitrogen (closed circles) and carbon (open circles) density in recently produced and fully expanded tissue (B) of *L. perenne* plants during dynamic labeling with ^{15}N and ^{13}C (semi-log scale).

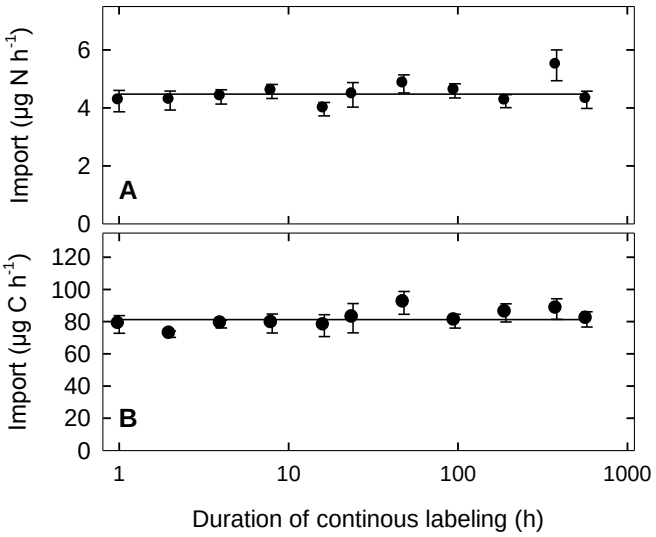

Fig. 5: Import flux over duration of labeling
Import flux of nitrogen (A) and carbon (B) into the leaf growth zone in plants of *L. perenne* during dynamic labeling with ^{15}N and ^{13}C (semi-log scale)

^{15}N AND ^{13}C LABEL INCORPORATION INTO THE LEAF GROWTH ZONE AND RECENTLY PRODUCED TISSUE

Quantitative dual ^{15}N and ^{13}C labeling was performed to asses the fluxes of nitrogen and carbon into the leaf growth zone, to help distinguish nitrogen and carbon derived from current assimilation and that from store mobilization, and to help infer carbohydrate-carbon (CH_2O-C) from amino-carbon (amino-C) fluxes. For this, label incorporation in the leaf growth zone and in the adjacent recently produced (fully expanded) tissue was measured after various labeling durations which were timed from 2 h to 24 d, so as to capture most components of the substrate supply system.

The resulting labeling time courses demonstrated that tracer arrived in the leaf growth zone with little delay, and that both nitrogen (Fig. 6A) and carbon (Fig. 6B) were rapidly exchanged by labeled material. Nitrogen was exchanged more slowly than carbon: while it took 80 h for half of growth zone nitrogen to become labeled, half of growth zone carbon was labeled after only 32 h. Labeling of recently produced tissue showed very similar kinetics, but first label appearance was delayed by a few hours relative to the growth zone. This was true for carbon and nitrogen (see insets in Fig. 6) and mainly stemmed from the fact that recently produced tissue originates exclusively from tissue displacement out of the growth zone (Fig. 1). So, label imported into the

growth zone appeared in recently produced tissue with some delay, which was dependent on the flux of tissue out of the growth zone and on the deposition pattern of nitrogen and carbon along the leaf growth zone (see Fig.1 in Lattanzi et al. 2005)). Preliminary tests revealed that all nitrogen and carbon deposition took place within the leaf growth zone, which was two-thirds the length of the enclosing sheath.

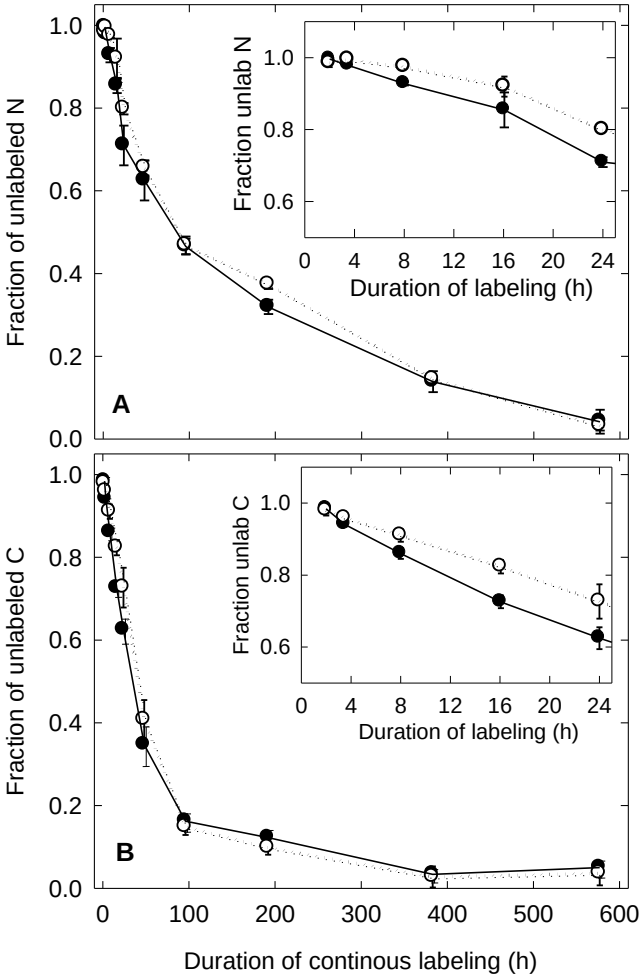

Fig. 6: Fraction of unlabeled nitrogen and carbon

Fraction of unlabeled nitrogen (A) and unlabeled carbon (B) in the leaf growth zone (closed circles) and recently produced leaf tissue (open circles) of *L. perenne* plants during dynamic labeling with ^{15}N and ^{13}C. Labeling started at 0 h and was continued for 575 h. Data are means of 4 replicates (± 1 SE). Insets expand the first 24 h of labeling. Lines are simple straight lines fitted to the data points.

KINETICS OF ^{15}N AND ^{13}C IN THE IMPORT FLUX INTO THE LEAF GROWTH ZONE

The amount of tracer in the nitrogen and carbon fluxes feeding the leaf growth zone were inferred from measurements of time rate changes in ^{15}N and ^{13}C contents of the leaf growth zone and of the fluxes leaving the leaf growth zone by way of respiration and tissue export. These calculations were made using eq. 3b, based on rationale and procedures presented by Lattanzi et al. (2004) and Lattanzi et al. (2005).

The labeling kinetics of the nitrogen import flux revealed three phases: a short lag (~0.9 h), followed by a phase of about 3 h in which tracer incorporation was very fast resulting in more than 50 % labeling of nitrogen in the import flux after only 4 h of labeling, and a period of very slow label incorporation which lasted until the end of the experiment. A residual 5 % of imported nitrogen remained unlabeled after 24 d of labeling.

The labeling kinetics of carbon also showed three phases: a first phase with rapid tracer incorporation in the first 2 to 4 h, a second phase of slower tracer incorporation until about 24 h of labeling and a third phase with even slower tracer incorporation until the end of labeling. There was no apparent lag of carbon tracer appearance in the import flux after the beginning of labeling. These phases were corroborated by compartmental modeling (see below).

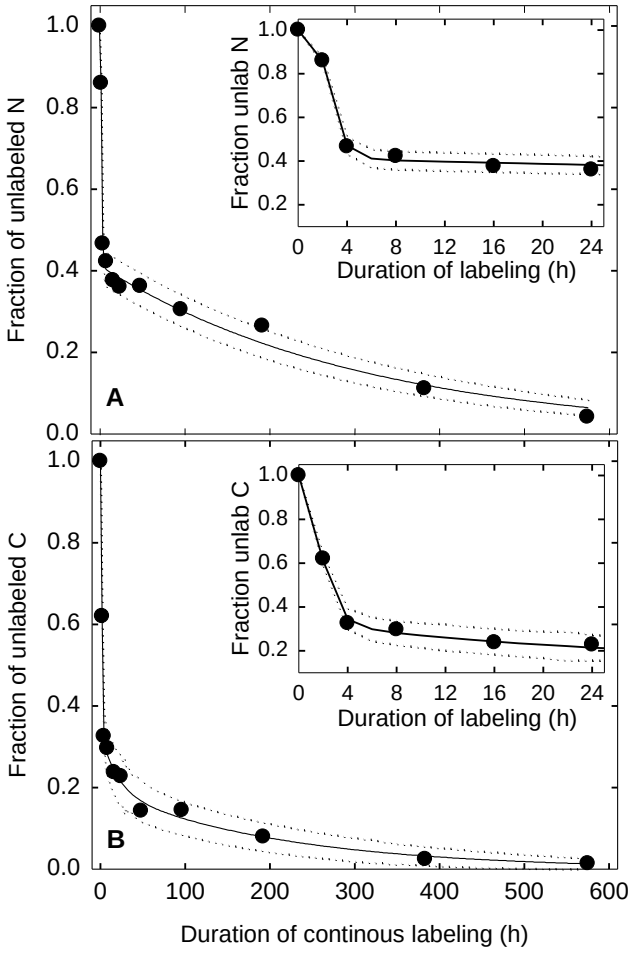

Fig. 7: Tracer time course of nitrogen and carbon in the import flux
Time course of the fraction of unlabeled nitrogen (A) and unlabeled carbon (B) in substrates imported into the leaf growth zone. Insets expand the initial 48 h of labeling. Solid lines display the fit of the two-pool nitrogen model (A) and the three-pool carbon model (B). Dotted lines display ±1 SE of the fit.

THE SUBSTRATE SUPPLY SYSTEM OF LEAF GROWTH

The time courses of ^{15}N and ^{13}C in the import flux (Fig. 4) were analyzed *via* compartmental modeling to infer structure (size, number and arrangement of pools) and kinetic properties (half life of pools) of the nitrogen and carbon supply systems feeding leaf growth. Alternative models of these systems were formulated in terms of compartmental models. Models were designed separately for nitrogen and carbon, and included the hypothetical 'minimum' two-pool model of nitrogen supply and the four-pool model of carbon supply (Fig. 2). Resulting in the models shown in Fig. 8.

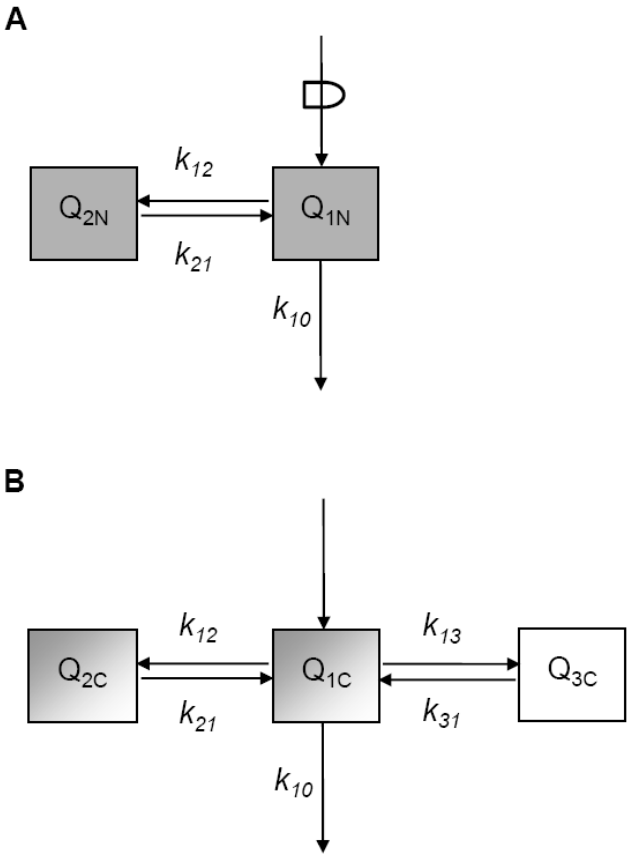

Fig. 8: Compartmental pool models for nitrogen and carbon
Two-pool model of nitrogen sources (A) and three-pool model of carbon sources (B) supplying the leaf growth zone of *L. perenne* plants. Newly acquired nitrogen or carbon enters a transport pool $Q_{1.}$ From there assimilates are either transported directly into the growth zone (k_{10}), or are exchanged with storage pools Q_2 or Q_3. Carbon can be supplied to leaf growth in forms of carbohydrate-C (white in B) or in forms of amino-C (grey in B). In solving the model, steady-state ($k_{01} = k_{10}$) and first order kinetics were assumed, that is, pool sizes are constant in time and fluxes are the product of pool size times a rate constant (k_{xy}, x and y referring to donor and receptor compartments, respectively).

All models were described in terms of pools obeying first-order kinetics, and fluxes between pools and the environment (uptake fluxes of nitrogen and carbon) and into the leaf growth zone. Each model was translated into a set of differential equations, which was then numerically solved to fit the time courses of ^{15}N or ^{13}C, iteratively estimating the rate constants for mass transfer between pools that minimized the difference between the measured values and the model prediction (Tab. 1).

Tab. 1: Model optimization results.
Optimized rate constants k_{xy} (h^{-1}, where x and y refer to donor and receptor pools, respectively) and delay in the two-pool nitrogen and three-pool carbon models describing the time course of ^{15}N and ^{13}C tracer incorporation into carbon and nitrogen substrates supplying leaf growth (Fig. 7).

	Nitrogen	**Carbon**
k_{10} (h^{-1})	0.690 ± 0.148	0.685 ± 0.067
k_{12} (h^{-1})	0.480 ± 0.110	0.157 ± 0.051
k_{13} (h^{-1})		0.155 ± 0.057
k_{21} (h^{-1})	0.0055 ± 0.0006	0.0058 ± 0.002
k_{31} (h^{-1})		0.063 ± 0.043
delay (h)	0.900 ± 0.064	-
RMSE	0.040	0.028

The ability of a model to fit the data (goodness-of-fit), as expressed by its root mean squared error (RMSE), was taken as a measure related to its biological realism. From the optimized rate constants, $t_{0.5}$ and proportional contribution of each pool to the import flux into the growth zone were estimated (Tab. 2). Measured import fluxes were then used to estimate pool sizes. The sensitivity of estimated parameters was investigated as described in Lehmeier et al. (2008) by plotting the response of the RSME to step-changes in optimized half lives. This allowed to avoid local minima during the iterative fitting process.

NITROGEN

A two-pool model (Fig. 8A) similar to that proposed in the 'minimum hypothesis' (Fig. 2) described well the ^{15}N kinetics in the import flux (RMSE = 0.040, a one-pool model exhibited a significant lack-of-fit RMSE = 0.220, and three-pool models could only improve the RMSE when introducing a 2nd delay, for which biological and empirical proof is so far not given). Tab. 1 summarizes the optimized values of the two-pool nitrogen model for each rate constant.
This model included a fast pool ($t_{0.5}$ 36 min), which received nitrogen from current uptake with a delay of 0.9 h (the delay means that tracer arrival into Q_1 lagged behind tracer being put in contact with roots) and supplied it to the leaf growth zone. This pool was termed 'transport pool' and represented only 0.2 % of the total nitrogen mass of a tiller. The 'transport pool' exchanged nitrogen with a second pool, named 'storage pool', which turned over more slowly ($t_{0.5}$ 127 h) and accounted for 18 % of the total tiller nitrogen. Of all nitrogen imported into the leaf growth zone, 60 % originated from direct transfer of absorbed nitrogen *via* the transport pool; the rest

cycled at least once through the storage pool (Tab. 2). Sensitivity analysis revealed that these characteristics of the nitrogen supply system, *i.e.* half life (Fig. 9A), size and relative contribution of each pool, were well constrained by the data.

Tab. 2: Derived pool characteristics in high N plants
Sizes (Q_n) and half lives ($t_{0.5}$) of pools, fluxes into (import) and out (F_{10}) of the system, and percent contribution to leaf growth of assimilates derived from current assimilation (direct transfer) or mobilization of short- and long-term stores. All these parameters were calculated from rate constants derived from the fit of the two-pool nitrogen model and the three-pool carbon model and measured import fluxes (Fig. 8).

	Nitrogen	**Carbon**
	µg	
Q_1	6.5 ± 1.4	119 ± 12
Q_2	566 ± 150	3198 ± 1578
Q_3		293 ± 231
tiller mass	3111	68850
	h	
$t_{0.5}$ Q_1	0.59 ± 0.05	0.69 ± 0.07
$t_{0.5}$ Q_2	127 – 133 ± 13	119 – 125 ± 43
$t_{0.5}$ Q_3		11 ± 8
	µg h^{-1}	
import = F_{10}	4.48	81.3
	%	
direct transfer	59 ± 4	69 ± 6
long-term store	41 ± 4	16 ± 4
short-term store		15 ± 5

CARBON

The hypothetical four-pool model pictured in Fig. 2 did not perform better than a simpler three-pool model (Fig. 8B, RMSE 0.038 and 0.028 respectively). In fact, the standard error for rate constants exceeded the value of the rate constant in five out of six cases in the four-pool model (data not shown), indicating that the model was over-parameterized and included redundant elements. The reason was that the two transport pools of the hypothetical ‘minimum-model’ shared similar kinetics, and could not be statistically resolved on the basis of labeling kinetics.

The three-pool model provided a clear distinction between a fast pool ($t_{0.5}$ 41 min) termed 'transport pool', a 'short-term store' ($t_{0.5}$ 11 h), and a 'long-term store' ($t_{0.5}$ 119 h). The transport pool represented 0.17 % of the total carbon mass of the tiller, and the short-term store and long-term store accounted for 0.43 % and 4.6 % respectively. Direct transfer of photosynthetically fixed carbon to the growth zone *via* the transport pool accounted for 70 % of all carbon supply to the growth zone, whereas short- and long-term stores routed 15 % each (Tab. 2). Again, the estimated characteristics of the carbon supply system were well constrained by the data (Fig. 9B).

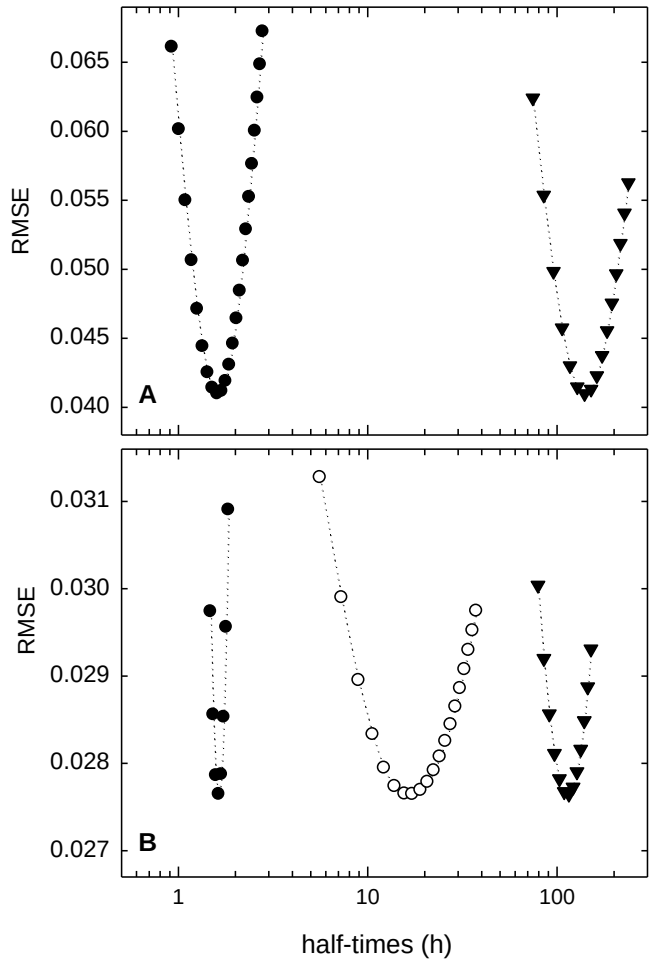

Fig. 9: Sensitivity analysis

Sensitivity of the fit of the nitrogen model (A) and the carbon model (B) to departures from optimized values of the half lives of the transport pool (closed circles), short-term carbon storage pool (open circles) and long-term storage pools (closed triangles). The minimum root mean squared error (RMSE) indicates the optimum half life, while a higher sensitivity (and, thus, confidence in optimized values) is expressed by a sharper increase in RMSE.

2.4 DISCUSSION

CHARACTERIZATION OF THE ASSIMILATE SUPPLY SYSTEM FOR LEAF GROWTH: THE DISTINCTION BETWEEN 'SINK SYSTEM' AND 'SOURCE SYSTEM' APPROACHES

This work presents a quantitative characterization of the assimilate supply system for leaf growth in a perennial cool-season (C3) grass growing in a near-steady-state in continuous light. This system was defined in terms of compartmental models of nitrogen and carbon. The experimental data and modeling yielded quantitative proof for the participation of several kinetically distinct pools (including stores) in the supply of nitrogen and carbon to leaf growth.

Any advance reported in this work is founded on the combined use of a specific set of approaches and methodologies: (i) the maintenance of constant growth conditions provided for a system in steady-state; (ii) measurements of growth zone state and process variables yielded the import fluxes of nitrogen and (amino- and carbohydrate-) carbon into the growth zone (Lattanzi et al. 2004); (iii) compartmental modeling of tracer time courses (Lattanzi et al. 2005) revealed the structure and kinetic properties of the supply system for leaf growth; (iv) sensitivity analysis (according to Lehmeier et al. 2008) helped to detect redundant elements and characteristics in more complex supply system models; (v) dual nitrogen and carbon dynamic labeling (*sensu* Ratcliffe and Shachar-Hill 2006) exposed the close kinetic identity of two of the nitrogen and carbon pools (vi) a wide range of labeling times (2 h to 24 d) was key to the identification of pools with half lives differing by more than three orders of magnitude (≤1 h to ≥4 d); and lastly, (vii) the investigation rested on the analysis of a large number of plants, ensuring representativeness of the data.

The present compartmental model is distinct from most of earlier studies in that it characterizes a sink supply system, rather than a source system. Examples of the later include compartmental systems of carbon fluxes in mature leaves (e.g. review by Farrar 1989b) or nitrogen fluxes in roots and mature leaves (review by Yoneyama et al. 2003). An important distinction between these and the present one concerns the nature of pools and pools' size. In source system studies, the pools are often defined physically (in terms of the localization in certain organs) and / or biochemically (e.g. starch, fructan or protein). In the sink system approach, as used here, physical and biochemical pool definitions are principally not possible: the existence and properties of pools is inferred solely from their function which is supplying nitrogen and carbon to the sink (i.e. leaf growth). Hence, as defined here, a pool comprises a set of biochemical compounds, potentially localized all over the plant, which i) exhibits practically the same proportion of tracer and thus the same labeling kinetics and ii) supplies leaf growth. Formally, a

pool reflects an amount of some material with a kinetically homogeneous behaviour and thus a uniform isotopic composition (Rescigno 2001).

Pools are hence identified based on the fact that they supply leaf growth: any assimilate pool that does not supply leaf growth is not grasped by the analysis. Further, a source pool (e.g. fructan stored in leaf sheaths) can in principle supply several sink functions (e.g. leaf growth and root respiration). It follows that pool size, as defined here, refers to a certain fraction of a given physical/biochemical pool, namely that fraction supplying leaf growth. The actual size may have been much bigger, if it also supplied other (competing) functions. This means that source and sink systems approaches do not necessarily yield congruent pool sizes.

THE IDENTITY OF POOLS

The results of the analysis generally agree with the hypothetical model (Fig. 2), supporting the existence of (i) rapidly turned over 'transport' pools, (ii) a short-term carbohydrate store, whose half life suggests vacuolar storage in source leaves, (iii) a long-term carbon store, whose half life longer than 4 d suggests fructans and proteins, and (iv) a nitrogen store whose half-time suggests proteins, although chloroplastic or vacuolar amino acids (and perhaps even nitrate) may also be part of this pool. Due to similar kinetics of the substrates comprising the carbon transport pool, the hypothesized CH_2O-C and amino-C transport pool could not be separated in the carbon model, resulting in a mixed transport pool.

Basis for this separation were essentially half lives of specific carbon and nitrogen compounds, in distinct plant parts and cellular spaces, estimated in previous studies (references see further on).

TRANSPORT POOL: A GATEWAY FOR THE DIRECT TRANSFER OF ASSIMILATED CARBON AND NITROGEN TO THE GROWTH ZONE

A notable distinction between nitrogen and carbon initial kinetics was the 0.9 h delay for ^{15}N tracer arrival in the transport pool (Tab. 1). This delay is too long to be explained by root to shoot xylem transport (Windt et al. 2006) and likely reflects the kinetics of nitrate uptake, plus the path from roots to photosynthetically active leaf laminae – the main site of nitrate reduction in *L. perenne* (Castle and Rowarth 2003) –, plus the possible storage of part of the nitrate flux in root vacuoles (Devienne et al. 1994). Otherwise, the half lives of the nitrogen and carbon transport pools were strikingly similar ($t_{0.5}$ 40 min, Tab. 2), and agree with those few reported for amino acids (cf. Yoneyama and Takeba 1984; Yoneyama et al. 1987, review by Yoneyama et al.

2003) and sucrose (cf. Farrar and Farrar 1986; Borland and Farrar 1988, review by Farrar 1989b) in the cytoplasmic and apoplastic spaces of photosynthetic leaves.

Inevitably, the transport pool must have also comprised phloem cytoplasmic and apoplastic spaces along the path from source leaves to the leaf growth zone. Indeed, the phloem appears to be an important component of this pool: (i) the ratio of transport carbon to transport nitrogen pools (18.3) was very close to that of imported substrates (18.1) and similar to that reported for phloem content in grass leaves (16 to 19: Hayashi and Chino 1990; Amiard et al. 2004); (ii) considering a 0.2 to 0.4 m distance from source cells to the leaf growth zone and a phloem velocity of 0.25 to 0.40 mm s^{-1} (Windt et al. 2006), the residence time of substrates in the phloem (10 to 20 min) can account for a substantial part of transport pools half lives.

STORAGE POOLS: VACUOLAR SUBSTRATES AND SENESCENCE RELATED MOBILIZATION OF RESERVES

A relatively long half life (~5 d, Tab. 2) supports the idea that the nitrogen store was made up of proteins. Such a half life is in line with that of RubisCo (~6 d from Friedrich and Huffaker 1980; Mae et al. 1983; Irving and Robinson 2006), generally regarded as a nitrogen store (Millard 1988), and with that of (some) soluble proteins (range 3.5 to 8 d; from Dungey and Davies 1982). However, we can not rule out the presence of amino acids in chloropasts and / or vacuoles in this pool (Winter et al. 1993): the facts that (i) splitting the nitrogen store into two pools improved the model fit, that (ii) the half-time of one of these splitted pools (48 h) would be close to short-term storage (cf. Yoneyama et al. 1987; Yoneyama and Takeba 1984), and that (iii) such improvement depended on simulating a temporal separation of the processes of synthesis and degradation of the protein-pool (as observed for RubisCo, Irving and Robinson 2006; Peterson et al. 1973), all call for a cautious interpretation of the biochemical and functional identity of the nitrogen storage pool.

The carbon long-term store most probably consisted partly of carbohydrate-carbon and partly of amino-carbon, as the comparatively low carbon to nitrogen mass ratio (w/w) estimate of the efflux of Q_2 (6.6) suggested the presence of amino acids derived from protein degradation. This has also been documented in other C3 grasses (Gebbing and Schnyder 1999). On the other hand this means that the larger part of carbon derived from long term stores might have originated from mobilization of fructans stored in the base of mature and senescing leaf sheaths. In vegetative cool-season grasses, this is one of the few carbohydrate pools with potentially low turnover rates (Volenec 1986) that could match the observed half life (Tab. 2), and the only pool of sufficient quantitative importance.

The half life of the short-term carbon storage pool (11 h) is consistent with the range 12 to 24 h cited for vacuolar stored Sucrose in mature, photosynthetically active leaves of several cool season (C3) grasses (Farrar 1989b) and other species (Geiger et al. 1983; Moorby and Jarman 1975; Rocher and Prioul 1987). Fructan turnover can also be in the order of several hours in leaf blades and sheaths (Borland and Farrar 1988; Farrar and Farrar 1986), but the number of studies is very scarce.

THE ROLE OF STORES IN SUPPLYING SUBSTRATES FOR LEAF GROWTH IN UNDISTURBED PLANTS OF A PERENNIAL GRASS

Forty percent of all nitrogenous material imported into the leaf growth zone and one-third of all carbon substrates were not delivered to the leaf growth zone directly after their assimilation but cycled (at least once) through other metabolic routes before being used in the leaf growth process. Clearly, leaf growth depended to a large extent on current nitrogen uptake and photosynthesis. But stores constituted a substantial, even co-dominant, component in both the nitrogen and carbon supply systems of perennial ryegrass. This is all more notable considering that plants grew in conditions allowing for continuous nitrogen uptake and carbon assimilation.

SHORT-TERM STORES: THE BUFFERING OF DIURNAL CYCLES IN PHOTOSYNTHESIS

Opposite to the marked day/night cycle in photosynthesis, leaf expansion continues almost unaltered during night, and related assimilate consumption by leaf growth zones is high (Schnyder et al. 1988). Starch-storing species (most dicots, all C4 grasses) rely on mobilization of chloroplastic starch synthesized with little or no turnover during light hours to buffer this imbalance. The proportion of assimilates diverted to starch synthesis is finely regulated and related to day-length (review by Smith and Stitt 2007). A similar understanding is lacking for C3 grasses that store vacuolar sucrose / fructans, in which day - night dynamics are less marked (Cairns et al. 2000). The present study shows that short-term stored carbon supplied 15 % of leaf growth, even in continuous light, meaning that (i) mobilization of carbohydrates from reserve pools also occurs in light, (ii) there must be a continuous turnover of carbohydrate stores, and (iii) this material is actually utilized for growth processes.

The proportion of sucrose passing through short-term vacuolar storage before being exported can be estimated from fitted (rate) constants in studies using compartmental analyses. In barley growing at 20°C with a 16 h photoperiod, this proportion averaged 40 % to 45 % (Baxter and Farrar 1999; Farrar and Farrar 1986) increasing to 65 % at 10°C and decreasing to 25 % at 30°C (Prosser and Farrar 1981). In wheat under 14 h photoperiod a similar value of 46 % was found

(Dilkes et al. 2004). In two *Poa* species this proportion was 35 % and 59 % (Borland and Farrar 1988). Vacuolar storage was important in *L. perenne* plants growing under a 12 h light photoperiod and 15°C (Lattanzi et al. 2005). Compared to these values, the 15 % observed in the present study is low (Tab. 2). This suggests that a lower proportion of assimilated carbon passes through vacuoles when the balance between carbon supply and demand determines a lesser need to buffer day/night cycles, as under continuous light. If the costs of vacuolar storage of carbon are substantial enough to reduce growth (as seems the case for starch storage, (Smith and Stitt 2007), such a strategy would be sensible. But this remains to be elucidated.

LONG-TERM STORES: WHAT ROLE DO THEY PLAY?

Long term stores provided one-sixth of all carbon used in leaf growth. This is similar to values observed in *L. perenne* plants growing under a 12 h photoperiod and 15°C (Lattanzi et al. 2005), suggesting that, contrary to the role of the short-term carbon store, continuous light had minor effects on the contribution of long-term stores. In contrast, mobilized nitrogen supplied 41 % of leaf growth demand. This is far more than the 24 % reported by Lattanzi et al. 2005). However, the fact that Q_2 might also comprise shorter-term storage calls for a cautious assessment. Indeed, splitting Q_2 into two storage pools gave contributions of 23 % and 18 % for the chloroplastic/vacuolar pool and the 'truly' long-term pool, respectively.

Assuming that the carbon to nitrogen ratio (w/w) of transported amino acids is 2.7 (Amiard et al. 2004; Fisher and Macnicol 1986) and that nitrate accounted for <10 % of nitrogen import (M. Kavanová et al. unpublished data), 15 % of all carbon imported into the leaf growth zone would have occurred in the form of amino-carbon. How much of this derived from protein recycling is uncertain. The reason is the ample opportunity for 'old' carbon to be lost, e.g. by respiration (Lehmeier et al. 2008), and to be replaced with 'new' (*i.e.* labeled) carbon during transamination (Deleens et al. 1994; Gebbing and Schnyder 1999). Hence, it is likely that the larger part of the carbon derived from long-term stores originated from fructan mobilization. In undisturbed plants, mobilization has been suggested to be associated with leaf senescence (Morvan-Bertrand et al. 2001; Volenec 1986).

Long term stores provided a significant proportion of all carbon and nitrogen used for leaf growth in plants growing undisturbed, with ample access to water and nutrients, and continuous photosynthesis. Why this is so, is unclear. Farrar 1989b) speculated that continuous turnover of reserves is the cost (diversion of assimilates away from growth and maintenance of enzymatic machinery) plants pay for having the ability to respond rapidly to perturbations of photosynthetic activity. We suggest such continuous turnover may be mediated by the turnover of organs, rather

than by any intrinsic regulatory mechanism, and hence may be a consequence of the general strategy that grasses have adopted to cope with environmental fluctuations (of temperature, water and nutrient supply, or grazing): a continual and rather rapid turnover of organs (leaves, in particular), leading to the replacement of old organs with new ones that are structurally and functionally prepared to perform better under the current (or ‘anticipated’) conditions.

2.5 CONCLUSION

It is a common view that substrates derived from current assimilation are used in preference to material derived from stores to sustain growth (Chapin F.S.3 et al. 1990). The present study provides experimental evidence in support of this: current assimilation supplied the greater part of nitrogen and carbon used for leaf growth (~60 % and ~70 %, respectively). Nonetheless, it definitely shows that storage constituted a substantial component in both the nitrogen and carbon supply systems of growing leaves in *L. perenne*, even under favourable growth conditions allowing for continuous photosynthesis / nitrogen uptake. Newly assimilated carbon and newly absorbed nitrogen provided for the immediate demand, for an 'anticipated' demand (*sensu* Smith and Stitt 2007) during the following day or so, and also for the maintenance of longer-term stores (leaf proteins and fructans) for an 'anticipated' demand in a more distant future. This argues against the idea of static reserve pools kept untouched for later use, at least in this perennial, fructan-storing C3 grass.

Clearly, from the source view there are several spatial compartments, which influence the kinetics of the nitrogen and carbon transport pools: cytoplastic and apoplastic space in photosynthetic mesophyll cells and the phloem. Studies of Britto and Kronzucker (2003), Devienne et al. (1994), Pitman (1963), Siddiqi et al. (1990), Siddiqi et al. (1991) and others were able to characterize the kinetics of nitrogen in different compartments in roots. And Cheeseman (1986) and co-workers as well as Schjoerring et al. (2002) and coworkers analyzed the kinetics of different solutes throughout the plant. Whereas tracer studies with ^{14}C on sucrose fluxes on source leaves were unable to separate apoplastic and cytoplastic compartments (Geiger et al. 1983; Rocher and Prioul 1987), even though they probably also exist. These kinetical differences within cytoplatic and apoplastic spaces are surely important, but compared to the big kinetic differences between transport and storage pools, which occur while observing the system from its sink side, they are hardly noticeable. And thus for the aim of this study we merge those small compartments (apoplastic and cytoplastic space) together in one bigger one called 'transport' pool. It should be mentioned, the term 'pool' as used in this study reflects an amount of some material with kinetically homogeneous behaviour (Jacquez and Simon 1993), which might well be located in different sites of a grass tiller, but nevertheless shows similar kinetics.

2.6 MATERIALS AND METHODS

$^{13}CO_2/^{12}CO_2$ GROWTH CHAMBERS FACILITY

The experiment took place in the $^{13}CO_2/^{12}CO_2$ gas exchange and labeling system described by Schnyder et al. (2003). Air supply to two growth chambers (E15, Conviron) was performed by mixing CO_2–free air and CO_2 with known carbon isotope composition ($\delta^{13}C$, with $\delta^{13}C = (R_{sample} / R_{standard}) - 1$, and R the $^{13}C/^{12}C$ ratio in the sample (air) and the international standard (VPDB)). CO_2 concentration in both chambers was kept constant at 360 µL L^{-1}. The rate of CO_2 supply was nine times larger than the stand CO_2 uptake rate, minimizing the effects of photosynthesis and respiration on concentration and $\delta^{13}C$ of CO_2 inside the chambers.

One chamber received CO_2 depleted in ^{13}C, the other, CO_2 enriched in ^{13}C (both CO_2 from Linde AG). Periodic adjustments of air flow maintained the $\delta^{13}C$ of CO_2 at $\delta^{13}C$ –1.7 ‰ (±0.7 ‰ SD, n=376) and $\delta^{13}C$ –28.8 (±0.6 ‰ SD, n=376). Custom-made air-locks allowed opening chamber doors for handling/sampling plants with minimal effects on the concentration and $\delta^{13}C$ of CO_2 inside the chambers (Lehmeier et al. 2008).

PLANT MATERIAL AND GROWTH CONDITIONS

Plant material and growth conditions were as described in Lehmeier et al. (2008). Briefly, seeds of perennial ryegrass (*Lolium perenne*, cv. Acento) were sown individually in plastic pots (350 mm high, 50 mm diameter) filled with quartz sand. Pots were arranged in plastic containers to form dense stands (378 plants m^{-2}). Plants grew in continuous light, supplied by cool white fluorescent tubes, at 275 µmol m^{-2} s^{-1} photosynthetic active photon flux density at the top of the canopy. Temperature was set at 20°C at the level of the leaf growth zone and relative humidity near 85 %. Plants were irrigated by brief flooding every 3 hours with a modified Hoagland nutrient solution containing 7.5 mM of nitrogen as nitrate (2.5 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 1.0 mM $MgSO_4$, 0.18 mM KH_2PO_4, 0.21 mM K_2HPO_4, 0.5 mM NaCl, 0.4 mM KCl, 0.4mM $CaCl_2$, 0.125 mM iron (Fe) as EDTA, and micronutrients). One of the chambers received nutrient solution with 1.0 atom% ^{15}N in nitrate, the other, nitrate with ^{15}N at natural abundance (0.37 atom%). The nutrient solutions were renewed frequently, and pots flushed with demineralized water to prevent salt accumulation.

^{15}N AND ^{13}C LABELING

Randomly selected individual plants were labeled by swapping them between chambers. Thus, plants growing in the chamber with ^{13}C-enriched CO_2 and 1 atom% ^{15}N in nitrate were transferred to the chamber with ^{13}C-depleted CO_2 and 0.37 atom% ^{15}N in nitrate, and *vice versa*. Pots were rinsed with the new nutrient solution prior to placement in the 'isotopically new' environment. In this way plants were labeled for 2, 4, 8, 16, 24, 48, 96, 192, 383 and 576 hours. Four replicate plants were used for each labeling interval. To minimize possible size- and development-related effects, a nested labeling pattern was used, so that the mean age of labeled plants for all labeling intervals only varied little (Fig. 10).

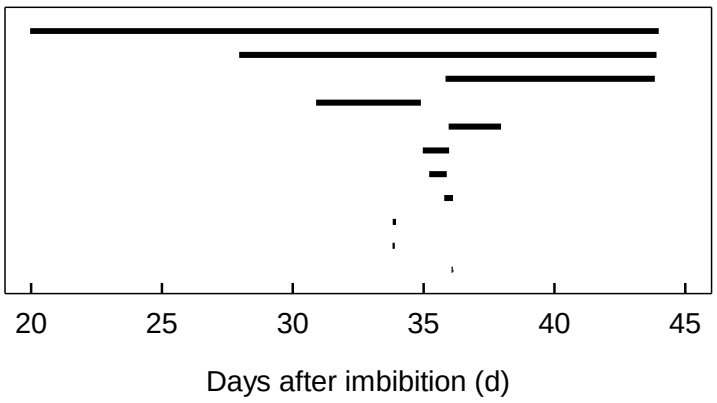

Fig. 10: Labeling pattern of high N grass plants for ^{15}N and ^{13}C.
Labeling was nested to minimize possible size- and development-related effects.

LEAF ELONGATION RATE MEASUREMENTS

Mature tillers (tillers having at least two fully expanded leaves) were selected for measurements of leaf elongation rate (LER, mm h^{-1}). LER was determined on the most rapidly growing blade, during the phase of maximal growth, when leaf elongation rate is near constant (Schnyder et al. 1990). Leaf length was measured with a ruler twice a day on three plants per chamber. LER was calculated as the rate of change of the distance between the tip of the elongating blade and the ligule of the youngest fully expanded leaf measured.

SAMPLING

As they grow, leaves undergo morphological changes along the blade and changes in the speed at which cells divide and expand in the leaf meristem (Maurice et al. 1997; Schnyder et al. 1990). Both these elements directly affect carbon and nitrogen import rates into the growth zone

(Lattanzi et al. 2004). Therefore, only leaves in which the growing blade had between 40 % and 60 % of its final length were sampled, as extensive work in grass leaf growth established that during this phase growth parameters (including leaf elongation rate, lamina width and thickness) vary little, both within an individual leaf and between successive leaves (Durand et al. 1999). In a sense, we sampled 'the same' leaf on each harvest date.

Plants were removed from the stands at the end of given labeling intervals. In each plant, the growth zone and an immediately adjacent piece of recently produced leaf tissue (RPT) were dissected from the most rapidly growing leaf of mature tillers. Preliminary tests showed that the leaf growth zone comprised two-third of the length of the enclosing sheath. The sampled adjacent recently produced tissue had a length of 3 mm (for labeling periods shorter than 1 d) or 5 mm (labeling periods longer than 1 d). On average, nine tillers were sampled from each plant (range three to 14 tillers).

Samples were combined by fraction, weighed, frozen in liquid nitrogen, freeze-dried for 72 h at -80°C, weighed again, ground to flour mesh quality with a ball-mill and stored at -30°C until elemental and isotopic analysis. Samples were processed swiftly: less than 30 min elapsed between removal of a plant from the stand and freezing of the samples in liquid nitrogen. In addition, unlabelled plants from both chambers were sampled and processed in the same way as labeled plants. These served as end-members in the mixing equation for assessment of ^{13}C and ^{15}N tracer kinetics (see below). Non-labeled plants were sampled at 2 d-intervals throughout the duration of the labeling experiment.

ELEMENTAL AND ISOTOPIC ANALYSES

Concentrations of carbon and nitrogen and isotopic composition ($\delta^{13}C$ and atom%^{15}N) were determined on samples of leaf growth zones and of recently produced tissue in 0.7 ± 0.05 mg aliquots by combustion in an elemental analyzer (NA 1110, Carlo Erba Instruments) interfaced to a continuous-flow isotope ratio mass spectrometer (CF-IRMS; Delta Plus, Finnigan). Each sample was measured against laboratory working standard gases (CO_2 and N_2), which were previously calibrated against IAEA secondary standards (IAEA-CH6, accuracy of calibration 0.06 ‰ SD; IEAE-N1 and IAEA-N2, accuracy of calibration 0.09 ‰). For ^{15}N no two-point calibration was done, but instead the isotopic composition of the nutrient solution was determined and served as end-members in the mixing model. Solid internal laboratory standards (SILS, wheat flour), with a carbon to nitrogen ratio of 21.6, similar to that of samples, were calibrated against these references. One SILS was measured after every tenth sample. The precision (SD) for sample repeats was 0.15 ‰ for $\delta^{13}C$ and 0.004 atom% for ^{15}N.

CALCULATION OF THE FRACTION OF LABELED AND UNLABELED CARBON AND NITROGEN IN A SAMPLE

The fraction of labeled carbon ($f_{lab\ C}$) was defined as the proportion of carbon derived from assimilation following transfer to the isotopically 'new' environment. For instance, for plants grown in chamber I and transferred to chamber II, chamber II meant the 'new' environment and chamber I was the 'old' environment. The fraction of unlabeled carbon ($f_{unlab\ C}$) is simply $1 - f_{lab\ C}$.

The isotopic composition ($\delta^{13}C$ and atom% ^{15}N) of end-members did not change during the course of the labeling experiment (data not shown). Also, the direction of the transfer for labeling (chamber I → II, or chamber II →I) had no effect on the kinetics of $f_{lab\ C}$ and $f_{lab\ N}$.

$f_{lab\ C}$ was assessed using a two-member mixing model (de Visser et al. 1997):

$$f_{labC} = \frac{\delta_{spl} - \delta_{old}}{\delta_{new} - \delta_{old}} \quad (1)$$

Where δ_{spl} is the $\delta^{13}C$ of the labeled sample, and δ_{old} and δ_{new} correspond to the $\delta^{13}C$ of the end-members of the mixing model. The fraction of labeled nitrogen ($f_{lab\ N}$) was obtained analogously.

ESTIMATION OF THE AMOUNT OF TRACER IN THE FLUXES OF CARBON AND NITROGEN IMPORTED INTO THE GROWTH ZONE

Import of labeled and non-labeled carbon and nitrogen into the growth zone were assessed as described by Lattanzi et al. (2004) and Lattanzi et al. (2005), except that here the time step size was 2 h (1 d in Lattanzi et al. 2005) and respiration in the growth zone was accounted for. For a growth zone in steady-state, the import of carbon (I_C, g growth zone^{-1} h^{-1}) equals its efflux by the displacement of newly produced tissue (termed export by tissue-bound efflux in Lattanzi et al. 2004; E_C, g growth zone^{-1} h^{-1}) and respiration (R, g growth zone^{-1} h^{-1}), thus $I_C = E_C + R$).

Displacement of carbon out of the growth zone (E_C) was estimated as the product of LER (mm h^{-1}) and the mass of carbon per unit length of recently produced tissue (ρ_C, g mm^{-1}): $E_C = LER * \rho_C$. Respiration was estimated to be 10 % of carbon imported into the leaf growth zone (Penning de Vries 1975; Schnyder and Nelson 1987). All relevant variables were almost constant (Fig. 3, Fig. 4), therefore average values were used.

Import of labeled carbon into the growth zone ($I_{lab\ C}$) was similarly assessed, but accounted for changes in the amount of labeled carbon within the growth zone during dynamic labeling. Thus, import of labeled carbon was assessed for 2 h–intervals (denoted with < >) as the displacement of labeled carbon in new tissue ($E_{lab\ C}$) and loss of labeled respired carbon ($R_{lab\ C}$), plus the

variation in mass of labeled carbon inside the growth zone ($G_{lab\ C}$) over the same time-interval t_{i-2} to t_i:

$$\langle I_{lab\,C} \rangle = \langle E_{lab\,C} \rangle + \langle R_{lab\,C} \rangle + (G_{lab\,C\,ti} - G_{lab\,C\,ti-2}) \quad (2)$$

Estimated $R_{lab\ C}$ assumed that the fraction of label in respired carbon was the same as in carbon in displaced tissue. Finally, the fraction of labeled carbon in the import flux into the leaf growth zone during the interval t_{i-2} to t_i ($<f_{lab\ CI}>$, with ($<f_{lab\ CI}> = < I_{lab\ C} >/<I_C>$) was given as:

$$\left\langle f_{lab\_IC} \right\rangle = \frac{E}{\left\langle I_C \right\rangle} + \frac{R}{\left\langle I_C \right\rangle} + \frac{(G_{lab\,C\,ti} - G_{lab\,C\,ti-2})}{\left\langle I_C \right\rangle} \quad (3a)$$

Since G_C, $<I_C>$, $<E_C>$ and $<R_C>$ were constant, and $<I_C> = <E_C> + <R_C>$, eq (3a) simplifies to:

$$\left\langle f_{lab\ IC} \right\rangle = \left\langle f_{lab\,EC} \right\rangle + (f_{lab\,GC\,ti} - f_{lab\,GC\,ti-2}) \times \frac{G_C}{\left\langle I_C \right\rangle} \quad (3b)$$

Eq. (3b) shows that estimation of $f_{lab\ IC}$ over a 2 h–interval ($t_{i-2} – t_i$) required estimation of (1) $f_{lab\ EC}$ over the same time-interval, (2) f_{labGC} at the lower (t_{i-2}) and upper (t_i) ends of the time-interval, and (3) G_C / $<I_C>$. (1) was estimated as the numeric integral of the fraction of labeled carbon in recently produced tissue over 2 h–intervals of the spline fitted to the time course of $f_{lab\ RPT\ C}$ ($<f_{lab\ E\ C}> = \int f_{lab\ RPT\ C}$). Similarly, splines fitted to the time course of the $f_{lab\ GC}$ were used to estimate values at t_{i-2} and t_i. Import of nitrogen (I_N) and the fraction of labeled nitrogen in the import flux ($<f_{lab\ I\ N}>$) were estimated in an analogous way.

COMPARTMENTAL MODELING OF CARBON AND NITROGEN TRACER TIME COURSES

The time course of nitrogen tracer into the leaf growth zone was described with a two-pool model. For carbon tracer, a three-pool carbon model was used (Fig. 8). These models were translated into sets of differential equations assuming a system in steady-state and obeying first order kinetics. This means that pool sizes and fluxes do not change over time (only the proportion of tracer does), and that fluxes are the product of pool sizes times rate constants k_{xy}, where x and y refer to donor and acceptor pool, respectively. Under these assumptions, the rate of change of each pool over time (t) is given by (shown only for carbon):

$$\frac{dQ_{1C}}{dt} = I_C - (k_{10} + k_{12} + k_{13})Q_{1C} + k_{21}Q_{2C} + k_{31}Q_{3C}$$

$$\frac{dQ_{2C}}{dt} = k_{12}Q_{1C} - k_{21}Q_{2C}$$

$$\frac{dQ_{3C}}{dt} = k_{13}Q_{1C} - k_{31}Q_{3C} \quad (4)$$

assuming that:

$$\frac{dQ_{1C}}{dt} = \frac{dQ_{2C}}{dt} = \frac{dQ_{3C}}{dt} = 0 \qquad \text{and } I_C = k_{10} Q_{1C} \tag{5}$$

where I_C is the measured import rate of carbon into the leaf growth zone, pool sizes are given by:

$$Q_{1C} = \frac{I_C}{k_{10}},\ Q_{2C} = \frac{k_{12}}{k_{21}k_{10}} I_C,\ Q_{3C} = \frac{k_{13}}{k_{31}k_{10}} I_C \tag{6}$$

Models were implemented in Modelmaker (version 3.0; Cherwell Scientific), as described in Lattanzi et al. (2005), except that predicted $f_{lab\ I\ C}$ and $f_{lab\ I\ N}$ were integrated over 2 h intervals, thus producing data comparable to measured values. The built-in Levenberg-Marquardt algorithm was used to optimize the values of fitted parameters that minimized the root of the mean of squared differences between measured and predicted $<f_{lab\ I\ C}>$ and $<f_{lab\ I\ N}>$. Then, sensitivity analyses were performed to determine the response of RMSE to departures from optimized pools half lives as described in Lehmeier et al. (2008). The higher the change in RMSE in response to these variations, the narrower the range of half lives providing similar goodness-of-fit, the higher the confidence in optimized values.

VALIDITY OF THE ASSUMPTIONS OF COMPARTMENTAL MODELING

Lattanzi et al. (2005) discussed in detail the validity of the assumptions on which the present analysis rests. The assumption that the metabolism and transport of tracers reflects that of unlabelled material was satisfied by assessing and correcting for isotope effects in the labeling experiment (see above). The assumption that pools obey first order kinetics and are well-mixed is probably untrue in a strict sense, but its practical validity has been supported in studies of carbon (Farrar 1989b) and nitrogen fluxes (Britto and Kronzucker 2003; Irving and Robinson 2006).

Regarding the assumption of a system in near steady-state, it is clear that carbon and nitrogen fluxes will vary in grass plants growing for 24 d. A nested labeling pattern (Fig. 10) was used to minimize size effects. Nevertheless, to determine if and how variations in carbon and nitrogen fluxes in individual mature tillers (the funtional unit in this study) would compromise the inferences made regarding the sources supplying import into the leaf growth zone (the ‘sink’ flux basis of our compartmental analysis), we assessed which fluxes would change, and to what extent.

First, carbon and nitrogen import into the leaf growth zone was considered in relation to the two sources of un-steadiness: diurnal rhythms (which affect photosynthesis, nitrate uptake, and carbon and nitrogen allocation) and leaf development (which affects leaf elongation rate, lamina

width, tissue density, and growth zone carbon and nitrogen masses). Diurnal rhythms were minimized by the use of continuous light, as in previous studies on carbohydrate and nitrogenous compounds fluxes into and within growth zones (Allard and Nelson 1991; Gastal and Nelson 1994; Lattanzi et al. 2004; Lehmeier et al. 2008; Schnyder and Nelson 1987), and frequent irrigation. Development was obviously not arrested. But growth conditions kept the shoot apical meristem vegetative, and we sampled leaves at a defined developmental stage in which growth and associated carbon and nitrogen fluxes were steady (see Results). These are the reasons behind the observed stability in measured rates of carbon and nitrogen import into the leaf growth zone (Fig. 5).

The size of mature tillers increased during the 24-d labeling period, and thus fluxes at the 'source' level must have varied in this period. However, such changes were i) brought about by increases in leaves' size, not number (thus, the balance between growing *vs*. mature or senescing leaves remained unchanged), and ii) marginal at short and medium time-scales. Both the half life of all but the slowest pools and the relative contribution of current assimilation *vs*. stores were largely defined within 48 h of labeling, because by that time tracer time-courses had already entered the third (final) phase (Fig. 7). Thus, changes in the size of mature tillers affected only the estimation of the half life of the slower pools (~120 h); and not at all the resolution of the fastest pools (half life: 0.7 h to 11 h) and the contribution of current assimilation and stores –the result most relevant to our conclusions.

3 THE CARBON AND NITROGEN SUPPLY SYSTEM OF LEAF GROWTH IN PERENNIAL RYEGRASS UNDER NITROGEN DEFICIENCY

3.1 ABSTRACT

In this study we analyzed the effect of nitrogen deficiency on the nitrogen and carbon supply system for leaf growth in the perennial ryegrass (*Lolium perenne*). Specifically, we studied the effect of nitrogen deficiency on i) the validity of the model structure supplying nitrogen and carbon to leaf growth evaluated under high nitrogen supply, ii) the half lives ($t_{0.5}$) of the pools and iii) the role of current assimilates *versus* stores. Individual plants, growing in continuous light with a low supply of nitrogen (1.0 mM) were dynamically labeled with $^{15}NO_3^-/^{14}NO_3^-$ and $^{13}CO_2/^{12}CO_2$ for 2 to 935 h. We analyzed the kinetics of tracer incorporation into the fluxes of carbon and nitrogen imported into the leaf growth zone by compartmental models - as done with plants grown under high nitrogen supply (7.5 mM) - and compared those results. Tracer kinetics of ^{15}N and ^{13}C under nitrogen deficiency could be described by the same two- and three-pool model valid under high nitrogen nutrition, meaning that the structure of the nitrogen and carbon supply system (*i.e.* number and arrangement of pools) was not affected by nitrogen deficiency. As well, current uptake/photosynthesis and stores supplied nitrogen and carbon to leaf growth. Half lives of the nitrogen and carbon transport pools, routing current assimilates in form of cytosolic and apoplastic amino acids and sucrose to the leaf growth zone, increased from ~50 min to ~1.2 to 3.0 h, but they still accounted for a substantial part of total nitrogen (50 %) and carbon (60 %) import. Hence, 50 % of nitrogen and 40 % of carbon substrates used for leaf growth first cycled through stores. The long-term nitrogen and carbon store, most likely existing of amino acids derived from protein mobilization, also showed a slower turnover ($t_{0.5}$ ~7 d). The carbon short-term store, assumed to contain vacuolar sucrose for buffering, showed a slightly increased $t_{0.5}$, but became more important under nitrogen deficiency and thus supplied 26 % of all carbon for leaf growth. As expected sizes of all carbon pools increased; but unexpectedly, the size of the nitrogen transport pool also increased. Results indicate that nutrient availability did not influence the structure of the supply system for leaf growth. It further showed that – even under nitrogen deficiency - stores are continuously synthesized and mobilized and, strikingly even nitrogen stores, are an important source for leaf growth.

3.2 INTRODUCTION

This study focuses on the effect of nitrogen deficiency on the nitrogen and carbon supply system of leaf growth in the forage grass *L. perenne*. It is known that nitrogen has a big effect on leaf growth of grasses (Gastal and Nelson 1994; Volenec and Nelson 1983; Vos et al. 2005), as it is an essential constituent of many biochemical compounds like amino acids, proteins, nucleic acids and secondary metabolites (Marschner 1995). Thus nitrogen deficiency has effects on the composition of mature leaf and root tissue and on the composition and the activity of the photosynthetic apparatus (Evans 1989; Vos et al. 2005). Growth of a grass leaf is confined to the basal part of the growing leaf – the leaf growth zone. As the leaf growth zone is completely heterotrophic (Schnyder et al. 2000), it depends on the supply of assimilates – mainly amino acids and sucrose (Amiard et al. 2004; Fisher and Macnicol 1986) – exported from source tissues (like mature photosynthesizing or senescing leaves, sheaths or roots). Evidently, current assimilation-derived substrates arrive faster in the growth zone than storage-derived material. This difference in the speed of arrival in the leaf growth zone, which is determined by the kinetic properties of the pools of the supply system, can be used to describe the whole-plant supply system *via* compartmental modeling (Lattanzi et al. 2005). In a previous study (see Chapter 2) we characterized the 'assimilate supply system' for leaf growth under favourable and steady growth conditions (*i.e.* at high nitrogen supply and in continuous light). Results showed that leaf growth was supplied by two distinct nitrogen and three distinct carbon pools (see Fig. 8, chapter 2), comprising transport and storage compartments for amino compounds and carbohydrates. Assimilate supply occurs in two forms: amino acids and sucrose. Those in turn can derive either from current assimilation (nitrogen up take or photosynthesis) feeding the growth zone directly *via* a 'transport pool' or can derive from mobilization from stores, which exchange assimilates with the transport pool.

Under favourable growth conditions (*i.e.* high nitrogen supply) current nitrogen assimilates (amino acids) as well as mobilization of long-term stored nitrogen compounds (leaf protein) had a similar importance for the nitrogen supply of leaf growth (59 % and 41 %, respectively). In the same plants current assimilation (sucrose and amino-C) supplied 69 % of all carbon, while the remaining carbon was equally supplied by a short-term (probably vacuolar sucrose and short-term stored fructans) and a long-term carbon store (fructans). Lattanzi et al. (2005) showed that the assimilate supply system for carbon in ryegrass was almost unaffected when changing hierarchical position in the canopy from dominant to subdominant; conversely, the assimilate

supply system for nitrogen was affected – mobilization of stores became more important. This opened the question if a change of nitrogen nutrition would also affect the supply system.

Effects of nitrogen deprivation on source tissues are well studied: the water-soluble carbohydrate content of grass shoots increased, due to an increase in fructan content, the main storage carbohydrate in temperate grasses (Jones et al. 1965; Lehmeier et al. 2008; Lewis et al. 2000; Nowakowski 1962), while soluble nitrogen content of shoots decreased dramatically (Lea and Morot-Gaudry J.-F 2001; Nowakowski 1962) as well as the content of total leaf protein and RubisCo, leading to a reduced photosynthetic capacity (Evans 1989). Further, the amino acid content in phloem exudates in wheat was closely related to the level of external nitrogen nutrition, while contrastingly, the amount of sucrose exported into the phloem was unaffected (Caputo and Barneix 1997). Thus the ratio of sugar : amino acids in the phloem increased under nitrogen deficiency. As these are the main assimilates supplying leaf growth, we expected similar relations in the import flux into the leaf growth zone: tissue of the leaf growth zone showed similar reactions to nitrogen deprivation as total shoot tissue (Gastal and Nelson 1994) – indicating that leaf growth under nitrogen deprivation is mainly limited by nitrogen supply than by carbohydrate supply.

So the effect of nitrogen deprivation on the composition of source and sink tissue is well known. Similarly the effect of nitrogen limitation on the cellular processes in the leaf meristem – leading to a reduced leaf elongation rate – have been elucidated (Kavanova et al. 2008). But still little is known about the effects of nitrogen deprivation on i) the structure of the supply system for nitrogen and carbon to leaf growth, ii) the turnover of pools and iii) the role of current assimilates *versus* stores for the substrate supply of leaf growth.

If nitrogen stores are turned over continuously - as results of high N plants indicated (see chapter 2) – and their mobilization is mediated by the turnover of organs rather than by a genetic program, then under nitrogen deprivation i) the nitrogen long-term storage pool should be turned over more slowly due to a slower leaf turnover rate and thus reveal a $t_{0.5}$, which is related to the leaf expansion duration or the leaf appearance rate and ii) stores should contribute relatively more nitrogen to leaf growth, because current-assimilation-derived nitrogen is limited.

For nitrogen deficient grass plants (1.0 mM) we also used compartmental modeling of the time course of tracer incorporation into carbon and nitrogen substrates being imported into the leaf growth zone (Moorby and Jarman 1975; Prosser and Farrar 1981) under quantitative dynamic labeling with ^{15}N and ^{13}C (de Visser et al. 1997) and compared results with the supply system for leaf growth of plants grown with ample nitrogen (7.5 mM) as characterized in chapter 2.

3.3 RESULTS

EFFECT OF NITROGEN DEFICIENCY ON PLANT GROWTH AND PROCESS VARIABLES

We grew plants under constant growth conditions with a continuous light regime coupled with a frequent supply of water and nutrients to obtain a constant growth rate and thus a (near-)steady-state of carbon and nitrogen uptake fluxes. When subjected to a lower nitrogen supply under the same light conditions, plants produced a set of responses typical of nitrogen deficiency: i) carbon use efficiency [growth / (growth + respiration)] changed from 0.68 (high N) (Lehmeier et al. 2008) to 0.61, which is in the range reported in literature in other herbaceous plants (Van Iersel 2003) (ii) the shoot to root ratio decreased from 2.8 (high N) to 2.1 (low N) (data not shown) showing a predicted reaction to limited nitrogen resources (Berendse and Moller 2009; Poorter and Nagel 2000) (iii) nitrogen concentrations decreased from 1.8 % to 0.9 % in shoots and from 1.3 % to 0.7 % in roots (in a dry weight basis; Lehmeier (2009) personal communication). This shows that results gained in this study are indeed comparable to plants grown under natural conditions.

Due to a nested labeling pattern and harvesting of tillers which were in the same developmental stage (length of growing leaf was 40 % to 60 % of its final length), process variables only changed marginally among plants. Leaves elongated constantly at a rate of 0.87 mm h^{-1} and also the length of the growth zone was constant during the labeling period (Fig. 11) as under high-N.

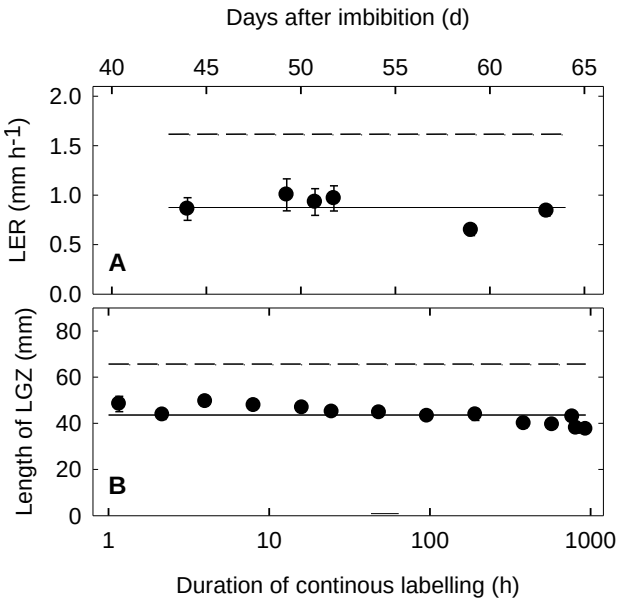

Fig. 11: Process variables low N plants– LER and length of LGZ
Leaf elongation rate (LER) (A) and length of leaf growth zones (B; semi-log scale) of plants of *L. perenne* grown under low nitrogen supply (1.0 mM) during the experimental phase of dynamic labeling with ^{15}N and ^{13}C. For comparison, values under high N conditions are shown as dashed line.

Similar to high-N plants, nitrogen and carbon mass in the growth zone and in recently produced (fully-expanded) leaf tissue did not change with duration of labeling (Fig. 12). These relationships indicated a biological system in near steady-state, in which the fluxes of nitrogen and carbon into and out of the growth zone were stable and only changed marginally during the experimental period (Fig. 13).

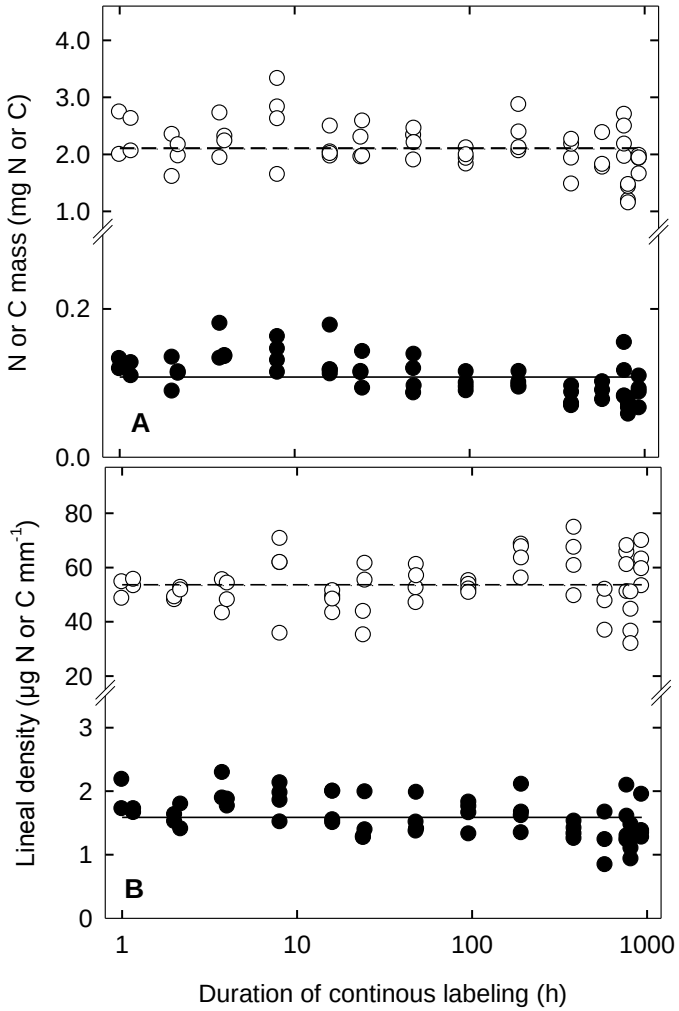

Fig. 12: Process variables in low N plants – N & C mass and lineal nitrogen and carbon density
Nitrogen (closed circles) and carbon (open circles) mass in the leaf growth zone (A) and lineal nitrogen (closed circles) and carbon (open circles) density in recently produced and fully expanded tissue (B) of *L. perenne* plants grown with low nitrogen (1.0 mM) during dynamic labeling with ^{15}N and ^{13}C (semi-log scale).

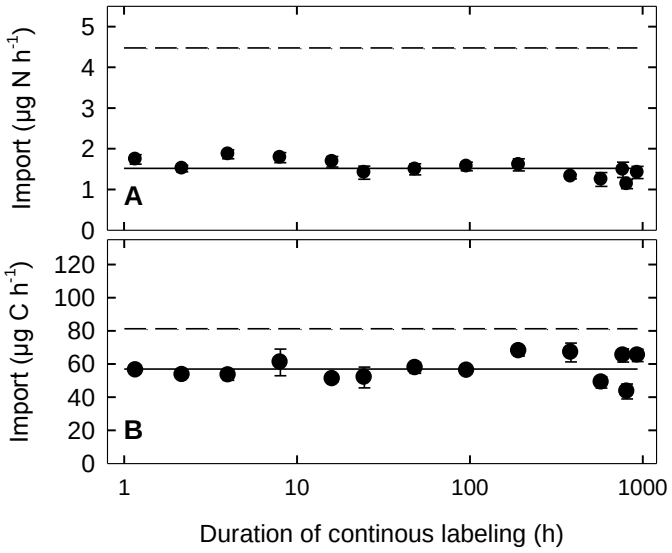

Fig. 13: Import flux over duration of labeling in low N plants
Import flux of nitrogen (A) and carbon (B) into the leaf growth zone in plants of *L. perenne* grown with low nitrogen (1.0 mM) during dynamic labeling with ^{15}N and ^{13}C (semi-log scale). For comparison, values under high N conditions are shown as dashed line.

As expected, the effect of nitrogen deficiency on process variables was significant ($P < 0.05$): i) relative growth rate during the experimental period from labeling until harvest was decreased by 51 % from 3.23 mg C g C^{-1} h^{-1} (high N plants) to 1.58 mg C g C^{-1} h^{-1} (low N plants) (Lehmeier, personal communication), ii) leaf elongation rate was reduced by 46%, length of the leaf growth zone by 35 %, iii) lineal nitrogen density of the growth zone and recently produced tissue was reduced by 31 % and 37 % as well as total tiller nitrogen while iv) lineal carbon density of the growth zone and recently produced tissue increased by 19 % and 31 % and total tiller carbon stayed constant (Tab. 3). But both, the rate of nitrogen and carbon imported into the growth zone decreased.

Tab. 3: State and process variables
State and process variables of plants grown under low N (1.0 mM) and high N (7.5 mM) and the effect of low nitrogen nutrition relative to high nitrogen nutrition with the significance based on a t-test (*P ≤ 0.05). Values are means ± 1SD. RPT: recently produced tissue.

		Low N			**High N**			**Effect of nitrogen deficiency**
variable	*unit*	*mean*	±	*1 SD*	*mean*	±	*1 SD*	*change rel. to high N*
Leaf elongation rate	mm h^{-1}	0.87	±	0.24	1.62	±	0.22	-46% *
Growth zone length	mm	43.59	±	4.88	65.70	±	7.30	-34% *
N mass of growth zone	µg LGZ^{-1}	0.11	±	0.03	0.23	±	0.04	-54% *
C mass of growth zone	µg LGZ^{-1}	2.11	±	0.42	2.67	±	0.44	-21% *
Lineal N density in RPT	µg mm^{-1}	1.59	±	0.04	2.51	±	0.39	-37% *
Lineal C density in RPT	µg mm^{-1}	53.67	±	0.04	41.09	±	5.75	31% *
Nitrogen import	µg h^{-1}	1.52	±	0.31	4.48	±	0.69	-66% *
Carbon import	µg h^{-1}	57.01	±	10.12	81.28	±	11.37	-30% *
Total tiller nitrogen	µg	1424			3111			-54% *
Total tiller carbon	µg	65616			68850			-5%

EFFECT OF NITROGEN DEFICIENCY ON THE ^{15}N AND ^{13}C LABEL INCORPORATION INTO THE LEAF GROWTH ZONE AND RECENTLY PRODUCED TISSUE

Label incorporation into the leaf growth zone and the recently produced tissue is shown in Fig. 14 as fraction of unlabeled nitrogen or carbon over a labeling period from 2 h up to 39 d.

Tracer time courses of labeled nitrogen and carbon showed that tracer arrived in the leaf growth zone with little delay after the onset of labeling. Labeling resulted in a rapid exchange of the previously unlabeled material by labeled nitrogen or carbon. In both nitrogen treatments tracer incorporation of carbon occurred faster than that of nitrogen. Labeling patterns of the recently produced tissue were similar as those of the leaf growth zone, except for a small delay. This was due to the fact, that all nitrogen and carbon deposition occurred within the leaf growth zone and the recently produced tissue was ‘fed’ by tissue-efflux from the leaf growth zone. In low N plants label incorporation of nitrogen and carbon was delayed in both tissues compared to high N plants. This indicated that the turnover of substrate pools was slower in low N plants and labeled substrate was cycling longer in the supplying pools before being transported to the leaf growth

zone. But still the pattern of label incorporation was similar compared to high N plants, implying a similar structure of the nitrogen and carbon supply system.

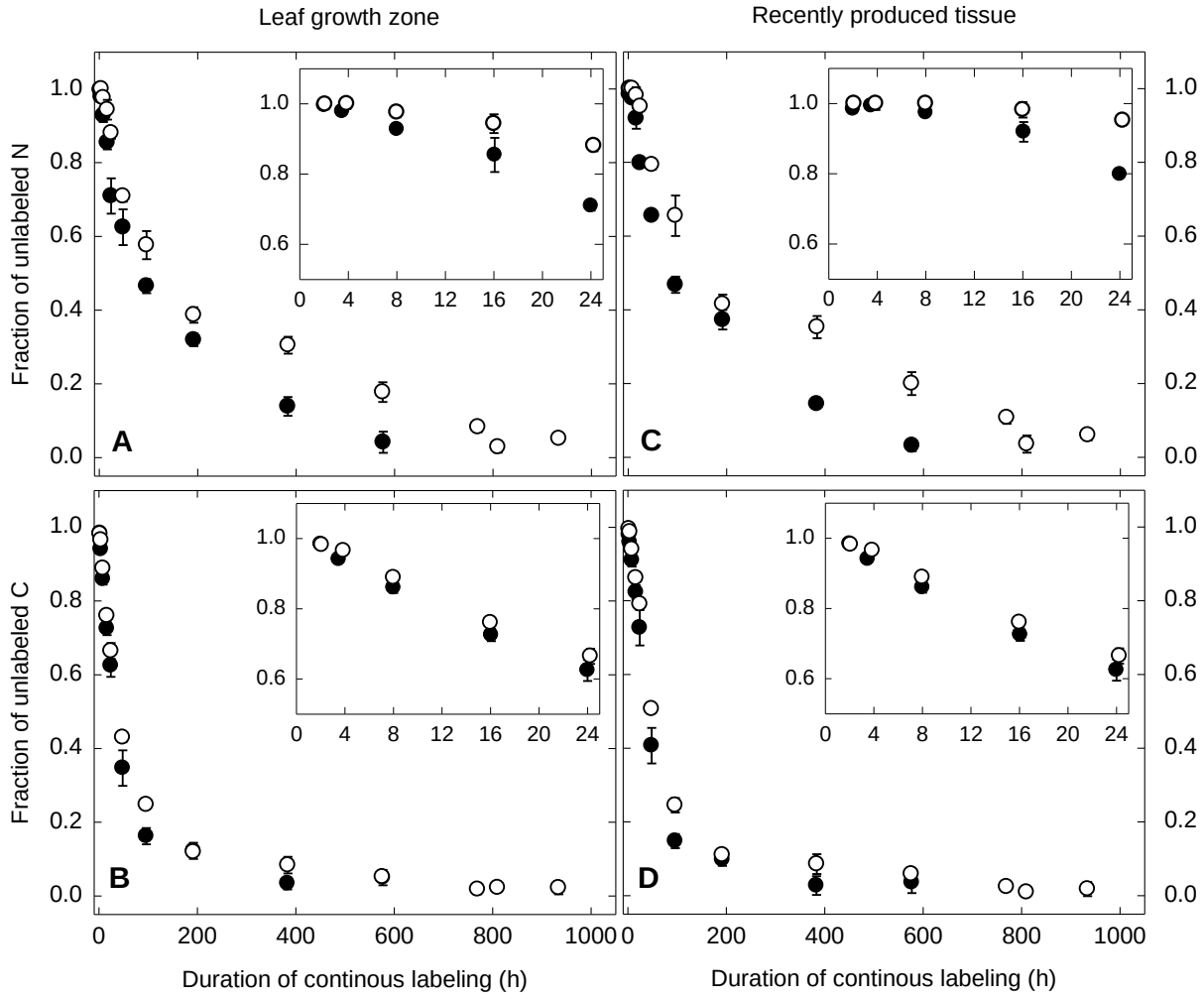

Fig. 14: Tracer time course of unlabeled nitrogen and carbon in high and low N plants
Fraction of unlabeled nitrogen (A & C) and carbon (B & D) in the leaf growth zone (A & B) and recently produced leaf tissue (C & D) of *L. perenne* plants grown under low N (open circles) or high N nutrition (closed circles) during dynamic labeling with ^{13}C and ^{15}N (±1 SE). Labeling started at 0 h and was continued for up to 575 h (high N plants) or 935 h (low N plants). Data are means of 4 replicates (±SE). Insets expand the first 24 h of labeling.

EFFECT OF NITROGEN DEFICIENCY ON THE KINETICS OF ^{15}N AND ^{13}C IN THE IMPORT FLUX INTO THE LEAF GROWTH ZONE

Based on the labeling kinetics of nitrogen and carbon in the leaf growth zone and adjacent recently produced tissue, the fraction of unlabeled nitrogen and carbon in the import flux into the growth zone was assessed (Fig. 15). In both nitrogen treatments the fraction of unlabeled

nitrogen decreased more slowly and with a different pattern than that of carbon. Low N nutrition delayed the import of labeled nitrogen and carbon compared to high N plants by some hours.

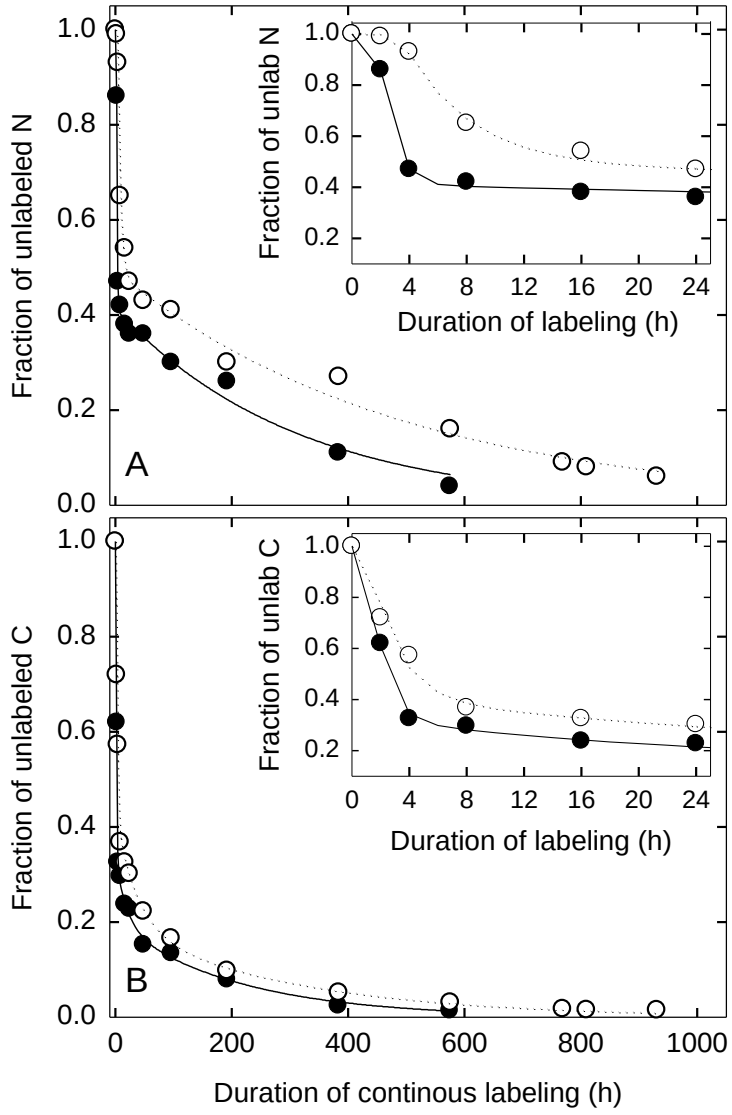

Fig. 15: Tracer time course of nitrogen and carbon in the import flux in high and low N plants
Fraction of unlabeled nitrogen (A) and carbon (B) in the import flux into the growth zone of plants grown under low N (open circles) and high N nutrition (closed circles) during the labeling period of 575 h (high N) or 935 h (low N). Dotted lines display the fit of models to data points of low N plants, solid lines that of high N plants. Insets expand the first 24 h of labeling.

NITROGEN

Labeling kinetics of nitrogen in the import flux of high and low N plants showed three explicit phases in both treatments: after a first lag period of ~0.9 h (high N) and ~ 2.2 h (low N) a second phase with rapid tracer import in the next 3 h and 15 h, respectively, followed. The last phase showed slow tracer import until the end of the labeling period. Under nitrogen deficiency tracer

appearance in the import flux was delayed and the rate of tracer import was slower compared to high N (see slope of model fits). In high N plants ~50 % of nitrogen was labeled already after 4 h, while in low N plants it took about 20 h.

CARBON

Labeling kinetics of carbon in the import flux also showed three phases with a slower tracer import in low N plants than in high N plants. After rapid tracer import in the first 4 h and 6 h, for high N and low N respectively, a second phase of rapid tracer import up to 50 h and 100 h and a third phase of slow tracer import until the end of labeling followed. In contrast to the nitrogen kinetics there was no lag in the import of labeled carbon into the growth zone. Nitrogen deficiency delayed tracer import of carbon, but to a lesser degree than for nitrogen. About 50 % of carbon import was labeled after 3 h in high N plants and after 5 h in low N plants. The strongest effect of nitrogen deficiency on the rate of tracer import into the growth zone was for nitrogen and carbon detectable in the first phase (see slope of model fits).

EFFECT OF NITROGEN DEFICIENCY ON THE SUPPLY SYSTEM FOR LEAF GROWTH

As labeling kinetics of the import flux into the leaf growth zone showed a similar pattern (number of phases) in low N plants as in high N plants, it was highly likely that the structure of the nitrogen and carbon supply system was similar. To test this, alternative model structures including the mentioned two-pool nitrogen and three-pool carbon model – as evaluated previously for high N plants (Fig. 8 in chapter 2) – were fit to the tracer time courses. We found that the same models describing the supply system of high N plants were also able to fit the labeling kinetics of the import flux of low N plants very well. The ability of a model to fit the data (goodness-of-fit), as expressed by its minimum root mean squared error (RMSE), was taken as a measure of its biological realism. From optimized rate constants the half-life ($t_{0.5}$) and proportional contribution of each pool were estimated. The two-pool nitrogen and the three-pool carbon model showed a RMSE of 0.02 and 0.02, respectively. A one-pool nitrogen and a two-pool carbon model showed a significant lack-of-fit (RMSE = 0.28 and 0.03) Increasing the number of pools did not improve the fit enough to accept the loss in degrees of freedom. Thus the previously evaluated two-pool N and three-pool C model showed the most sensible fit to the data in both treatments. Fits of the models from low N plants (dotted lines) are shown in comparison to the fits of high N plants (solid lines) in Fig. 15. System parameters obtained by model optimization runs and the effect of nitrogen deficiency on each parameter are displayed in Tab. 4.

Tab. 4: Comparison of derived pool characteristics in high and low N plants

Parameters of the supply system for leaf growth of low N (1.0 mM) and high N (7.5 mM) plants as obtained by model optimization runs, and the effect of nitrogen deficiency relative to high nitrogen nutrition with the significance based on a t-test (*P ≤ 0.05; ns: not significant P > 0.05). Displayed are absolute sizes of pools Q_1, Q_2 and Q_3 (in µg), pool sizes relative to total tiller nitrogen or carbon (in %), half lives of pools ($t_{0.5}$), the delay between the start of labeling and first nitrogen tracer appearance in the import flux and the contribution of the transport pool (p_Q_1), the short-term store (p_Q_2) and the long-term store (p_Q_3) to the substrate supply to leaf growth.

		Nitrogen			**Carbon**		
		Low N	***High N***	***Effect of nitrogen deficiency***	***Low N***	***High N***	***Effect of nitrogen deficiency***
	unit	*mean ± 1se*	*mean ± 1se*	*Difference (%)*	*mean ± 1se*	*mean ± 1se*	*Difference (%)*
Q_1	µg	13.7 ± 2.7	6.5 ± 1.4	+ 111 *	183 ± 22	119 ± 12	+ 54 *
Q_2	µg	352 ± 74	566 ± 150	ns	3037 ± 2267	3198 ± 1578	ns
Q_3	µg				644 ± 488	293 ± 231	ns
Q_1	%	1.0	0.2	+360	0.3	0.2	+ 62
Q_2	%	24.7	18.2	+36	4.6	4.6	0
Q_3	%				1.0	0.4	+130
$t_{0.5}\,Q_1$	h	3.24 ± 0.29	0.59 ± 0.05	+ 449 *	1.4 ± 0.2	0.69 ± 0.07	+ 97 *
$t_{0.5}\,Q_2$	h	174 ± 16	127 ± 13	+ 37 *	173 ± 84	119 ± 43	ns
$t_{0.5}\,Q_3$	h				19 ± 12	11 ± 8	ns
delay	h	2.2	0.9	+ 144			
p_Q_1	%	0.52 ± 0.05	0.59 ± 0.04	ns	0.61 ± 0.07	0.69 ± 0.06	ns
p_Q_2	%	0.48 ± 0.04	0.41 ± 0.04	ns	0.13 ± 0.06	0.16 ± 0.04	ns
p_Q_3	%				0.26 ± 0.07	0.16 ± 0.05	ns

EFFECT ON POOL SIZES

The size of the nitrogen and carbon transport pool increased by more than 100 % and 50 %, respectively, due to nitrogen deficiency. The effect was even more pronounced when looking at relative pool sizes - considering that total tiller nitrogen decreased and total tiller carbon stayed constant: the relative size of the nitrogen transport pool increased by 360 %, the carbon transport pool by 60 % under nitrogen deficiency. The absolute size of the nitrogen long-term store tended to decrease under low N nutrition (not statistically significant), but in relative terms there was an increase by one third. In contrast, there was no effect on the size of the carbon long-term store, while the size of the carbon short term store more than doubled. So under nitrogen deficiency relatively more nitrogen and carbon of the whole tiller was involved in leaf growth.

EFFECT ON HALF LIVES AND THE CONTRIBUTION OF EACH POOL TO LEAF GROWTH

Half lives of all pools increased due to nitrogen deficiency (Tab. 4). The most pronounced effect was on the transport pools. Interestingly, nitrogen and carbon long-term stores, which already revealed similar half lives in high N plants (~170 h), again were turned over with a similar speed (~120 h) at low N. The delay for first nitrogen tracer arrival in the growth zone, which likely reflected the behaviour of the nitrogen uptake system along the transport path from roots to the photosynthetically active leaf laminae, more than doubled. In contrast, labeled carbon appeared in the leaf growth zone with nearly no delay in both nitrogen treatments, supporting a direct path for current carbon assimilates to leaf growth. Under nitrogen deficiency mobilization-derived substrates tended to become a bit more important than current assimilation-derived substrates, but this effect was not statistically significant.

EFFECT ON THE DIFFERENT SOURCES OF CARBON SUPPLY

Nitrogen deficiency reduced the total import flux of carbon into the leaf growth zone by 30 %. Mainly affected were the assimilation- and long-term storage-derived carbon fluxes, which were reduced by 38 % and 42 %, respectively, while the short-term storage-derived flux increased by 16 % (Tab. 5). Under the assumption that all carbon imported into the growth zone was in the form of sucrose and / or amino acids with a C:N (w/w) ratio of 2.74 for amino acids (Amiard et al. 2004), CH_2O-C was the main source of carbon, supplying 93 % of the total carbon flux. Nitrogen deficiency increased the contribution of CH_2O-C in the total, and in both the assimilation- and storage-derived fluxes into the growth zone.

Tab. 5: Contributions of amino- and sucrose-C
Estimated contributions of amino-C and sucrose-C within the total carbon flux and to current assimilation-derived and storage-derived carbon fluxes into the growth zone of low N and high N plants.

	Total flux		**Assimilation-derived flux**		**Long-term store derived flux**		**Short-term store derived flux**	
	Low N	***High N***	***Low N***	***High N***	***Low N***	***High N***	***Low N***	***High N***
Flux (mg C h^{-1}) [a]	57.0	81.3	34.9	55.9	7.4	12.8	14.7	12.6
Amino-C [b]	7 %	15 %	6 %	13 %	27 %	39 %	0 %	0 %
CH_2O-C	93 %	85 %	94 %	87 %	73 %	61 %	100 %	100 %
CH_2O-C:amino-C	12.7	5.6	15.1	6.7	2.7	1.6	-	-

[a] The amount of the direct and storage-derived fluxes were calculated by the total flux times the contributions of the individual pools.
[b] Amino-C was calculated as nitrogen flux times 2.74 (assumed C:N ratio (w/w) of amino acids according to Amiard et al. (2004)).

The sucrose-C: amino-C ratio in the import flux into the growth zone was 5.6 in high N plants and 12.7 in low N plants (Tab. 5). The estimated sucrose-C: amino-C ratio of the transport pools of high N plants (5.7) agreed well with that of the import flux, while in low N plants this ratio was 3.9 and thus differed from that of the import flux. It is not clear where this discrepancy comes from. Results indicated that in low N plants relatively more sucrose-C (93% *vs.* 85%) was routed to leaf growth than under high nitrogen supply.

3.4 DISCUSSION

GENERAL VALIDITY OF THE NITROGEN AND CARBON SUPPLY SYSTEM

This study showed that the structure of the nitrogen and carbon supply system for leaf growth was not affected by differences in nitrogen fertilizer supply. Under nitrogen deficiency nitrogen was supplied by two pools and carbon by three pools, the same as in high-N. This was true although the concentration of nitrogen in the nutrient solution differed by a factor of 7 – this corroborates the general validity of the supply system model presented in Chapter 2 over a wide range of external nitrogen supply. Even if grass plants are very adaptive to changes in environmental conditions and change their allometric relations, it seems, that the major pools supplying leaf growth always stay the same. This could be due to a permanently high sink strength of leaf growth under both high and low nitrogen nutrition. Lattanzi et al. (2005) observed a similar relationship for the supply system of ryegrass growing in different hierachical positions in a canopy.

Nitrogen deprivation had an increasing effect on the half life of all pools: the turn over of the transport and short-term storage pools was nearly 2-fold slower, which could be related to the fact that growth processes also were slowed down. LER and RGR decreased by half and the flux of nitrogen and carbon through the whole system was also decreased. Considering this circumstances the nature of pools, as suggested for high N plants might still be the same. It still is highly likely, that the transport pools were derived from uptake or photosynthesis (*i.e.* amino acids and sucrose) and consisted of compounds cycling in the cytosol and phloem. Half life of the carbon short-term storage pool (19 h) was within the range of 12 to 24 h as found for vacuolar stored sucrose or fructans in C3 grasses in sheaths by Borland and Farrar (1985) and Farrar (1989a). Labeling kinetics of fructans isolated from the source leaves showed one pool with a half life of 3 to 6 d, which agreed well with the half-life of the long-term store, supporting the suggestion that fructans could form part of that pool. Thus it seems that fructans could act as both: as long-term and short-term store to supply carbon to leaf growth.

As low N plants were suffering severe nitrogen limitation, the nitrogen long-term store most probably was fed by compounds, which had a physiological function and were afterwards recycled, rather than by previously accumulated or stored material (for definition of terms see Chapin F.S.3 et al. 1990)). In low N plants the only nitrogen pool, which had a considerable size to provide 50 % nitrogen for leaf growth are leaf proteins (mainly RubisCo), as already suggested for high N plants (Evans 1996; Millard 1988).This was also corroborated by the half life of ~7 d (Friedrich and Huffaker 1980; Irving and Robinson 2006; Mae et al. 1983).

From this perspective the supply system of plants – or at least of temperate C3 grasses – in terms of the structure and the identity of pools did not change due to changing external conditions as long plants retained in vegetative growth. But, relatively more nitrogen and carbon of the whole tiller stood in contact with leaf growth.

ROLE OF STORES UNDER NITROGEN DEFICIENCY

Under favourable and stable growth conditions stores supplied 40 % of nitrogen and 30 % of carbon (half by the short-term- and half by the long-term store, see Tab. 2 chapter 2) for leaf growth and were turned over continuously, which was markedly in consideration of the widespread idea that stores are laid down only to be mobilized following a perturbation such as grazing or mowing, or during spring regrowth, and whenever possible, plants would use current assimilation instead of stores (Chapin F.S.3 et al. 1990).

Under nitrogen deprivation the importance of stores for the supply of leaf growth indeed increased: 50 % of nitrogen and 40 % of carbon was storage-derived. It is known, that plants accumulate more carbon (mainly fructans) under nitrogen deficiency as the supply exceeds demand (Pollock et al. 2003), but there is hardly any literature about the fate and usage of those carbohydrates.

Under nitrogen deficiency and under a relative excess of light (where there is no need to buffer day-night cycles) a substantial portion of short-term stored carbohydrates supplied leaf growth (26 %). As building up and mobilizing fructans is connected with costs, there must be an advantage of such a metabolic pattern. Either, building fructan stores is a genetically fixed program to minimize the risk of carbon shortage (in case of darkness) or, as sucrose accumulation might have a regulatory role on photosynthesis (Farrar et al. 2000), it might be a way to prevent down-regulation of photosynthesis by feedback signaling, if sucrose concentration becomes to high. The higher contribution of stored carbohydrates and thus of estimated CH_2O-C from long-term stores(see Tab. 5) to leaf growth under low nitrogen nutrition is not completely clear. But it is interesting that Gebbing and Schnyder (1999) found an efficiency of only 56 % for mobilized carbon of vegetative plant parts recovered in grain proteins. This means that losses in the range of 44 % must have occurred during protein mobilization and re-synthesis. In case these losses also occurred during the turnover of leaf proteins, then ~1.8 times more carbon would be connected to the turnover of leaf proteins. This could explain the CH_2O-C : amino-C ratio of the long-term store of 1.6 – 2.7.

Even if the shoot: root ratio decreased under nitrogen deficiency, corroborating a common respond of allocation to nitrogen deficiency (Hermans et al. 2006 and refs therein), plants

allocated a higher percentage of total tiller N to leaf growth in this stress situation. Under high N conditions we suggested that the nitrogen long-term storage pool most likely consisted of mobilized leaf proteins. In nitrogen limited plants relatively more nitrogen was derived from this pool. This corroborates its identity, as in nitrogen limited plants leaf proteins (RubisCo) constitute most of the plant's nitrogen and as nitrogen is scarce and the nitrogen concentration in shoots and roots was only about 50 % of high N plants, grasses may mobilize as much as possible from senescencing leaves and reinvest most (if not all) into leaf growth. Under suitable external conditions, nitrogen resorption from senescing leaves was found to be close to 80-90 % (Hortensteiner and Feller 2002; Liu et al. 2008; Tabuchi et al. 2007). Thus it seems that grasses grown under nitrogen deprivation must reuse relatively more nitrogen previously fixed in proteins than grasses grown under ample nitrogen supply.

TURNOVER OF LONG-TERM STORES IS MEDIATED BY THE TURNOVER OF ORGANS

If mobilization of long-term stores is mediated by the turnover of organs rather than by a genetic program - as results of high N plants indicated (see chapter 2) – and if long-term stored carbon derives mainly from protein mobilization then i) under nitrogen deprivation the long-term storage pools should be turned over more slowly due to a slower leaf turnover rate and thus reveal a $t_{0.5}$, which is related to the leaf expansion duration or the leaf appearance rate and ii) both nitrogen and carbon long-term stores should reveal a similar $t_{0.5}$.

Half lives of long-term nitrogen and carbon stores increased by about 40 % due to nitrogen deprivation and had nearly identical half lives. This means that either substrates cycling in this pool were affected in a similar way by nitrogen deficiency or that the biggest part of nitrogen and carbon in the long-term store shared similar identity. Half lives (5 and 7 d, for high N and low N respectively) and the high contribution to leaf growth, support a predominant protein nature (RubisCo) of the long-term storage pool, which is mobilized during leaf senescence. But pure proteins would reveal a carbon to nitrogen mass ratio (w/w) of ~ 3. Yet, under high external N supply the ratio in the long-term store was 5.7, while under low N supply it diverged to 8.6. Thus, 'non-nitrogenous' carbon (probably 'CH_2O-C') must have formed a substantial part of the carbon in both cases. Estimations of the contribution of amino-C and CH_2O-C in the storage-derived flux (Tab. 5) corroborated this suggestion and showed that the contribution of CH_2O-C increased under nitrogen deficiency. As we found a fructan content of 85 % in total water soluble carbohydrates in the shoot tissue of high N plants and of 91 % in low N plants (Lehmeier et al. 2008) and the half life of the fructan pool (3 to 6 d, see Fig. 17B) agreed well with the long-term store, we suggest that fructans stored in sheaths supplied leaf growth to a substantial part. The

stunning similarity and the concomitant increase of half lives hints to a strong relation of leaf protein and fructan mobilization mediated *via* the turn over of leaves and sheaths. The life span of a *L. perenne* leaf was about 500 degree-days (personal communications, Schleip 2009), which is approximately three times the half life of the nitrogen and carbon long-term store. Leaf appearance rate was 5 d and 11 d under high and low nitrogen supply, respectively (data not shown), close to the half lives of the long-term storage pools.

As leaf growth is a continuous process and needs a continuous supply of substrate, mobilization of long-term stores and thus the turnover of proteins and fructans in older leaves and sheaths must also be a continuous process - at least seen integrated over all organs of a grass plant.

NITROGEN DEFICIENCY INCREASED THE SIZE OF THE NITROGEN TRANSPORT POOL – A CONTRADICTION?

Against all expectations the absolute and relative size of the nitrogen transport pool increased under nitrogen deprivation, although nitrogen uptake was reduced. If leaf growth was limited by nitrogen availability, then one might expect a positive relationship between leaf growth rate and the quantity of nitrogen in the transport pool (*i.e.* amino acids, see Caputo and Barneix 1997)) as this directly supplies the growth zone with nitrogen. But this was not the case. Thus, the amount of nitrogen substrate cycling in the cytosol and phloem was not directly controling leaf growth rate, implying that another factor was involved in the control of leaf growth.

One factor which could control the rate of amino acid delivery to the growth zone *via* phloem is the effect of other (non-amino) phloem solutes on phloem transport. According to the pressure flow hypothesis of phloem transport of Münch (1930) mass flow of phloem sap from the source to the sink region is (to a great part) dependent on the concentration gradient between sources and sink (Lalonde et al. 2003). The later is to a big part dependent on the unloading capacity of sucrose in the sink. Thus the ability of sinks to lower the osmotic potential in the cytosol by transforming osmotically active substrates or by transferring them into the vacuole, might co-determine the bulk flux of substrate into the sink cells. Sucrose is the main phloem sap osmoticum (Turgeon 1995; Winter et al. 1992) and its concentration in the shoot is greatly increased while in the phloem it is unchanged by low nitrogen nutrition (Caputo and Barneix 1999; Jones et al. 1965; Nowakowski 1962), leading to an increasing sucrose: amino acid ratio in the phloem under this situation. Under nitrogen deprivation, the utilization rate of sucrose in growth-related biosynthetic processes in the growth zone and hence the rate of sucrose unloading could be greatly limited by low nitrogen availability, decreasing the pressure gradient and slowing phloem flow. Such a mechanism could well cause an increase of nitrogen at the source

end of the nitrogen transport pool (*i.e.* cytosol in leaves), as amino acids export into the phloem might be related to the flux of sucrose into the phloem (Winter et al. 1992). The elevated nitrogen concentration in turn would help to increase phloem loading of amino acids, and thus counterbalance the effect of slowed phloem sap translocation on amino acid delivery to the leaf growth zone. Additionally the leaf growth zone could also counterbalance the reduced phloem flow and accompanied reduced amino acid import by sequestration of sucrose imported *via* the phloem to sustain phloem transport and import of 'non-sucrose' solutes, like amino acids (Schnyder et al. 2000). Gastal and Nelson (1994) and Volenec and Nelson (1984) have shown, that leaf growth zones of cool-season grasses can synthesize large quantities of fructan, which has been interpreted in terms of sequestration of sucrose (Schnyder et al. 1988).

3.5 CONCLUSION

Results gained in grass plants grown under nitrogen deficiency provide evidence that leaf growth draws on the same substrate pools as plants grown under ample nitrogen supply. Only contributions of single pools to the supply of leaf growth changed. This strongly affirms the general validity of the supply system model presented in chapter 2 over a range of external nitrogen supply. The results further showed that the contributions of mobilized nitrogen and carbon compounds from stores to leaf growth even increased under nitrogen deficiency (50 % and 40 %, respectively). Thus – integrated over all leaves and sheaths – this result gave strong evidence that a certain portion of stores (mainly fructans and leaf proteins) must be turned over continuously. As half lives of nitrogen and carbon long-term stores in both cases (high and low nitrogen nutrition) were similar and also fitted to the particular leaf appearance rate, this indicated that storage mobilization was controlled by the appearance of leaves and thus in turn by their senescence.

Stores in this studies need to be interpreted in the sense of Chapin F.S.3 et al. (1990): nitrogenous long-term stores – mainly leaf proteins/ RubisCo are laid down to primary full fill physiological functions and are then recycled to sustain leaf growth. Carbon stores may be of several kinds: accumulated material due to an excess of carbohydrates or formed reserves, which were actually built for an ‘anticipated’ demand.

The increased importance of stores under nitrogen deficiency suggests that the efficiency of protein mobilization for leaf growth increases or that allocation to other sinks (like tillering) decreases under this stress situation. Implementing that nitrogen resorption is not genetically fixed but dependent on the external nitrogen supply and the interplay between costs and benefits of remobilization.

3.6 MATERIALS AND METHODS

The experimental setup has been described in detail in chapter 2 for high N plants. Low N plants were grown simultaneously with high N plants in two extra growth chambers under the same growth conditions, except for a lower nitrogen supply. Thus, here we give only a brief description emphasizing differences between the two applied nitrogen treatments. For more detail see chapter 2.

PLANT MATERIAL AND GROWTH CONDITION

Seeds of perennial ryegrass (*Lolium perenne*, cv. Acento) were sown individually in plastic pots filled with quartz sand. Pots were arranged in plastic containers at a density of 378 plants m^{-2}. Plants were grown under constant growth conditions (continuous light, 20°C at the level of the leaf growth zone and a relative humidity near 85 %). The stands were irrigated by briefly flooding the boxes every 3 hours with a modified Hoagland nutrient solution containing either 1.0 mM nitrogen as NO_3^- ('low N') or 7.5 mM nitrogen as NO_3^- ('high N'). The composition of the nutrient solution was: basic supply: 1.0 mM $MgSO_4$, 0.18 mM KH_2PO_4, 0.21 mM K_2HPO_4, 0.5 mM NaCl, 0.4 mM KCl, 0.4mM $CaCl_2$, 0.125 mM Fe-EDTA and micronutrients. Additional for high N: 2.5 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, and 1.0 mM KNO_3 (low N) or 2.5 mM $Ca(NO_3)_2$ and 2.5 mM KNO_3 (high N). One chamber of each nitrogen treatment received nutrient solution with NO_3^- with natural abundance of ^{15}N (0.37 atom% ^{15}N; later called '^{15}N-depleted'), the other with NO_3^- isotopically enriched in ^{15}N (1 atom% ^{15}N, later called '^{15}N-enriched'). The nutrient solutions were renewed frequently, and pots flushed with demineralized water to prevent salt accumulation.

$^{13}CO_2/^{12}CO_2$ GROWTH CHAMBERS FACILITY

Four growth chambers (Conviron E15, Conviron, Winnipeg, Canada) formed part of the $^{13}CO_2/^{12}CO_2$ gas exchange and labeling system as described by Schnyder et al. (2003). One chamber of each nitrogen treatment received ^{13}C-depleted CO_2 ($\delta^{13}C$ -28.8 ‰), the other ^{13}C-enriched CO_2 ($\delta^{13}C$ -1.7 ‰; Linde AG, Höllriegelskreuth, Germany). CO_2 concentration in all four chambers was kept constant at 360 µL L^{-1}.

^{15}N AND ^{13}C LABELING

Labeling began 5 weeks (high N) or 8 weeks (low N) after imbibition of seeds, when plants had established three tillers. Plants were labeled by swapping randomly selected individual plants between chambers of the same nitrogen treatment. Thus, plants growing in the '^{13}C- and ^{15}N-

enriched' chamber were transferred to the '^{13}C- and ^{15}N- depleted' chamber and *vice versa*. Low N plants were labeled for intervals of 2, 4, 8, 16, 24, 48, 96, 192, 383, 576 and 975 hours, high N plants only up to 576 hours. Four replicate plants were used for each labeling interval. A nested labeling pattern was applied to minimize size- and development-related effects (see Fig. 16).

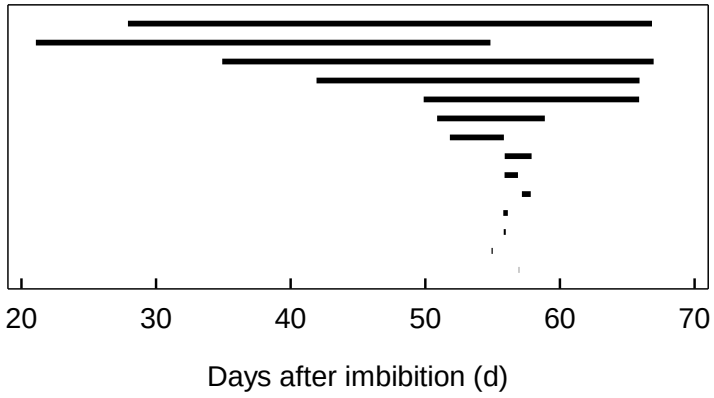

Fig. 16: Labeling pattern of low N plants.
Labeling was nested to reduce size- and development-related effects.

LEAF ELONGATION RATE MEASUREMENTS

Mature tillers (tillers having at least two fully expanded leaves) were selected for measurements of leaf elongation rate (LER, mm h^{-1}). LER was determined on the most rapidly growing blade, during the phase of maximal growth, when leaf elongation rate is near constant (Schnyder et al. 1990).

SAMPLING

Plants were removed from the stands at the end of given labeling intervals. In each plant the growth zone and an immediately adjacent piece of recently produced leaf tissue was dissected from the most rapidly growing leaf of mature tillers. The adjacent recently produced tissue had a length of 3 mm (for labeling periods shorter than 1 d) or 5 mm (for labeling periods longer than 1 d). Samples of replicates were combined by fraction, weighed, frozen in liquid nitrogen, freeze-dried, weighed again, ground to flour mesh quality with a ball-mill. In addition, unlabelled plants from both chambers were sampled and processed in the same way as labeled plants. These served to determine ^{13}C and ^{15}N discrimination in the respective chambers, and were used as 'end-members' in the mixing equation for assessment of ^{13}C and ^{15}N tracer kinetics (see below). Non-labeled plants (as controls) were sampled at 2 d-intervals throughout the duration of the labeling experiment.

ELEMENTAL AND ISOTOPIC ANALYSES

Concentrations of nitrogen and carbon and isotopic composition ($\delta^{13}C$ and atom%^{15}N) were determined on each leaf growth zone and recently produced tissue by combustion in an elemental analyser interfaced to a continuous-flow isotope ratio mass spectrometer. Each sample was measured against laboratory working standard gases (CO_2 and N_2), which were previously calibrated against IAEA secondary standards. The precision (SD) for sample repeats was 0.15 ‰ for $\delta^{13}C$ and 0.004 atom% for ^{15}N.

CALCULATION OF THE FRACTION OF LABELED CARBON AND NITROGEN IN A SAMPLE

The fraction of labeled carbon (f_{lab_C}) was defined as the proportion of carbon that was taken up following transfer to the (isotopically) 'new' environment. For instance, for plants grown in the 'enriched' chamber and transferred to the 'depleted' chamber for labeling, the 'depleted' chamber meant the 'new' environment, whereas the 'enriched' chamber was the 'old' environment. f_{lab_C} was assessed using a two-source mixing model (as used by Schnyder and de Visser 1999 and Lattanzi et al. 2005):

$$f_{lab\_C} = \frac{\delta_{spl} - \delta_{old}}{\delta_{new} - \delta_{old}}, \quad (1)$$

where δ_{spl} is the $\delta^{13}C$ of the labeled sample, and δ_{old} and δ_{new} correspond to the $\delta^{13}C$ of the 'end-members' of the mixing model. Fraction of unlabeled carbon was assessed as $1\text{-}f_{lab\_C}$.
The fraction of labeled nitrogen (f_{lab_N}) was obtained using analogous procedures.

ESTIMATION OF THE AMOUNT OF TRACER IN THE FLUXES OF CARBON AND NITROGEN IMPORTED INTO THE GROWTH ZONE

The import of labeled and non-labeled carbon and nitrogen (assigned in the following as 'substrate') into the growth zone was assessed using principles presented by Lattanzi et al. (2004 and 2005) except that here the time step size was 2 h and respiration in the growth zone was accounted for. In brief: for a growth zone in the steady-state, the import of substrate equals its export by tissue-bound mass efflux and its loss by respiration (only for carbon). All relevant features of the growth zone and adjacent recently produced tissue were constant (see results). Respiration in the leaf growth zone was estimated to be 10 % of carbon import (Penning de Vries et al. 1974; Schnyder and Nelson 1987). Export of substrate out of the growth zone was estimated as the product of LER and the mass of substrate per unit length of recently produced tissue.

Import of labeled substrate into the growth zone was similarly assessed, but considered the fact that the amount of labeled substrate in the growth zone changed during dynamic labeling. Thus, import of labeled substrate was assessed for 2 h-intervals as the export of labeled substrate *minus* the loss of labeled respired carbon *plus* the variation in mass of substrate inside the growth zone over the same time-interval. For formulas see materials and methods section in chapter 2.

COMPARTMENTAL MODELING OF CARBON AND NITROGEN TRACER TIME COURSES

The time course of nitrogen tracer into the leaf growth zone was described with a two-pool model. For carbon tracer, a three-pool carbon model was used (Fig. 8, chapter 2). These models were translated into sets of differential equations assuming a system in steady-state and obeying first order kinetics. This meant that pool sizes and fluxes did not change over time (only the proportion of tracer did), and that fluxes were the product of pool sizes times rate constants k_{xy}, where x and y refer to donor and acceptor pool, respectively. The setup of the models was based on former studies using compartmental analyses in plant systems (Lattanzi et al. 2004; Moorby and Jarman 1975; Rocher and Prioul 1987). For details see chapter 2.

ASSUMPTIONS AND DEFINITIONS IN THE SENSE OF COMPARTMENTAL MODELING

Lattanzi et al. (2005) discussed in detail the validity of the assumptions on which the present analysis rests. Also in materials and methods (chapter 2) this assumptions regarding steady-state are discussed. Briefly, i) the assumption that the system was in near steady-state was assured by growing plants in constant conditions and affirmed by the constancy of growth and state variables (see Fig. 11, Fig. 12 and Fig. 13), ii) the assumption that the metabolism and transport of tracers reflects that of unlabelled material was satisfied by assessing and correcting for isotope effects in the labeling experiment (see above) and (iii) the assumption that pools obey first order kinetics and are well-mixed is probably untrue in a strict sense, but its practical validity has been supported numerous times (e.g. Britto and Kronzucker 2003; Farrar 1989b; Irving and Robinson 2006).

The term ‘pool’ as used in this study reflects an amount of some material with kinetically homogeneous behaviour (Jacquez and Simon 1993), which might well be located in different sites of a grass tiller, but nevertheless shows similar kinetics.

4 SUMMARIZING AND CONCLUDING DISCUSSION

Competition for light is one of the main driving forces for leaf growth. It ensures the ability for light capture and thus for photosynthesis and carbon uptake. The process of leaf growth is highly regulated and very sensitive to nutrient supply. Thus this study elucidated potential substrate pools (*i.e.* sources) for leaf growth and their reaction to nitrogen deficiency.

The first part of the presented study analysed the structure of the nitrogen and carbon supply system of leaf growth by an approach consisting of dynamic labeling with ^{15}N and ^{13}C of newly assimilated nitrogen and carbon and compartmental analysis of the tracer kinetics of nitrogen and carbon imported into the growth zone. Those kinetics revealed that both nitrogen and carbon were supplied by current assimilates and long-term stores, while carbon additionally was supplied by short-term stored material. In both cases current assimilation provided most of the supply, but storage mobilization contributed also a substantial part. The second part of the study evaluated the influence of nitrogen deficiency on the structure of the substrate supply system, its kinetic properties and the importance of current assimilates *versus* stores. Revealing that a similar number of pools supplied nitrogen and carbon to leaf growth under low nitrogen supply as under high nitrogen supply. Importance of short and long-term storage pools slightly increased under low nitrogen supply.

4.1 IDENTITY OF CARBOHYDRATE POOLS OR "CAN BIOCHEMICAL FRACTIONATION HELP TO IDENTIFY THE POOLS?"

In both studies (chapter 2 & 3) the identity of pools was discussed and assessed by comparison of pool half lives with literature data on half lives of substrate pools in sources like leaves or roots, or by knowledge about metabolic processes or substrates transported in the phloem. Since the used method of compartmental modeling aggregated the surely very complex substrate supply of the whole plant only into 3 carbon pools it seems obvious that observed pools most probably are biochemically heterogeneous. But they have one thing in common: they have the same metabolic behaviour (in terms of turn-over) and thus are isotopically similar. Thus it is highly likely that those pools reflect major constituents of chemical compounds, which are known to contribute a considerable portion to the supply of leaf growth and which are tightly involved in metabolism (like sucrose or amino acids).

To clarify the suggested identity of carbon pools, a biochemical separation of water-soluble carbohydrates into fructan, sucrose, glucose and fructose, which were thought to be main constituents of carbon pools and a following analysis of tracer kinetics may help. As sucrose and

fructans made up for most of the water-soluble carbohydrates in both nitrogen treatments (sucrose: N+: 32 % ± 7, N-: 15 % ± 4; fructans: N+: 53 % ± 11, N-: 74 % ± 6; data not shown), their labeling kinetics in source leaves of perennial ryegrass were analyzed. As sucrose is the main transport form of carbon, cytosolic sucrose was thought to be a main constituent of the transport pool, while vacuolar sucrose and fructans highly likely are constituents of the storage pools. By analysing mature labeled leaves of the same grasses, which are sources for leaf growth, it could be detected that sucrose in those leaves was compartmented into three pools, while fructans only showed one pool (for results see Fig. 17). If sucrose and/or fructans were constituents of the carbon supply system, one would expect similar half lives as derived from the analysis of carbon import flux into the growth zone – plus some delay due to phloem transport. Compartmental analysis of the kinetics of sucrose from leaves showed that half lives of the two fast pools corresponded well with findings of Borland and Farrar (1988) of transport (*i.e.* cytosolic) and vacuolar sucrose of source leaves of two grass species. As expected, in both nitrogen regimes the half lives of sucrose-C pools were shorter than those detected in the import flux into the growth zone. It is unclear how much delay was due to phloem loading, transportation and phloem unloading. But considering that sucrose also passed leaf sheaths on its way to the growth zone and likely stood in contact with the sheath's carbohydrate metabolism, this may explain an additional off-set between half lives of pools measured directly in leaves and those inferred from the import flux into the growth zone. Conclusively, biochemical fractionation of water-soluble carbohydrates in source leaves strongly encourages the suggestion that cytosolic and vacuolar sucrose was the main constituent of the carbon transport and short-term storage pool.

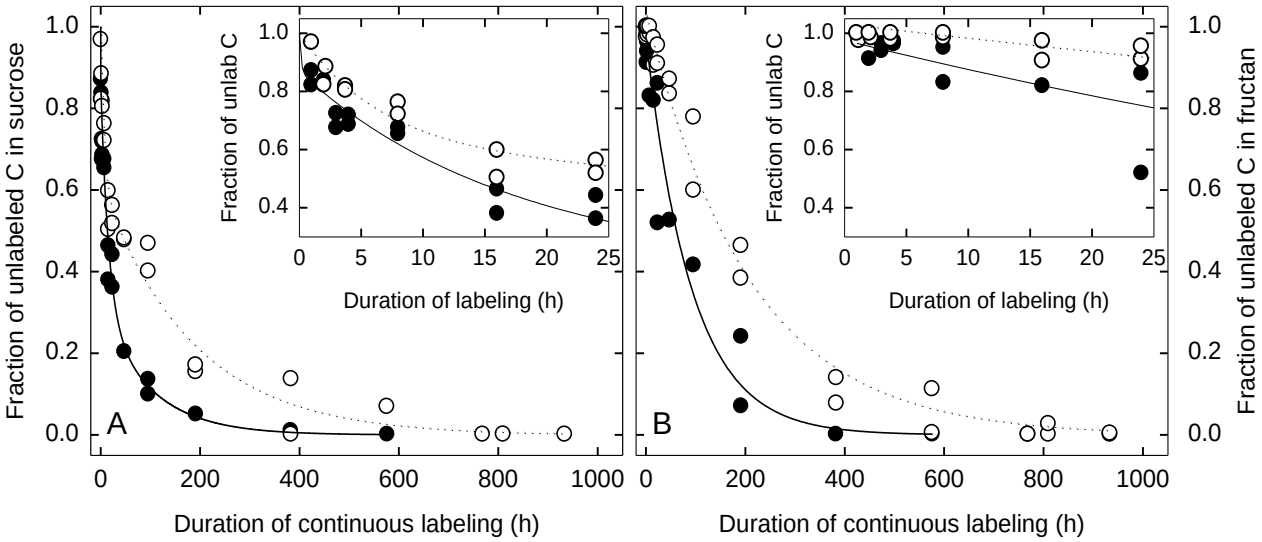

Fig. 17: Labeling kinetics of carbon in leaf sucrose and fructans

Labeling kinetics of carbon in sucrose (A) and fructans (B) of the youngest fully expanded leaves of perennial ryegrass plants grown with a nitrogen supply of either 7.5 mM (closed circles) or 1.0 mM (open circles). Growth and labeling conditions were as described in chapter 2 & 3. Insets expand the first 24 h of labeling. Lines show fits of compartmental models to the data, which were obtained as described in chapter 2.

Fructan and sucrose were extracted as described by Morvan-Bertrand et al. (1999) with exception of a NaP-Buffer and acetone purification step instead of water/ethanol extraction. The isotopic composition (∂^{13}C) of carbon in sucrose and fructan was determined by combustion of the samples in an elemental analyser (NA 1110, Carlo Erba Instruments, Milan, Italy) interfaced to a continuous-flow isotope ratio mass spectrometer. The fraction of unlabeled carbon was assessed *via* equation 1 in chapter 2. Under both nitrogen supplies tracer time course of carbon in sucrose showed three distinct phases, which could be described by a three-pool model similar to the carbon model (see chapter 2 & 3). Half lives were Q_1= 0.15 ± 0.1 h; Q_2= 71 ± 94 h; Q_3= 4.2 ± 2.4 h for high N plants (RMSE= 0.05) and Q_1= 0.09 ± 0.09 h; Q_2= 53 ± 18 h; Q_3= 2.2 ± 1.4 h for low N plants (RMSE= 0.03). In both cases three-pool models performed better than simpler two-pool models (RMSE= 0.06 and 0.05, respectively). Under both nitrogen supplies tracer incorporation into fructans showed one phase and thus was described by a one-pool model. Half lives were in high N Q_1= 63 ± 7 h and in low N Q_1= 144 ± 9 h. Data are means ± 1SE.

Interestingly under both nitrogen regimes sucrose showed a third slow phase, which rather showed a half life in the range of the fructan pool. As there are several isomeric transformations in the hexose pool (Buchanan 2000), this could be sucrose-C which was derived from glucose split-off during fructan synthesis and was reincorporated into sucrose during its synthesis.

The half-life of the fructan pool under both nitrogen regimes fitted well with the half-life of the carbon long-term store. Considering the contribution of CH_2O-C to the storage-derived flux (see

discussion chapter 3 and Tab. 5), carbon derived from fructan turn over thus may well be part of the long-term carbon storage pool.

Thus biochemical fractionation with following compartmental analysis of tracer kinetics in single biochemical compounds is a suitable tool to further elucidate pool identities. To explain possible delaying effects of phloem transport or leaf sheaths on sugar pool half lives, it would be necessary to analyze also the tracer time course of carbohydrates in sheaths.

4.2 STRENGTHS AND CONSTRAINTS OF THE APPROACH

Compartmental analysis is a comparatively simple means of investigating a difficult and highly complex system (Dale et al. 1981). It was used in many biological studies like movement of drugs through animals (Atkins 1972) or to study translocation dynamics of carbon (Moorby and Jarman 1975) and nitrogen (Yoneyama and Takeba 1984) in source systems. Compartmental models are a valuable system approach for inferring number of pools of a system, pool sizes and their kinetic properties (*i.e.* turnover rates) by means of feeding a traced compound (like ^{13}C or ^{15}N) to the system and analysing the saturation kinetics in the components or the outflow of the system. This method is effective for comparing responses of the system to experimental treatments (Dale et al. 1981) like the response of the supply of leaf growth to nitrogen deprivation in our case.

Principally each steady-state system can be analyzed by this means. But knowledge about its biology and structure and a hypothetical model structure makes the resulting analysis more meaningful, because even when assuming the same number of pools, the topology can vary a lot. A three-pool model for example consisting of one transport and two storage pools can be arranged in different ways: the transport pool exchanges i) with two separate storage pools, ii) only with one storage pool directly to which the 2^{nd} storage pool is connected or iii) exchanges with both storage pools, which are also interconnected. Thus each model structure assumed implies differences of metabolic pool properties. We have chosen the simplest model with biological consistency able to fit the observed tracer kinetics (according to the ‘principle of parsimony’).

Pools, as described here, are a mixture of compounds with similar metabolic behaviour (as defined by Jacquez and Simon 1993). The number of pools, which are detectable, corresponds to the number of phases with different slopes in a semi-logarithmic plot of residuals. Thus pools can be separated from each other when their turnover differs at least by a factor of two (Jacquez 1972). This means that pools with nearly similar half lives are seen as one pool. Certainly a high time resolution increases the discriminatory power of the analysis. So to make the experimental

setup realistic, it is necessary to have some expectations and estimations about putative pool half lives to choose the most suitable time resolution.

Strength of the comprehensive approach analyzing the supply system of a sink *via* compartmental modeling is clearly that all pools contributing carbon or nitrogen to leaf growth are captured by their 'footprint' in the labeling kinetics - unrestricted of their physical origin or location in the plant. Thus the complexity of the plant´s metabolism can be simplified and broken down to the major contributing pools, which are characterized by their function.

4.3 DO SIMILAR POOLS FEED THE TWO BIG SINKS - LEAF GROWTH AND RESPIRATION?

Respiration and leaf growth are the two main sinks for carbon in growing vegetative plants. As plants for the present study were grown together with plants used for the analysis of the respiratory carbon supply system (Lehmeier et al. 2008) the two datasets gained under high N were completely comparable. The respiratory substrate pool system of the shoot was characterized by 3 distinct pools with half lives in the range of > 0.1 h up to 33 h (Tab. 6). Collectively, the respiratory substrate pool system constituted 13.2 % of total carbon mass of plants, while the carbon supply system for leaf growth comprised 5.2 % of total shoot carbon. In comparison, water-soluble carbohydrates accounted for 28% of total plant carbon (Lehmeier et al. 2008), meaning that both sinks could well be fed from them. The question rises if the carbon supply of both sinks is fed by the same metabolic pools.

Tab. 6 shows a comparison of the results of both studies on grasses under high N nutrition.

Tab. 6: Comparison of the supply system for leaf growth and respiration

Half lives and contributions of pools supplying leaf growth and respiration in plants of *L. perenne* grown under 7.5 mM external nitrogen (growing conditions as described in chapter 2). Pool characteristics were evaluated with compartmental analysis of tracer-time courses of ^{13}C in the import flux into the growth zone and in CO_2 respired of shoots and roots (data of respiration according to Lehmeier et al. 2008).

		Growth	**Respiration**	**Growth**	**Respiration**
		half life (h)		*contribution (%)*	
curr. assimilation 1	h		< 0.2		0.15
curr. assimilation 2	h	0.7	3	0.69	0.28
short-term store	h	11	33	0.16	0.57
long-term store	h	119		0.16	

First, there is a marked difference in the structures of both supply systems: while growth was fed by one transport pool consisting of sucrose in the cytosol, apoplasm and phloem space, respiration was approvably fed by a very quick malate pool and a transport sucrose pool. Both transport pools exchanged carbon with a short-term storage pool, comprising vacuolar sucrose and eventually fructans.

Even if the half lives of the transport pools differ by a factor of 4, it still is reasonable to consider a common identity in form of cytosolic sucrose, considering that the study of Lehmeier et al. (2008) comprises respiration at the whole plant level, which means, that tissues of very different developmental status contribute to respiration (*i.e.* growing, mature and senescing leaves, sheaths and roots), while on the other hand mainly photosynthetic active leaves contribute to the current carbon supply of leaf growth. So when arranging half lives of transport sucrose, detected in different sites of grass plants, according to their metabolic distance to the off-spring of cytosolic sucrose in photosynthetic active cells, differences in half times seem to become obvious: 0.15 h for sucrose in the youngest fully expanded leaf of *L. perenne* (Fig. 17), 0.7 h for sucrose imported into the growth zone of *L. perenne* (Tab. 2), 3 h for sucrose respired in *L. perenne (*Lehmeier et al. 2008), 1-2 h for sucrose located in cytoplasmic and apoplastic space in mesophyll, bundle-sheath and phloem cells of photosynthetic leaves (Farrar 1989a) and 1-4 h for total tissue sucrose in leaf growth zones of *Festuca arundinacea* (Schnyder and Nelson 1987). To which extend short-term stored carbohydrates were derived from similar sources is hard to say. Sucrose in leaves showed half lives between 2 and 50 h and fructans > 60 h (Fig. 17). So short-term stores used for both growth and respiration might rather derive from vacuolar sucrose than from fructan turnover. A careful interpretation would be that carbon derived from fructan break down was rather used for leaf growth than for respiration.

To summarize, current assimilation-derived carbon was the main source for leaf growth (69 %), while respiration drew mainly on short-term stores. As in continuous light there is no need to buffer day-night-cycles and carbohydrate stores could rather be seen as actively built reserves than as accumulations (in the sense of Chapin F.S.3 et al. 1990), this even suggests an underlying genetic program to minimize the risk of fluctuating carbon availability for respiration, implementing a higher metabolic priority.

4.4 CONCLUSION

This study shows that mobilized substrates from stores were an important source for leaf growth under a wide range of external nitrogen supply. Further, these stores supplying growth were turned over continuously - contradicting a common view of stores as fixed reserves laid down once and only mobilized for the regrowth of tillers after winter or defoliation. This further suggests at least two possible different patterns of storage: continuously cycled stores and case-of-need stores.

In perennial C3 grasses, water-soluble carbohydrates which are not used in growth either are respired or invested into other metabolic processes like defense. In a situation of nutrient shortage - like under nitrogen deficiency - grasses invest relatively more nitrogen and carbon into growth and respiration (Lehmeier et al. 2010) giving experimental evidence for the trade-off between growth and defense as described in the ‘growth-differentiation balance’ theory of Herms and Mattson (1992), which is the central hypothesis of the joint project SFB 607.

LIST OF ABBREVIATIONS

Tab. 7: List of used abbreviations

Symbol	*Description*	*Units*
C	*carbon*	
N	*nitrogen*	
$\delta^{13}C$	*carbon isotope composition*	
atom% ^{15}N	*nitrogen isotope composition*	
f	*fraction of…*	
lab C, lab N	*labeled C or N*	
unlab C, unlab N	*non-labeled C or N*	
G	*leaf growth zone*	
RPT	*recently produced leaf tissue adjacent to the LGZ*	
LER	*leaf elongation rate*	*mm* h^{-1}
G	*mass of (C or N) in the growth zone*	μg
I	*import into the growth zone (of C or N)*	*g growth zone*$^{-1}$ h^{-1}
E	*export (of C or N) out of the growth zone in form of newly produced tissue*	*g growth zone*$^{-1}$ h^{-1}
R	*respiration*	*g growth zone*$^{-1}$ h^{-1}
ρ	*lineal density of C or N in recently produced tissue*	*g* mm^{-1}
t	*time*	*h or d*
<variable>	*average of 2 h-intervals*	$2h^{-1}$
kxy	*first order rate constants for flux from pool x (donor pool) to pool y (acceptor pool)*	d^{-1}
Q	*sizes of pools*	*g*
$t_{0.5}$	*half life*	*h or d*
RubisCo	*ribulose-1,5-bisphosphat-carboxylase/-oxygenase*	
RGR	*Relative growth rate*	*g* g^{-1}

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