Lehrstuhl für Grünlandlehre

Technische Universität München

# Sources of Carbon and Nitrogen for Leaf Growth in Grasses

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Agrarwissenschaften

genehmigten Dissertation.

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| Vorsitzender: | Univ.-Prof. Dr. D. Treutter |
| Prüfer der Dissertation: | 1. Univ.-Prof. Dr. J. Schnyder |
| | 2. Univ.-Prof. Dr. R. Matyssek |
| | 3. Univ.-Prof. Dr. U. Schmidhalter |

Die Dissertation wurde am 21. 10. 2004 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 09. 11. 2004 angenommen.

## ABSTRACT

**Aims:** The subject of the present study was the use of carbon and nitrogen substrates for leaf growth in a $C_3$ (*Lolium perenne* L.) and $C_4$ (*Paspalum dilatatum* Poir.) grass. Specifically, the interests were, first, to explore how carbon and nitrogen substrates are used to produce leaf area, and second, to determine which sources supply them. In order to do this, a novel methodological approach to estimate C and N import into leaf growth zones was developed and coupled with steady-state labelling of photosynthesis ($^{13}CO_2/^{12}CO_2$) and N uptake ($^{15}NO_3^-/^{14}NO_3^-$). Time-courses of tracer incorporation into leaf growth zones were analyzed with compartmental models thus to resolve the number and kinetics of distinct pools supplying leaf growth.

**Materials & Methods:** Plants of *L. perenne* and *P. dilatatum* were grown in mixed stands at 15°C (leading to $C_3$ dominance) and 23°C ($C_4$ dominance). Thus manipulated, individual plants grew in different environments and attained contrasting status, largely associated with their hierarchical positions within the stand. Compared to subordinate –shaded– plants (the $C_3$ grass at 23°C, the $C_4$ grass at 15°C), dominant –well-lit– plants (the $C_3$ grass at 15°C, the $C_4$ grass at 23°C) were bigger, taller, intercepted a greater proportion of incoming light, and had higher photosynthesis and N uptake rates.

**Results & Discussion:** In response to severe defoliation, leaf area production was buffered from C shortage by an increased efficiency of substrates use in leaf area expansion. This was based on two mechanisms: mobilisation of C and N stores located within the growth zone, and decreases in the density of produced tissue. This response was evident in all plants, but its magnitude varied greatly between treatments, being directly related to the plant C status at the moment of defoliation. Thus, dominant *L. perenne* plants were able to maintain unaltered leaf area expansion rates for up to 2 d after defoliation, while leaf expansion rate decreased abruptly in subordinate *P. dilatatum* plants. In a second step, the sources of C and N supplying leaf growth were explored in undisturbed plants. Leaf growth relied largely on photoassimilates delivered either directly after fixation or short-term storage. Short-term C stores were equally important in dominant and subordinate plants. Hence, no link was found between the importance of stores and C acquisition rate. Conversely, compared to dominant plants, leaf growth in subordinate plants relied more on mobilized N from long-term stores, being largely independent of external N. These differences correlated well with the ratio of C to N in growth-substrates, and were associated with responses in N uptake.

**Conclusions:** the present study demonstrates that (*i*) refoliation of these $C_3$ and $C_4$ species is sustained by identical mechanisms: short-term mobilisation of reserves within the growth zone, and reduced costs of produced leaf area, however (*ii*) the expression of these mechanisms depend strongly on the growth zone C status prior to defoliation. The second part of the study showed that (*iii*) the importance of C stores is not influence by photosynthesis capacity but largely associated to buffering light/dark cycles in both *L. perenne* and *P. dilatatum*. However, (*iv*) it revealed a negative association between the ability of these grasses to acquire external N and the relative importance of long-term internal stores in supplying N for leaf growth suggesting a common control mechanism may be operating.

## ZUSAMMENFASSUNG

**Zielsetzung:** Die vorliegende Arbeit befasst sich mit der Nutzung von C- und N-Substraten im Blattwachstum einer $C_3$ (*Lolium perenne* L.) und einer $C_4$ Grasart (*Paspalum dilatatum* Poir.). Es soll insbesondere geklärt werden wie C- und N-Substrate in Blattflächenwachstum umgesetzt werden, und aus welchen Quellen diese Substrate stammen. Diese Fragestellungen wurden mit neuen methodischen Ansätzen in Kombination mit ‚steady-state' Markierung der Photosyntheseprodukte ($^{13}CO_2/^{12}CO_2$) und des aufgenommenen N ($^{15}NO_3^-/^{14}NO_3^-$) untersucht. Der zeitliche Verlauf des Einbaus der Tracer in die Blattwachstumszonen wurde mit kompartimentellen Modellen untersucht, mit dem Ziel die Zahl und kinetischen Eigenschaften verschiedener Substratpools für das Blattwachstum zu erfassen.

**Material und Methoden:** *L. perenne* und *P. dilatatum* Pflanzen wurden in gemischten Beständen bei 15°C und 23°C angezogen. Erwartungsgemäß führten diese Verfahren bei 15°C zur Dominanz von *L. perenne* ($C_3$) und bei 23°C zur Dominanz von *P. dilatatum* ($C_4$), während die jeweils andere Art eine subordinate Position einnahm. Die dominanten Pflanzen waren größer, erhielten mehr Licht, und zeigten höhere Photosynthese- und N-Aufnahmeraten.

**Ergebnisse und Diskussion:** Entblätterung führte zu einer kurzfristigen drastischen Minderung des C-Imports in die Blattwachstumszonen. Die Blattflächenentwicklung wurde jedoch durch diesen Effekt nur wenig beeinflusst, weil die verminderte C-Verfügbarkeit durch eine erhöhte Effizienz der Substratnutzung im Blattflächenwachstum gepuffert wurde. Diese erhöhte Effizienz beruhte auf zwei Mechanismen: Mobilisierung von C- und N-Reserven innerhalb der Wachstumszonen, und Produktion von Blattfläche mit (temporär) verminderter Gewebedichte. Diese Reaktionen traten in allen Pflanzen auf, die Stärke der Reaktion war jedoch vom C-Status der Pflanzen zum Zeitpunkt der Entblätterung – und damit von den verschiedenen Verfahren– abhängig. Dominante *L. perenne* Pflanzen zeigten bis 2 Tage nach Entblätterung eine unverminderte Blattflächenentwicklung, während diese bei subordinaten *P. dilatatum* Pflanzen nach Entblätterung unmittelbar reduziert war.

In einem zweiten Schritt wurde untersucht welche Quellen das Blattwachstum während seiner ungestörten Entwicklung mit C- und N-Substraten versorgen. Diese Untersuchungen zeigten, dass das Blattwachstum auf C-Substraten beruht welche entweder unmittelbar nach Fixierung oder nach kurzzeitiger Zwischenspeicherung den Wachstumszonen zugeführt wird. Die Bedeutung kurzzeitiger Speicher war in dominanten und subordinaten Pflanzen ähnlich, und somit nicht von der Photosyntheserate der Pflanze abhängig. Andererseits war die N-Versorgung von Blattwachstumszonen in subordinaten Pflanzen stärker von Langzeitspeichern abhängig als in dominanten Pflanzen. Diese Effekte standen in enger Beziehung mit dem C:N Verhältnis im Substrat welches den Wachstumszonen zugeführt wurde, und waren von der N-Aufnahmerate abhängig.

**Schlussfolgerungen:** Die vorliegenden Untersuchungen belegen, dass (*i*) die Wiederbeblätterung der beiden $C_3$- und $C_4$-Gräser von denselben Mechanismen abhängt: kurzfristige Mobilisierung von Reserven innerhalb der Blattwachstumszonen, und reduzierte Kosten der Blattflächenentwicklung. (*ii*) Das Leistungsvermögen dieser Mechanismen ist jedoch vom C-Status der Pflanze zum Zeitpunkt der Entblätterung abhängig. (*iii*) Im ungestörten Wachstum leisten kurzfristige C-Speicher einen Beitrag zum Blattwachstum der unabhängig von der Photosynthesekapazität der Pflanzen ist. Diese Speicher

dienen offensichtlich der Pufferung der Licht/Dunkel-Diskontinuität in der Bereitstellung von aktuellen Assimilationsprodukten. (*iv*) Es besteht eine negative Beziehung zwischen dem Vermögen externen N aufzunehmen und dem Beitrag langfristiger N-Speicher zum Blattwachstum. Dieses Ergebnis kann als Hinweis für einen Mechanismus aufgefasst werden welcher den Beitrag von N-Speichern zum Blattwachstum von der aktuellen N-Aufnahme abhängig macht.

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# Chapter I. GENERAL INTRODUCTION

The primary role of leaves is photosynthesis and the subsequent provision of assimilates. Hence, leaf growth involves, on one hand, an instantaneous demand for C and N substrates, and on the other, it sets up the future capacity for providing assimilates for growth, nutrient uptake, storage, and plant defence. Leaves play a further role in grazed ecosystems, as they are not only the producers of photoassimilates needed for growth and maintenance, but also constitute the food of the many heterotrophic organisms that thrive on them, including cattle (Schnyder *et al.* 2000). In contrast to other crops, the way a grass plant is harvested, *i.e.* defoliated, profoundly affects its productivity (Parsons & Chapman 1998).

Knowledge on the sources of C and N substrates supplying leaf growth, and on leaf growth responses to defoliation, are therefore important steps in understanding the functioning of grazed ecosystems from both agronomical (yield) or ecological (fitness) perspectives.

## STATE OF THE ART

### *The leaf growth zone*

In grass leaves, growth –*i.e.* the conversion of substrates into tissue structure– is confined to a tissue segment, the *growth zone*, usually no longer than one-half the length of the sheath tube, located at the base of elongating leaves (Davidson & Milthorpe 1966; Kemp 1980). Cells are produced at the very base of the leaf by an intercalary meristem[1], and thereafter undergo expansion and maturation (Volenec & Nelson 1981). Hence, the growth zone comprises the sequential arrangement, along a longitudinal axis, of a cell division, a cell elongation and a cell maturation zone.

Cells stay in the division zone for relatively long times, typically more than 48 to 72 h, elongating at very low rates while going through successive division cycles (MacAdam, Volenec & Nelson 1989). Therefore, the velocity of displacement of a cell away from the meristem is initially low. However, once a cell stops dividing, a phase of rapid expansion begins and its displacement velocity increases abruptly because of the increasing number of expanding cells at more basal positions. Within 24 to 36 h, the cell attains its final size, leaves the elongation zone, and its displacement velocity equals the leaf

[1] This meristem is divided in two parts by the ligule, which are activated in sequence during leaf development to produce, first, the leaf blade, and then, the leaf sheath, respectively (Schnyder *et al.* 1990; Skinner & Nelson 1994). The present study is confined to the growth of leaf blades.

elongation rate (Schnyder *et al.* 1990; Schäufele & Schnyder 2000). The maturation zone, whose size is about 50 to 75% of that of the elongation zone (Allard & Nelson 1991; Gastal & Nelson 1994; Maurice, Gastal & Durand 1997), is characterized by a lack of further changes in cell dimensions, a continued accumulation of dry weight, and further functional differentiation (Maurice *et al.* 1997; Rademacher & Nelson 2001; MacAdam & Nelson 2002).

***Fluxes of tissue, carbon, and nitrogen in the growth zone***

The flux of tissue generated by continuous production of cells at basal position and their subsequent expansion brings about an associated flux of tissue-bound C and N. Efflux of tissue-bound C and N out of the growth zone, and of respired C, are balanced by the concurrent import of substrates, mainly sucrose and amino acids (Volenec & Nelson 1984; Schnyder & Nelson 1988a; Schnyder & Nelson 1988b; Gastal & Nelson 1994). Deposition of C and N present different spatial patterns. N import is closely associated with cell division and early cell elongation (Gastal & Nelson 1994), whereas the greatest C import rates occur concomitantly with maximal cell expansion (MacAdam & Nelson 1987; Schnyder & Nelson 1988a; Schnyder & Nelson 1988b). Deposition rates of C and, particularly, N decrease drastically as cell elongation rates slow down, becoming virtually zero at the end of the maturation zone. Thus, by the end of the growth zone and before emerging from the whorl into the light, leaf tissue is structurally mature and functionally differentiated, and most of C and N deposition has already taken place (Davidson & Milthorpe 1966; Boffey, Selldén & Leech 1980; Allard & Nelson 1991; Gastal & Nelson 1994; Maurice *et al.* 1997).

***Relationship between substrates supply and tissue production***

Based on the previous description, the leaf growth zone can be conceived as a 'place' where C and N substrates are imported, transformed, and then exported as tissue whose structural and functional features are largely determined. There are strong indications of significant plasticity in the relationship between tissue production and mass deposition, and hence on the efficiency of substrate conversion into new leaf. For instance, stimulation of water influx –tissue expansion– relative to C deposition within the growth zone has been observed in response to treatments leading to C deficits, such as the dark part of diurnal cycles (Schnyder & Nelson 1988b), low light intensity (Schnyder & Nelson 1989),

and severe defoliation (de Visser, Vianden & Schnyder 1997). In opposition, under N deficiency or saline stress, the ratio of C to water deposition increases (Gastal & Nelson 1994; Hu, Schnyder & Schmidhalter 2000). Despite this, the relationship between substrate supply and tissue efflux has rarely been examined critically. In consequence, little is known about putative control mechanisms, and of eventual effects on relevant parameters of produced tissue, such as its C and N density on an area basis. Partly, this is due to a lack of a suitable methodology to analyze responses at the whole growth zone level. In the present study, an approach for determining the fluxes tissue, and of C and N is presented (Chapter II).

Understanding defoliation responses is of particular interest because it may point to mechanisms sustaining the exposure of photosynthetic area following leaf removal. In this sense, studies have indicated the potential importance of C stored within the growth zone to support efflux immediately after defoliation (Davidson & Milthorpe 1966; Morvan-Bertrand *et al.* 2001; Schäufele & Schnyder 2001). However, information is largely restricted to a few $C_3$ species growing as isolated plants. No information is available on $C_4$ grasses, nor for plants growing within mixed stands. Structural and functional differences between $C_3$ and $C_4$ leaves (Dengler *et al.* 1994; Anten, Schieving & Werger 1995), and between well-lit and shaded leaves (Prioul, Brangeon & Reyss 1980), may modulate the expression of known responses. This is explored in Chapter II.

***Sources of substrates supplying the leaf growth zone***

C and N imported into the leaf growth zone may derive from either current assimilation/uptake or mobilization from stores. Tracer techniques are central to the study of sources supplying growth. Thus, pulse-chase $^{14}C$-labelling studies have shown that current assimilation in (older) mature leaves and in the exposed part of the growing leaf can both supply C for leaf growth (Anderson & Dale 1983; Allard & Nelson 1991; Brégard & Allard 1999). Further, using steady-state $^{13}C$-labelling, Schnyder & de Visser (1999) showed that less than 10% of C accumulating in regrowing leaves after severe defoliation derived from assimilates older than 3 d. These results support the notion that leaf growth in grasses relies largely on recent photosynthesis. On the contrary, recent N uptake seems to be of lesser importance in supplying growth. $^{15}N$-labelling studies consistently indicate that 'old' N may contribute substantially to leaf growth, although its importance can vary depending on species and

growth conditions (Bausenwein, Millard & Raven 2001; Thornton *et al.* 2002; Santos, Thornton & Corsi 2002). Also, they show that N stores would have a more sustained role than C stores in supplying leaf growth, at least following defoliation (Avice *et al.* 1996; Schnyder & de Visser 1999).

The available information thus demonstrates that both relatively 'new' and 'old' C and N may be used in leaf tissue production. However, interpreting these results in terms of distinct sources, *e.g.* current assimilation/uptake *vs.* mobilization from stores, is less clear. One reason is unaccounted tracer mixing. Often, tracers move and mix with several pools before being used for growth. In consequence, the origin of tracer imported into the growth zone changes along a labelling period (Farrar & Gunn 1998). Compartmental modelling provides a means to address this by resolving the number and kinetics of present pools through quantitative analysis of tracer time-courses. This technique, extensively used for studies of C and N fluxes in source systems (*i.e.* photosynthetic leaves and active roots, Moorby & Jarman 1975; Devienne, Mary & Lamaze 1994a), has received less attention in sink systems.

Furthermore, the aforementioned limitation with respect to information being confined to single plants of $C_3$ species, is also evident regarding the role of current assimilation/uptake and mobilization from stores in supplying leaf growth. In fact, opposite to knowledge on mobilization from C and N stores (Feller & Fischer 1994; Stitt & Krapp 1999; Farrar, Pollock & Gallagher 2000), an understanding of putative controls of the relative importance of stores in providing substrates for growth is lacking. In the present study, a novel approach for analyzing tracer kinetics is presented and used to establish the role of C and N stores in supplying leaf growth in a $C_3$ and a $C_4$ grass growing in mixed dense stands but otherwise undisturbed (Chapter III).

**AIMS**

The first aim of this research was to develop an approach to study fluxes of tissue, C, and N at the whole leaf growth zone level to investigate how C and N substrates are used to construct leaf area. It was of particular interest to analyze the relationship between import of C and N and tissue production, and the effects of contrasting growth zone C status on defoliation responses (Chapter II).

In a second step, the method was further developed to account for fluxes of labelled and non-labelled C and N, and tracer time-courses were analyzed with compartmental models. This allowed to define

the sources supplying C and N for leaf growth in terms of distinct pools with respective turnover rates. In particular, the effects of contrasting plant status on the role of current acquisition *vs.* mobilization from stores in supplying leaf growth were investigated (Chapter III).

Two explicit hypothesis were tested: (*i*) that C status of the growth zone affects the relationship between substrate import and tissue efflux, and (*ii*) that the importance of stores as sources of C and N substrates depends on actual acquisition rates. In both cases, a particular experimental set-up was used in order to produce plants with different status. Individual plants of a $C_3$ and a $C_4$ grass were grown in mixed stands at moderately low and high temperatures. This led to $C_3$- or $C_4$-dominated stands, and thus to hierarchically dominant (well-lit) and subordinate (shaded) individuals, while avoiding any below-ground interaction between the plants. We selected *L. perenne* and *P. dilatatum* as the $C_3$ and $C_4$ grass, respectively, due to their morphological similarity, and to the availability of basic information on the establishment and temperature response of *L. perenne*/*P. dilatatum* mixed stands (Harris, Forde & Hardacre 1981a; Harris, Forde & Hardacre 1981b).

# Chapter II. DEFOLIATION EFFECTS ON CARBON AND NITROGEN SUBSTRATE IMPORT AND TISSUE-BOUND EFFLUX IN LEAF GROWTH ZONES OF GRASSES [2]


## ABSTRACT

Grassland plants suffer regular defoliation, causing loss of photosynthetic activity and internal resources. Consequently, re-foliation may be substrate-limited. We tested the hypothesis that decreased C import in leaf growth zones is (partially) compensated by (i) mobilisation of substrate within growth zones and (ii) increased efficiency of substrate use in leaf area expansion, but (iii) that these processes depend on the C status of growth zones at defoliation. Mixtures of a $C_3$ (*Lolium perenne* L) and a $C_4$ grass (*Paspalum dilatatum* Poir.) grown at 15°C ($C_3$ dominance) and 23°C ($C_4$ dominance). Individual plants thus grew in contrasting (light and temperature) environments before being defoliated. Defoliation caused a drastic and immediate decrease in C import, but effects on leaf area expansion were buffered by biomass mobilisation in the growth zone and increases in specific leaf area of produced tissue. Thus, over the first 2 d post-defoliation, the amount of leaf area produced per unit imported C increased by 39% to 102% depending on treatment. The magnitude of these buffering responses was correlated with the concentration of water soluble carbohydrates in the growth zone at defoliation. Similar responses were observed for N, although defoliation effects were smaller and delayed relative to those on C import. This study demonstrates refoliation is sustained by short-term mobilisation of reserves within the growth zone and reduced costs of produced leaf area, but that these mechanisms depend on growth zone C status at defoliation.


[2] Lattanzi F.A., Schnyder H. & Thornton B. (2004) *Plant, Cell & Environment* **27,** 347-356.

## INTRODUCTION

Defoliation is an ubiquitous factor shaping vegetation state in grassland ecosystems. It is also a dramatic event in the life of grassland plants, as it deprives them of (part of) the photosynthetic apparatus and of internal resources such as leaf N. Despite regeneration of lost leaf area (*i.e.* re-foliation) is an essential component of the suite of plant responses leading to recovery, it is still a poorly understood process (Richards 1993). Indeed, the effect of defoliation on substrate supply to leaf growth zones has not been analysed. Also, little is known about putative plasticity in the efficiency of substrate conversion into new leaf area.

In grass species, the production of leaf tissue is confined to a short zone at the base of growing leaves, the *growth zone*, where an intercalary meristem produces cells which then undergo expansion and maturation (MacAdam & Nelson 1987; MacAdam *et al.* 1989; Rademacher & Nelson 2001). The continuous production of cells at basal positions and their subsequent expansion gives rise to a flux of tissue and tissue-bound mass out of the growth zone. This export is balanced by import of substrate (Figure II.1). The *growth zone* thus refers to the composite of cell division, expansion and maturation zones and may be conceived as a 'place' where substrates are imported, transformed, and exported as structurally and functionally differentiated tissue.

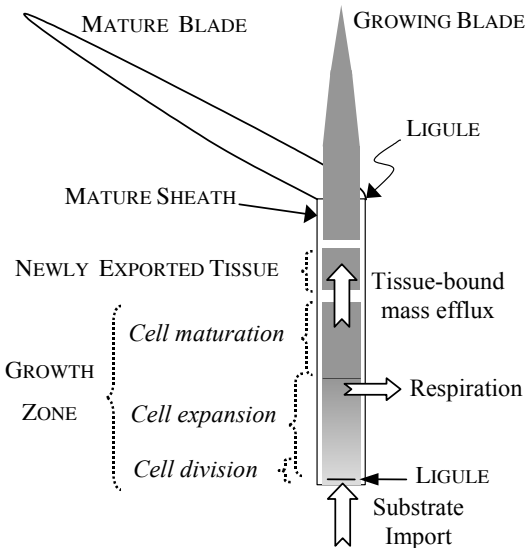


Figure II.1. Schematic of the growth zone of an elongating leaf in a grass tiller showing the disposition of the cell division, expansion and maturation zones, as well as the fluxes of substrate import, tissue-bound mass efflux, and respiration. The location of the sampled piece of newly exported tissue is indicated.

Defoliation effects on substrate import and use in leaf growth zones have been scarcely studied. de Visser *et al.* (1997) observed a rapid and substantial reduction of C, N and water mass of the growth zone of severely defoliated *Lolium perenne* L. plants. This phenomenon involved mobilisation of non-structural carbohydrates and decreases in the concentration of structural biomass, and was termed *dilution* (Schnyder & de Visser 1999). Dilution was suggested to arise from imbalances between substrate influx and tissue-bound mass efflux, and serve to sustain the exposure of photosynthetically active tissue under conditions of substrate shortage. This would imply changes in relevant morphophysiological parameters of newly exported tissue, such as increases in specific leaf area (SLA, $m^2$ kg $C^{-1}$) and decreases in leaf N content ($N_L$, g N $m^{-2}$). These hypotheses have not been tested, for defoliation effects on actual rates of C and N substrate import into, and tissue-bound efflux out of, the growth zone have not been analysed.

One of the predictions from studies of growth zone responses to defoliation is that the potential for growth zone dilution and maintenance of leaf area expansion rate shortly after defoliation is less when the concentration of non-structural carbohydrates within the leaf growth zone is low (de Visser *et al.* 1997; Morvan-Bertrand *et al.* 2001). Low concentrations may be brought about by growth at low irradiance (Schnyder & Nelson 1989). Hence, plants growing in contrasting light environments should

respond differentially to defoliation. Specifically, within a canopy, subordinate (shaded) plants would have a lesser scope for adaptation in response to defoliation than dominant (well-lit) plants. Notwithstanding this, post-defoliation leaf area expansion and associated C and N fluxes in the growth zone has not been explored in plants growing in different hierarchical positions in stands.

In addition to being scarce, studies on growth zone responses to defoliation have been confined to temperate grasses, with no $C_4$ species studied. Leaves of $C_3$ and $C_4$ species differ in a number of structural and functional properties, including the proportion of mesophyll and bundle-sheath tissues (Dengler *et al.* 1994), and the C to N ratio and $N_L$ (Anten *et al.* 1995). Therefore, it is questionable whether responses to defoliation are the same in both species groups. $C_3$ and $C_4$ grasses coexist in grazed grasslands of North and South America, Southern Africa, Asia, and Australia (Collatz, Berry & Clark 1998).

This study assessed component processes of growth zone responses to defoliation in *L. perenne*, a $C_3$ species, and *Paspalum dilatatum* Poir., a $C_4$ species. Plants were grew in mixtures at moderately low or high temperatures (leading to $C_3$- or $C_4$-dominated canopies). This design produced a large range of growth conditions, and thus of plant status at defoliation. The analysis included short- (days) and long-term (weeks) effects of defoliation on C and N substrate import and tissue-bound efflux, and production rate and relevant morphophysiological characteristics of mature leaf area (SLA and $N_L$). A method for the estimation of substrate import and tissue-bound mass efflux in leaf growth zones is presented.

## MATERIALS AND METHODS

### *Plant material and growth conditions*

Seeds of *Lolium perenne* cv "Racolta" and *Paspalum dilatatum* cv "Grasslands Raki" were sown in sand and germinated in the dark at 25°C and 35°C, respectively. After four days, a 12 h light period was supplied by cool-white fluorescent lamps. Photosynthetic photon flux density (PPFD) was gradually increased from 250 μmol $m^{-2}$ $s^{-1}$ to 550 μmol $m^{-2}$ $s^{-1}$ over the following 10 d. Seedlings of similar size and phenological stage were then transplanted into tubes (i.d. 50 mm, height 350 mm) filled with 760 g quartz sand (0.3 mm to 0.8 mm particle size) at a density of one plant per tube. The base of each tube had a drainage hole covered by a fine nylon net.

Within plastic boxes (560 mm x 760 mm x 370 mm), 89 tubes of each species were arranged in a hexagonal design where each plant had six equidistant neighbours, two of them conspecifics. Two of these *L. perenne*/*P. dilatatum* mixtures (420 plants $m^{-2}$) were allocated to each of four growth cabinets (E15, Conviron, Winnipeg, Canada). Mixtures were irrigated daily by flooding the boxes three times during the light period and once during the dark period with modified half-strength Hoagland's solution (2.5 mM $KNO_3$, 2.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM $KH_2PO_4$, 0.5 mM NaCl, 0.125 mM Fe-EDTA, 23 µM $H_3BO_3$, 4.5 µM $MnSO_4$, 0.38 µM $ZnSO_4$, 0.16 µM $CuSO_4$, 0.05 µM $Na_2MoO_4$). At each flooding event, the nutrient solution remained in the boxes for 30 min, so each tube became saturated, then drained to field capacity.

***Temperature and defoliation treatments***

Immediately after transplanting, temperature was set to 25°C/23°C (light/dark) in two growth cabinets and to 15°C/14°C (light/dark) in the two other cabinets. Vapour pressure deficit was controlled at 0.5/0.3 kPa (light/dark) in all growth cabinets. These treatments resulted in daily average sand temperatures (15 mm below surface) of 23°C and 15°C, respectively, and to which they referred to hereafter. Six weeks after transplanting, half the mixtures growing in the high temperature regime were defoliated at the beginning of the dark period. Ten days later, half the mixtures growing in the low temperature regime were similarly defoliated. *L. perenne* and *P. dilatatum* plants were cut at 90 mm and 120 mm, respectively, at 23°C, and 65 mm and 41 mm at 15°C. The adjustment of the cutting height resulted in similar defoliation severities between treatments: a reduction of shoot biomass by *c.*70%, and the removal of virtually all lamina area. Immediately before defoliation, PPFD was recorded at 15 cm increments from the top to the bottom of the mixtures. Thereafter, leaf area of 12 plants per treatments was separated into the 15 cm strata and measured on a planimeter (LI-3100, Licor, NE, USA).

***Sampling and C and N analysis***

The length of the leaf elongation zone (the composite of cell division and expansion zones) was determined 1 d before defoliation on six tillers per treatment by the pin-pricking method (Davidson & Milthorpe 1966; Schnyder & Nelson 1989). The length of the maturation zone was then estimated as

two-thirds (15°C) or equal (23°C) to that of the elongation zone. Thus defined, the growth zones (50 mm and 60 mm in *L. perenne*, and 41 and 70 mm in *P. dilatatum*, at 15°C and 23°C, respectively) comprised the leaf segment where the bulk of growth-associated C and N deposition occurred (see below).

Plants were sampled from the centre of undefoliated (control) and defoliated mixture at 0, 1, 2, 5, 12 & 18 d after defoliation at 15°C, and 0, 1, 2, 4, 8 & 15 d after defoliation at 23°C. Growth zones were dissected out of two or three mature tillers in each of eight plants per treatment. A further piece of *newly exported tissue* was sampled immediately adjacent to the growth zone (length: 35 mm and 50 mm in *L. perenne* and *P. dilatatum* at 23°C, and 25 mm and 8 mm at 15°C; see Fig. 1). Only leaves whose blade length was approximately half the length of the blade of the youngest fully expanded leaf were sampled. This ensured only blade tissue was present in the growth zone, and all sampled leaves were at a similar developmental stage. Harvests began 1 h after the lights went off. Plants were kept in a dark room at 10°C and processed within 4 h.

Tissue samples were placed in aluminium trays, immediately heated to 100°C for 1 h, dried at 65°C for a further 48 h, and then weighed. Four to seven replicates out of the eight harvested plants were ground to a fine powder. Total C and N content were determined on 1 mg dry matter (DM) using a elemental analyser (NA1500, Carlo Erba Instruments, Milan, Italy).

Procedures for extraction of water soluble carbohydrates (WSC) followed Schnyder & De Visser (1999). In brief, 10 mg DM of ground material was weighed into 2.2 ml Eppendorf tubes, and 2 ml water added. Tubes were sealed and transferred to a water bath at 93°C for 10 min, shaken for 45 min, and centrifuged at 10,000 g for 15 min. The supernatant was removed and an aliquot was hydrolysed in 0.1 M $H_2SO_4$ for 20 min at 93°C. Reducing power of the hydrolysed carbohydrates was detected photometrically at 425 nm after reduction of a potassium ferricyanide solution. Fructose (biochemical grade from Merck) was used as a standard. Analysis of WSC was restricted to growth zone of control plants due to having insufficient material in the other samples.

***Estimation of C and N substrate import and tissue-bound efflux***

Assimilate flux into and out of the growth zone was estimated using a simple model of mass fluxes through the growth zone (Figure II.1). A substance (*X*) is imported ($I_X$, μg X zone$^{-1}$ d$^{-1}$) into the

growth zone ($G_X$, μg X zone$^{-1}$) as substrate, and exported by tissue-bound efflux ($E_X$, μg X zone$^{-1}$ d$^{-1}$). Thus,

(Eq. II.1) $$dG_X / dt = I_X - E_X$$

For a particular time interval $t_0 - t_1$, $I_X$ can be approximated as:

(Eq. II.2) $$I_X = E_X + ( G_{X \text{ at } t1} - G_{X \text{ at } t0} )$$

The growth zone is heterotrophic, thus influx occurs only through import. Further, it was assumed that no export occurred other than by tissue-bound efflux. Respiration in the growth zone was ignored, thus underestimating actual C import by the amount lost through respiration. This measure of import is analogous to a *net* deposition rate (*e.g.* Schnyder & Nelson 1989; Allard & Nelson 1991; Maurice *et al.* 1997; Schäufele & Schnyder 2001).

Following (Eq. II.2), computation of import of C ($I_C$) and N ($I_N$) required estimates of growth zone C ($G_C$) and N mass ($G_N$) at two times, and of the efflux of C ($E_C$) and N ($E_N$) over that time-interval. The former were measured directly (see above), while $E_C$ and $E_N$ were estimated by measuring their two components: the rate of leaf length ($L_L$, mm d$^{-1}$) or leaf area production ($L_A$, mm$^2$ d$^{-1}$), and the density of C and N in exported tissue on a per unit length ($\rho_L$, g mm$^{-1}$) or area basis ($\rho_A$, g mm$^{-2}$). The rate of leaf length production was estimated by measuring leaf elongation rate (LER) on 12 leaves per treatment every 1 d to 3 d. Only data from leaves equivalent to those selected for growth zone C and N analysis were used. Similarly, since final leaf width is determined shortly after cell extension ceases (MacAdam *et al.* 1989; Maurice *et al.* 1997), leaf area production rate was estimated by the leaf area expansion rate (LAER) defined as the product of LER times leaf width measured at the end of the growth zone. Finally,

(Eq. II.3a) $$E_C = L_L \rho_{LC}, \text{ and}$$

(Eq. II.3b) $$E_N = L_L \rho_{LN}$$

where subscripts C and N refer to carbon and nitrogen. Density of C and N per unit leaf length or leaf area were estimated in the piece of newly exported tissue. Eqs. (Eq. II.3a) and (Eq. II.3b) thus become:

(Eq. II.4a) $$E_C = L_A \rho_{AC}, \text{ and}$$

(Eq. II.4b) $$E_N = L_A \rho_{AN}$$

Note that $\rho_{AC}$ is equivalent to the inverse of SLA, if mass is represented in units of C, and $\rho_{AN}$ corresponds to $N_L$. The apparent efficiency of C and N substrate conversion into leaf area was defined as the ratio of mature leaf area production per unit imported C ($L_A/I_C$ ) or N ($L_A/I_N$).

Computation of $I_C$, $I_N$ and $E_C$ and $E_N$ were straightforward in control plants because LER, leaf width and C and N densities were nearly constant. Defoliation introduced non-steadiness, therefore polynomial functions were fitted to describe the time-course of these variables in defoliated plants. To do this, the *mean* time-interval over which the sampled piece of newly exported tissue was produced must be estimated since densities of C and N were averages of that piece of tissue. In control plants, the time-interval was simply: length divided by LER, since LER was constant. In defoliated plants, the integral of the function fitted to describe the time-course of LER between $t_1$ (sampling time) and $t_0$ (unknown) was numerically solved for $t_0$. Estimated time-intervals were similar in both species and temperature regimes: 18.8 h (SE 0.7) and 26.8 h (SE 0.3) for control and defoliated plants, respectively. Variations of $G_X$ were insignificant relative to $E_X$ in control plants, thus $I_X \approx E_X$.

*Method evaluation*

The presented method has one main assumption: deposition of X within the piece of tissue where density of X was estimated is negligible relative to the flux of X through it. Therefore, density of X was assumed constant along the piece of sampled tissue, and no deposition of X is assumed to occur outside the growth zone. This assumption could not be tested with the present data. Therefore, actual values of $E_{DM}$ by Allard & Nelson (1991) and Maurice *et al.* 1997(1997), and of $E_N$ by Gastal & Nelson (1994), were compared to values estimated with this method using the same criteria for defining growth zone length (*i.e.* 1.67 or 2.0 times the length of the leaf elongation zone depending on temperature), and the same ratio of LER to newly exported tissue length of control plants (*i.e.* 0.8 = 18.8 h / 24 h). $I_{DM}$ estimates were compared to actual values, derived by integration of local net deposition rates, only for Maurice *et al.* (1997) as no other data set allowed changes in growth zone mass to be computed. Regressions of estimated against observed values did not differ from the $y = x$ line in either variable nor data set ($P > 0.5$; Figure II.2).

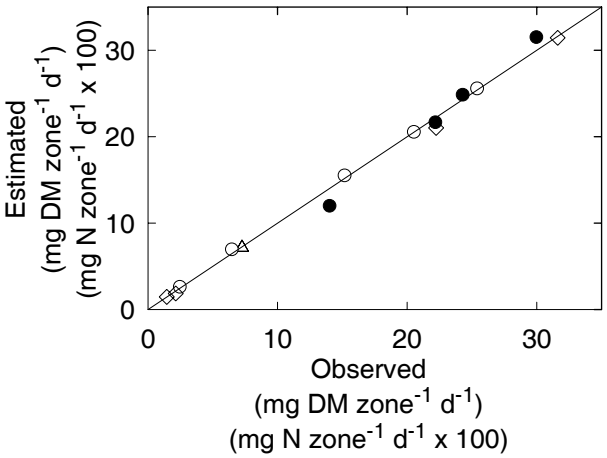


Figure II.2. Comparison of estimated vs. observed values of tissue-bound efflux (○◊Δ) and import (●) of dry matter (DM, ●○ Δ) and N (◊) in leaf growth zones of grasses. Δ Allard & Nelson 1991), ◊ Gastal & Nelson 1994), and ○● Maurice *et al.* 1997).

An analysis of sensitivity revealed halving or doubling the length of the piece of newly exported tissue did not change estimated $E_X$ and $I_X$ by more than 10% as long as the sampled leaf was within the phase of steady growth. The method could not be evaluated for non-steady conditions caused by defoliation because no data set was available. However, since sampled tissue was the piece of tissue produced over the last 26.8 h, estimated daily tissue-bound efflux rates should be accurate.

***Statistical analysis***

Experimental units (plants) were arranged in a sub-plot design with two blocks (growth chambers) and two to four replicates per block. Effects of harvest date (*i.e.* time), defoliation, temperature regime and their interactions were tested by ANOVA, for each species. For LER data, the covariance structure of the error (repeated measures) was taken into account in a mixed model (PROC MIXED in SAS® v.6.12, SAS Inc., NC). SE of mass fluxes ($E_X$ and $I_X$) were estimated by the error propagation technique as in Schäufele & Schnyder (2001). LER and leaf width variances were similar for all sampling dates and were therefore pooled. Tissue-bound C and N efflux, which included SE of LER and density of X in per unit leaf length were estimated more precisely than import rates, which additionally included the SE of the difference between two $G_X$ values.

## RESULTS

### *Canopy structure and composition at defoliation*

At defoliation, all mixtures had the same total amount of leaf area. But, at 15°C, *L. perenne* individuals were larger than their *P. dilatatum* neighbours, and had more leaf area in the upper half of the canopy. The opposite occurred at 23°C (Figure II.3). For ease of designation, the larger component in each mixture ($C_3$ at 15°C, $C_4$ at 23°C) will be referred to as dominant, the other as subordinate in the following.

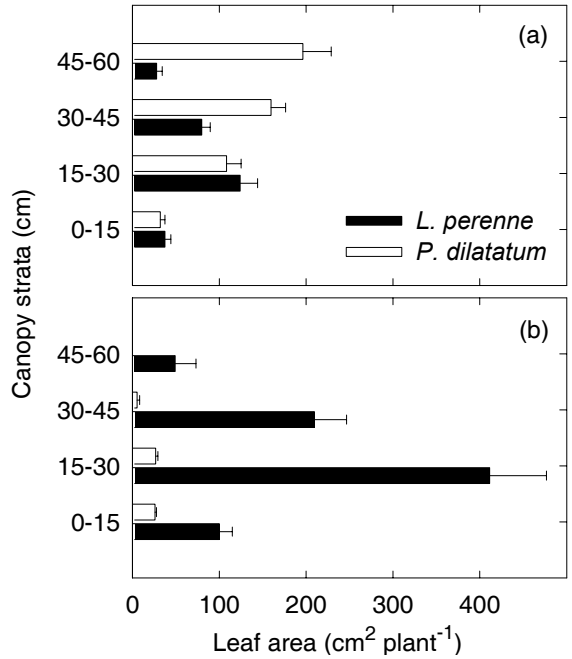


Figure II.3. Vertical distribution of the leaf area of undefoliated (control) mixed stands of *Lolium perenne* and *Paspalum dilatatum* growing at 23°C (a) and 15°C (b). Bars indicate 1 SE ($n$ = 4).

### *Growth zone state and process variables in control plants*

The total amount of C and N present in the growth zone of control plants, as well as the concentration of WSC, showed little or no change during the experimental period. Similarly, SLA and $N_L$ of newly produced leaf tissue were stable with time, therefore only time-weighted averages are shown (Table II.1). Likewise, the rates of length and area production, estimated by LER and LAER respectively, were constant in control plants (time-effect $P > 0.05$; Figure II.4). Accordingly, import of C and N into the growth zone, and efflux of tissue-bound mass were virtually constant in control plants because state and process variables involved in their estimation were also steady. Thus, average values for the

whole experimental period are shown in Table II.1. Comparison of such steady-state values indicated that, on one hand, growth zones of dominant plants had a greater C and N mass, higher WSC concentrations, and exported C and N at higher rates bound in tissue of lower SLA and higher $N_L$, than growth zones of subordinate plants. On the other hand, *L. perenne* showed consistently higher WSC concentrations in the growth zone, and lower SLA and higher $N_L$, than *P. dilatatum*. Since leaf width at the end of the growth zone was always greater in *P. dilatatum* than in *L. perenne* (Table II.1), differences between species in LAER were larger than differences in LER at 23°C, but smaller at 15°C.

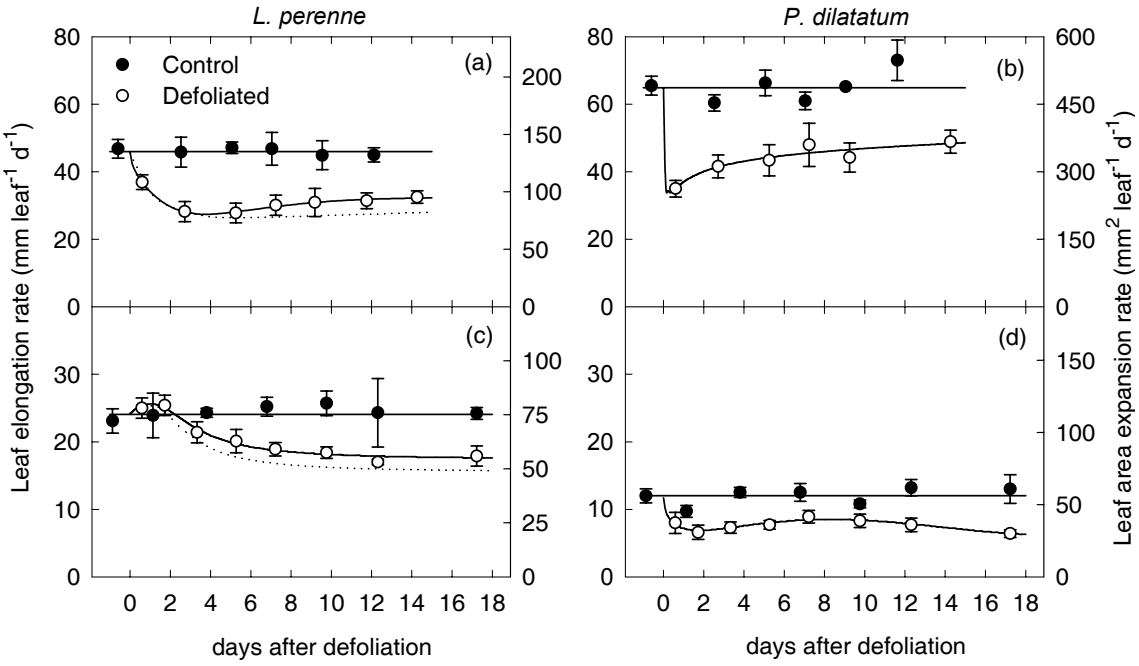


Figure II.4. Defoliation effects on the leaf elongation rate (LER, —), and leaf area expansion rate (LAER, ····) of the most rapidly elongating leaf of *Lolium perenne* and *Paspalum dilatatum* tillers. Plants grew in mixtures at 23°C (a & b) and 15°C (c & d), and thus in hierarchically dominant (b & c) or subordinate positions (a & d). Bars indicate ±1 SE ($n$ = 3 to 9). Lines correspond to the slope of linear regression of cumulative elongation against time (control plants), and the polynomial curve fitted to the time-course of LER and LAER (defoliated plants).

Table II.1. State and process variables of leaf growth zones in undefoliated (control) plants of *Lolium perenne* and *Paspalum dilatatum*. Average (and SE) of C and N mass and water soluble carbohydrates concentration (WSC) of the growth zone, specific leaf area (SLA), leaf nitrogen content ($N_L$), and leaf width of recently produced tissue, and C and N import and tissue-bound efflux rates†. Plants grew in mixtures at 23°C and 15°C, and thus in hierarchically dominant or subordinate positions.

| | *Lolium perenne* | | *Paspalum dilatatum* | |
|---|---|---|---|---|
| | 23°C Subordinate | 15°C Dominant | 23°C Dominant | 15°C Subordinate |
| C mass, mg zone$^{-1}$ | 1.10 (0.06) | 2.04 (0.06) | 3.30 (0.12) | 0.72 (0.03) |
| N mass, mg zone$^{-1}$ | 0.15 (0.008) | 0.21 (0.006) | 0.44 (0.017) | 0.10 (0.006) |
| WSC, mg C g C$^{-1}$ | 178 (20) | 319 (18) | 80 (6) | 26 (3) |
| SLA, m$^2$ kg C$^{-1}$ | 134 (6) | 66 (3) | 137 (11) | 187 (21) |
| $N_L$, g N m$^{-2}$ | 0.76 (0.03) | 1.13 (0.04) | 0.58 (0.04) | 0.49 (0.02) |
| Width, mm | 2.80 (0.08) | 3.14 (0.07) | 7.45 (0.20) | 4.48 (0.11) |
| C import, μg zone$^{-1}$ d$^{-1}$ | 972 (75) | 1136 (66) | 3488 (327) | 286 (23) |
| N import, μg zone$^{-1}$ d$^{-1}$ | 98 (6.5) | 85 (5.0) | 278 (26.4) | 26 (2.6) |

† Tissue-bound efflux equals substrate import in control plants (see Materials and Methods).

***Effects of defoliation***

Defoliation rapidly decreased import and efflux of C and N ($P < 0.05$; Figure II.5 & Figure II.6). Initially, substrate import was affected more than concurrent tissue-bound efflux, leading to decreases of growth zone C and N mass (Figure II.7). Defoliation also decreased the rates of leaf length and area production ($P < 0.05$, Figure II.4). But in all treatments such reductions were much less than those of C efflux. Therefore, newly produced tissue had an increased SLA (Figure II.8). C import was proportionally more decreased than that of N on day 1. Hence, the C:N ratio of imported substrates diminished after defoliation (Figure II.9). This effect counterbalanced the increase in SLA, and therefore $N_L$ was affected little (*L. perenne*) or not at all (*P. dilatatum*) (Figure II.8).

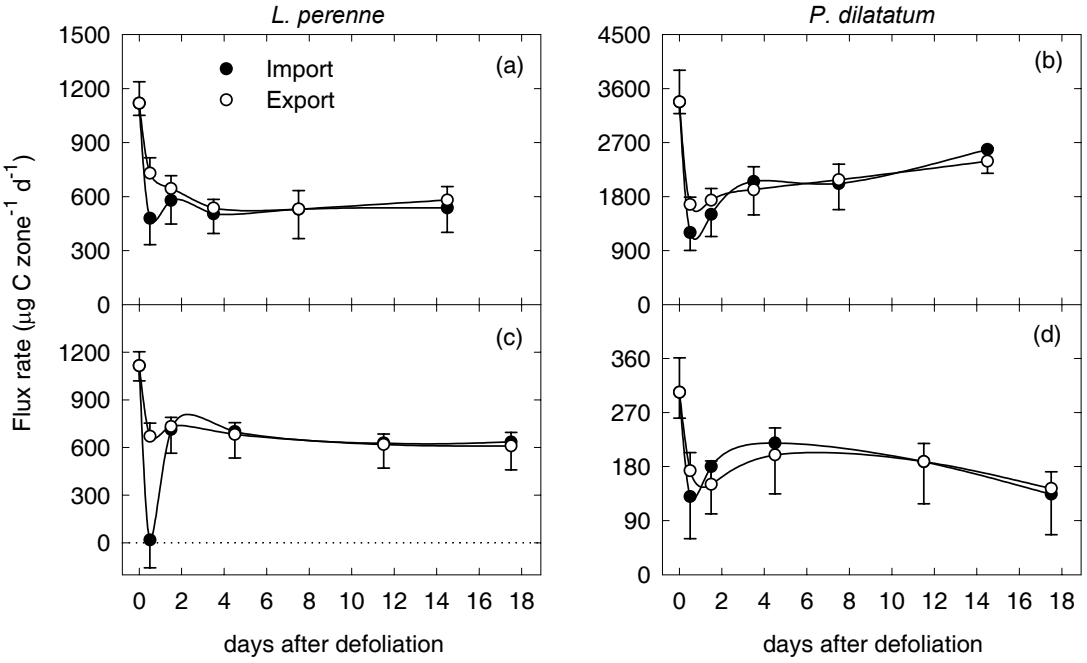


Figure II.5. Rates of C import (net of respiration) into, and tissue-bound C efflux out of, the leaf growth zone of the most rapidly elongating leaf of *Lolium perenne* and *Paspalum dilatatum* tillers following severe defoliation. Growth conditions as in Figure II.4. Bars indicate 1 SE.

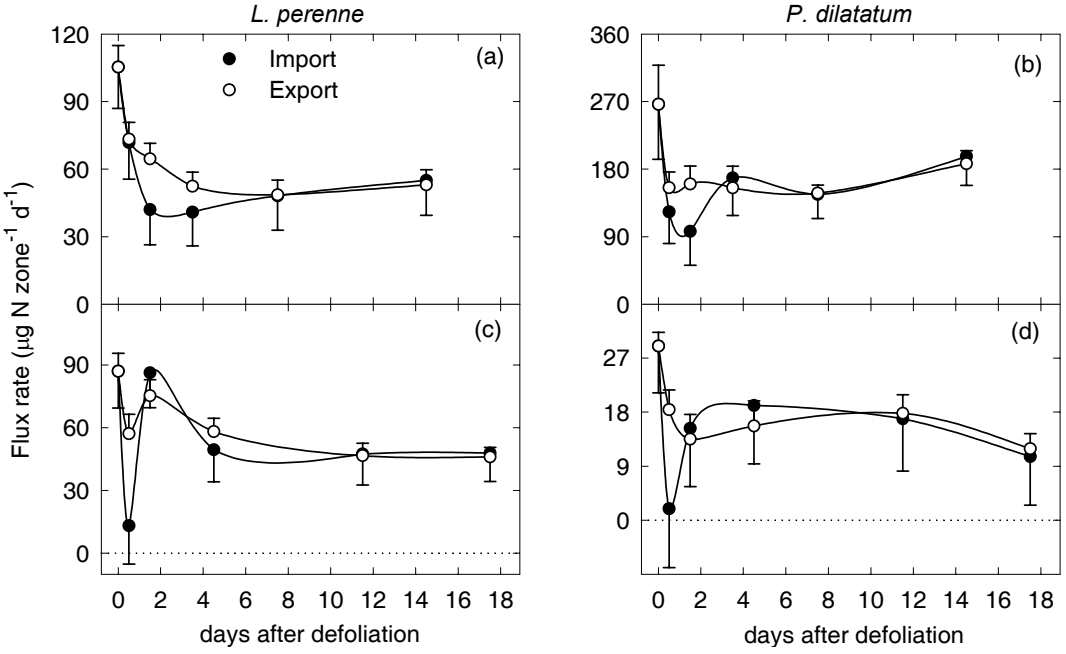


Figure II.6. Rates of N import into, and tissue-bound efflux out of, the leaf growth zone of the most rapidly elongating leaf of *Lolium perenne* and *Paspalum dilatatum* tillers following severe defoliation. Growth conditions as in Figure II.4. Bars indicate 1 SE.

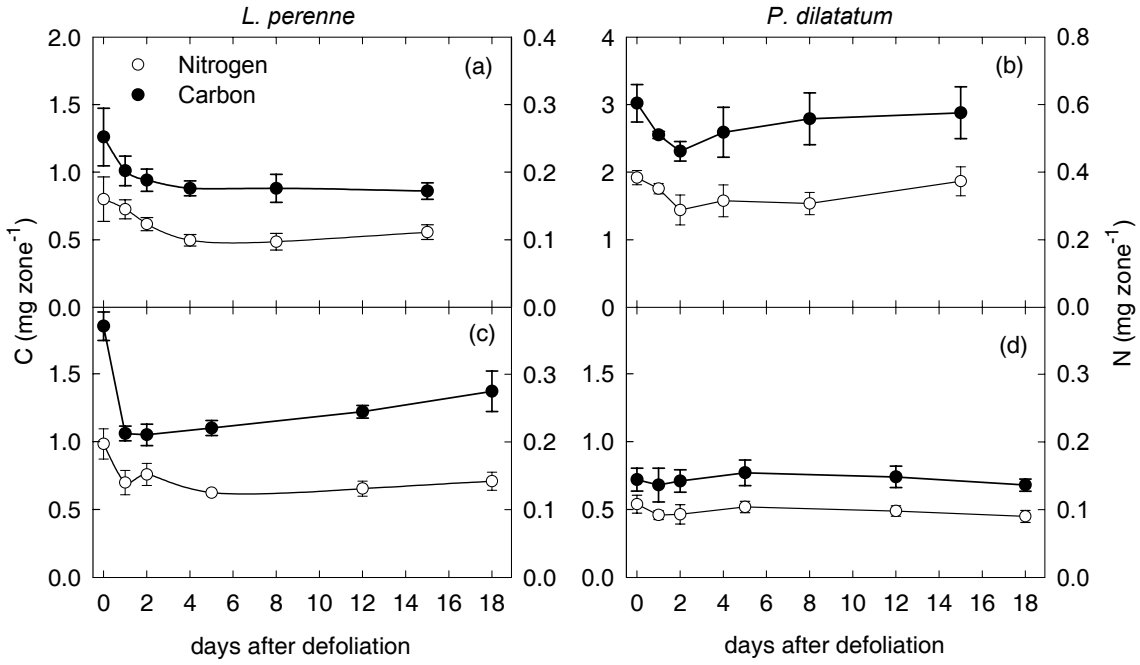


Figure II.7. Defoliation effects on the total amount of C and N in the growth zone of the most rapidly elongating leaf of *Lolium perenne* and *Paspalum dilatatum* tillers. Growth conditions as in Figure II.4. Bars indicate ±1 SE ($n$ = 4 to 7).

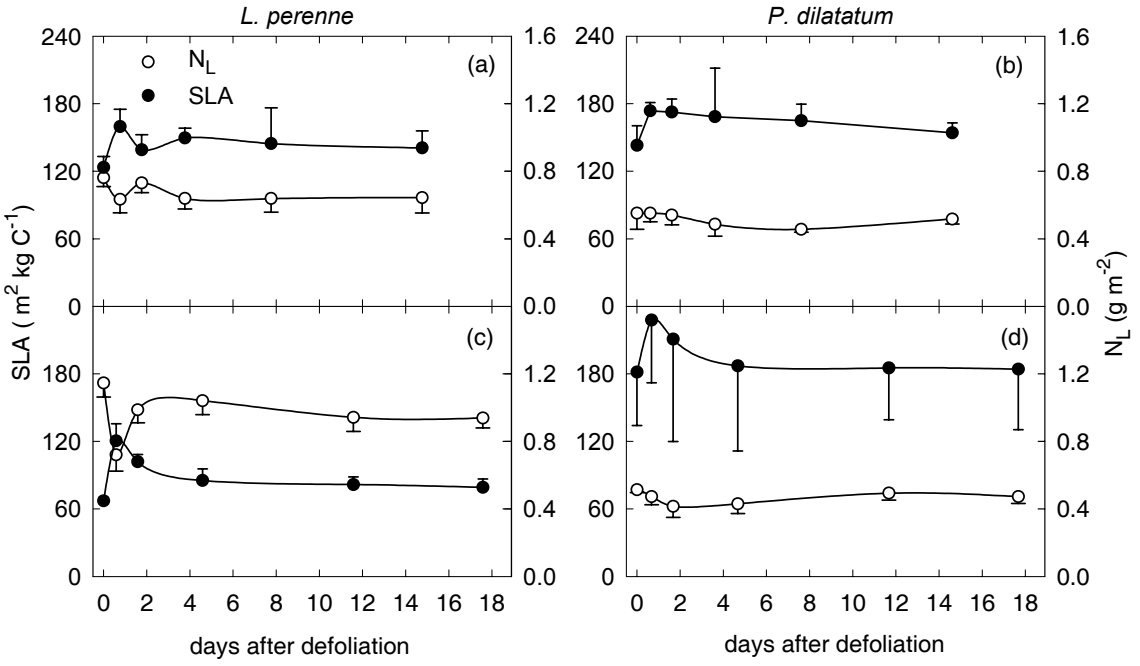


Figure II.8. Defoliation effects on the specific leaf area (SLA) and leaf nitrogen content ($N_L$) of newly exported tissue (cf. Fig. 1) in the most rapidly elongating leaf of *Lolium perenne* and *Paspalum dilatatum* tillers. Growth conditions as in Figure II.4. Bars indicate 1 SE ($n$ = 4 to 7).

After 2 d (C) to 4 d (N) of regrowth, import and tissue-bound efflux became balanced once again, at levels 38% to 50% lower than those of control plants (defoliation-effect: $P < 0.05$; time-effect $P > 0.1$; Figure II.5 & Figure II.6), and the C:N ratio of imported substrates recovered close to pre-defoliation values (Figure II.9). Also after day 4, SLA and $N_L$ of newly exported tissue, and the rates of tissue length and area production were close to or at steady-state (Figure II.4 & Figure II.8). Since a slight reduction of leaf width appeared in *L. perenne* plants by day 4 (subordinate plants: -7%, dominant plants: -11%. $P < 0.05$, data not shown), defoliation reduced LAER to a slightly greater extent than LER in this species (Figure II.4). Compared at these new steady-states, LER was 27% lower than pre-defoliation values in dominant plants, and 36% lower in subordinate ones, while LAER was 31% and 42% lower, respectively.

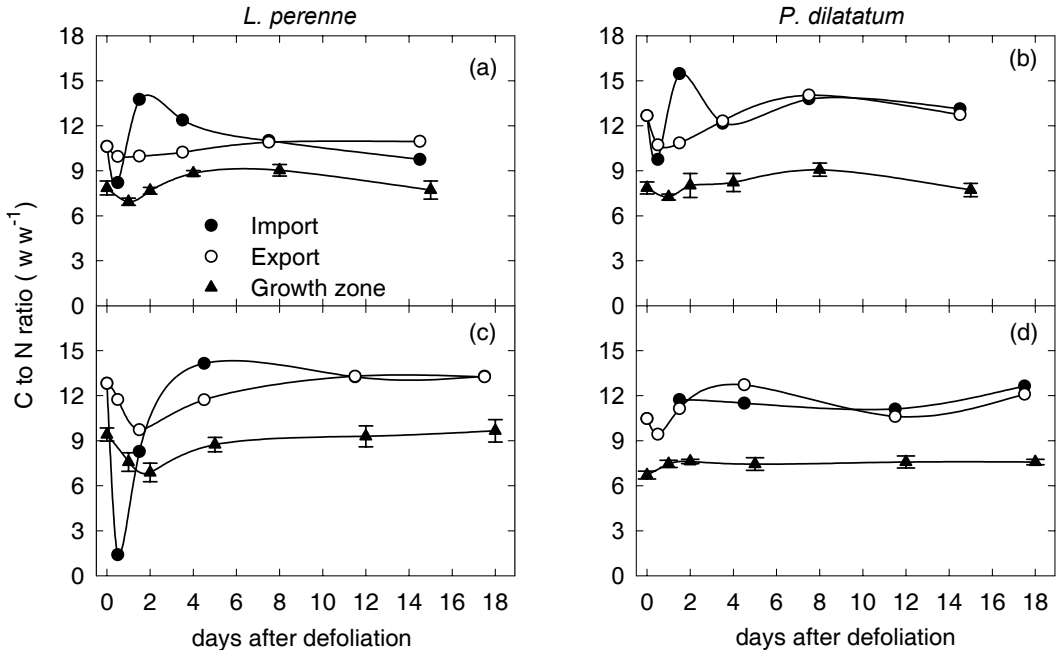


Figure II.9. C to N ratio (w $w^{-1}$) of imported substrates, newly exported tissue, and growth zone biomass in the most rapidly elongating leaf of *Lolium perenne* and *Paspalum dilatatum* tillers following severe defoliation. Growth conditions as in Figure II.4. Bars indicate ±1 SE (n=4 to 7).

Despite the general defoliation-response pattern observed in all treatments, distinct effects of species and growth condition were evident in the magnitude of these responses. First, decreases in growth zone C and N mass and changes in SLA of newly exported tissue were of greater magnitude and persisted for longer in dominant than subordinate plants, and in *L. perenne* than in *P. dilatatum*. Thus,

for example, C mass decreased by 47% in *L. perenne* at 15°C and by 28% in *P. dilatatum* at 23°C (dominant plants), but only by 19% in *L. perenne* at 23°C and 2% in *P. dilatatum* at 15°C (subordinate plants). These differential responses were detected as significant *defoliation* x *temperature regime* interactions ($P < 0.05$).

Secondly, although defoliation decreased C and N efflux to a similar extent in all treatments, the effects on their components differed (Eq. II.4). LER remained unaltered in dominant *L. perenne* plants, decreasing only after day 2. Conversely, LER reductions were immediate in subordinate *L. perenne*, and even more abrupt in both *P. dilatatum* treatments. Therefore, while shortly after defoliation lower efflux was largely associated with decreased LER in *P. dilatatum* plants, in dominant *L. perenne* it was entirely related to changes in SLA and $N_L$. Both components were affected in subordinate *L. perenne* plants. After day 5, lower LER was the main cause of lower tissue-bound efflux in all plants.

## DISCUSSION

### *Buffering of mature leaf area efflux from defoliation-induced decreases in substrate import*

Our data demonstrate a considerable short-term flexibility of the relationship between substrate import into the growth zone and mature leaf area production. During the first 2 d of regrowth, C import into the growth zone was reduced by 49% to 67% in the different treatments (Figure II.5). But leaf area expansion was maintained at rates equivalent to 56% to 101% of control plants (Figure II.4). Hence, the apparent efficiency of conversion of imported C into new leaf area ($L_A/I_C$) increased by 27% to 140%. Essentially the same response was observed for N, $L_A/I_N$ increasing by 16% to 76%. These responses were evident in both *L. perenne* and *P. dilatatum* plants growing in either dominant or subordinate positions within the mixtures, suggesting they are a general feature of the defoliation response of grasses.

Two mechanisms contributed to increase the ratio of mature leaf area produced per unit imported substrate. On one hand, C and N present in the growth zone at defoliation was mobilised, leading to negative balances for both elements (Figure II.7). On the other, less mass was deposited during tissue expansion and maturation, leading to increased SLA and decreased $N_L$ of newly exported tissue (Figure II.8). Mobilisation of growth zone biomass was the product of a transient phase (< 2 d) during

which import of C and N was reduced more than their efflux (Figure II.5 & Figure II.6). A negative growth zone mass balance could also result from the synthesis of tissue with lesser lineal density than the tissue being displaced out of the growth zone. However, the celerity (1 d) of the response means that it must have been due at least in part to mobilisation of non-structural material within the growth zone. Previous studies suggest this was mainly WSC, chiefly fructans in the case of *L. perenne* (Davidson & Milthorpe 1966; Morvan-Bertrand, Boucaud & Prud'homme 1999; Schnyder & de Visser 1999; Morvan-Bertrand *et al.* 2001). Processes underlying N mobilisation within the growth zone are less well understood than those of C compounds, although relatively small changes in soluble protein concentrations have been reported (Morvan-Bertrand *et al.* 1999; Morvan-Bertrand *et al.* 2001). Other labile pools such, as free amino-acids, might be important. Post-defoliation use of non-structural carbohydrates and soluble organic N within growth zones has not been analysed previously in $C_4$ grasses.

The second mechanism, alteration of the structural characteristics of produced tissue, operated over a longer period. Defoliation effects on SLA and $N_L$ of newly produced tissue have not been analysed before. But higher SLA and lower $N_L$ must result from decreases in either tissue density and/or tissue thickness (Witkowski & Lamont 1991). The immediate response likely involved changes in tissue density because tissue exported out of the growth zone during day 1 was mostly maturing –already expanded– tissue (Figure II.1). In the longer term, decreases of tissue thickness, associated to a general adaptation of leaf morphology (Groot, Neuteboom & Deinum 1999), may have also contributed to higher SLA and lower $N_L$ values.

The apparent efficiency of substrate conversion into mature leaf area started to recover towards pre-defoliation values soon after the initial response. By day 4 to day 5, the balance between substrate import and tissue-bound mass efflux was restored. But rates were 40% to 50% lower than pre-defoliation levels (Figure II.5, Figure II.6 & Figure II.7). By that time, production rate and structural characteristics of new tissue were also becoming stable (Figure II.4 & Figure II.8). The duration of this transitional phase roughly corresponds to the residence-time of cells within the growth zone (compare MacAdam *et al.* 1989; Schnyder *et al.* 1990). Consequently, mobilisation of C and N and initial responses in SLA, $N_L$ and LER were physiological responses of cells already present in the growth zone at defoliation (see also Schäufele & Schnyder 2001). But new steady-states in growth

zone and produced tissue state and process variables reflected the activity of cells produced after the defoliation event. Hence, persistent lower rates of substrate import and leaf area production, and thus sustained higher $L_A/I_C$ ratios, involved not only physiological but also morphological adjustments of the growth zone (*e.g.* cell number, Schäufele & Schnyder 2000).

***Buffering capacity is influenced by growth zone C status prior to defoliation***

Although growth zone responses to defoliation were similar in the different treatments, their magnitude differed. Thus, C import per unit mature leaf area produced during the first 2 d after defoliation decreased more in *L. perenne* than in *P. dilatatum* (-49% *vs.* -28%), and more when plants were dominant than when they were subordinate (-50% *vs.* -28%). Consequently, decreases in leaf area production were proportionally greater in *P. dilatatum* than in *L. perenne*, and in subordinate than in dominant plants (Figure II.4). These differences were closely correlated with the concentration of WSC in the growth zone at defoliation (Figure II.10). Notably, correlations were strong not only with the amount of mobilised C, as would be expected (*e.g.* Morvan-Bertrand *et al.* 1999), but also with changes in SLA. Growth zones with high WSC levels mobilised proportionally more C *and* increased proportionally more the SLA of produced tissue in response to defoliation (Figure II.10). These observations support a causal link between the buffering response and the initial growth zone C status. Further, they indicate longitudinal expansion was prioritised in detriment of processes determining SLA (*e.g.* axial expansion) when leaf growth confronted a decreased C supply after defoliation. In this context, a lesser scope for reserve mobilisation and for (further) increases of SLA would be expected when C import is largely supply-limited before defoliation. This was probably the cause of the lesser capacity to buffer leaf area expansion from decreases in substrate import of subordinate compared to dominant plants.

Temperate grasses store a large proportion of WSC in the growth zone as fructans (Volenec & Nelson 1984; Schnyder & Nelson 1989), which are rapidly and extensively mobilised in response to defoliation (Davidson & Milthorpe 1966; Volenec 1986; Morvan-Bertrand *et al.* 1999; Morvan-Bertrand *et al.* 2001). $C_4$ grasses do not synthesize fructan (Chatterton *et al.* 1989). Yet, leaf growth zones of $C_4$ grasses may contain starch (Dengler, Donnelly & Dengler 1996). Additionally, leaves are generally thinner in $C_4$ than in $C_3$ grasses (Dengler *et al.* 1994). Hence, the lesser magnitude of

responses in *P. dilatatum* compared to *L. perenne* may also reflect morphophysiological constraints inherent to $C_4$ species: a lack of C reserves compounds in the growth zone, and a leaf anatomy limiting the scope for adjustments in leaf thickness. Clearly, the roles of starch (in $C_4$ species) and of limits in the plasticity of SLA in leaf growth zone responses to defoliation need further study.

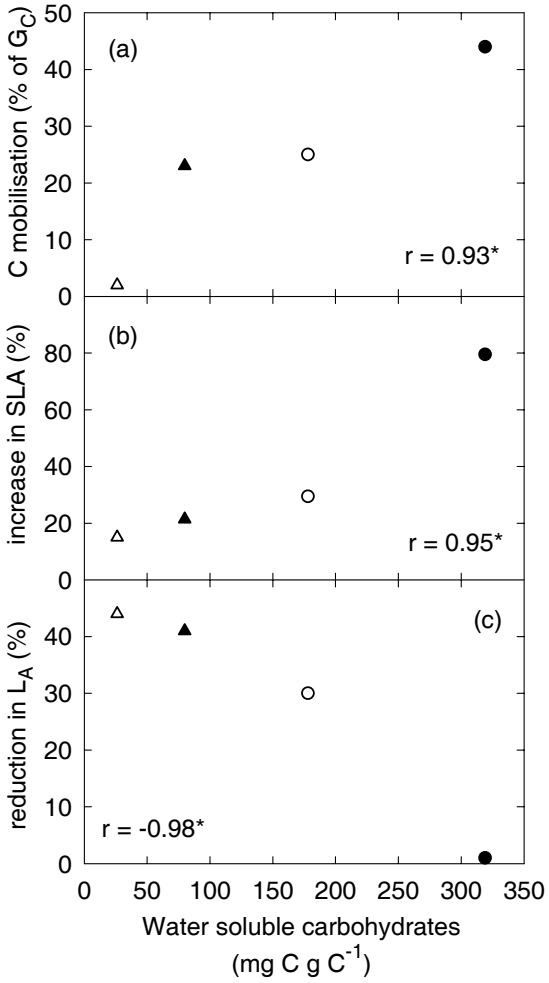


Figure II.10. Correlation between the concentration of water soluble carbohydrates (WSC) in the growth zone at defoliation and (a) C mobilisation, (b) increase in specific leaf area (SLA), and (c) reduction in leaf area production rate ($L_A$), expressed as a proportion (percentage) of initial values. Data points correspond to mean values ($n$ = 4 to 7). *Lolium perenne* (●○) and *Paspalum dilatatum* (▲Δ) plants grown in mixtures at 23°C and 15°C, and thus in hierarchically dominant (●▲) or subordinate (○Δ) positions. * indicates $P < 0.05$.

In conclusion, the present study revealed a general mechanism by which leaf area expansion and exposure is buffered from the effect of defoliation-induced decreases of substrate import into the growth zone. This mechanism involved short-term transient mobilisation of C and N present in the

growth zone, and more longer-term decreases of C and N deposition per unit produced leaf area. As it sustained refoliation, this mechanism contributed to a rapid re-establishment of plant photosynthetic activity. By reducing the demand of substrates for leaf growth, it would also contribute to a more rapid restoration of whole-plant growth and maintenance processes during the initial post-defoliation phase.

# Chapter III. THE SOURCES OF CARBON AND NITROGEN SUPPLYING LEAF GROWTH – ASSESSMENT OF THE ROLE OF STORES WITH COMPARTMENTAL MODELS [3]


## ABSTRACT

Patterns of synthesis and breakdown of C and N stores are relatively well known. But the role of mobilized stores as substrates for growth remains less clear. In this paper, a novel approach to estimate C and N import into leaf growth zones was coupled with steady-state labelling of photosynthesis ($^{13}CO_2/^{12}CO_2$) and N uptake ($^{15}NO_3^-/^{14}NO_3^-$), and compartmental modelling of tracer fluxes. The contributions of current C assimilation/N uptake and mobilization from stores to the substrate pool supplying leaf growth were then quantified in plants of a $C_3$ (*Lolium perenne* L.) and $C_4$ grass (*Paspalum dilatatum* Poir.) manipulated thus to have contrasting C assimilation and N uptake rates. In all cases, leaf growth relied largely on photoassimilates delivered either directly after fixation or short-term storage (turnover rate=1.6 to 3.3 $d^{-1}$). Long-term C stores (turnover rate<0.09 $d^{-1}$) were in general of limited relevance. Hence, no link was found between the role of stores and C acquisition rate. Short- (turnover rate=0.29 to 0.90 $d^{-1}$) and long-term stores (turnover rate<0.04 $d^{-1}$) supplied most N used in leaf growth. Compared to dominant (well-lit) plants, leaf growth in subordinate (shaded) plants relied more on mobilized N from long-term stores, being largely independent of external N. These differences correlated well with the ratio of C to N in growth-substrates, and were associated with responses in N uptake. Based on this, we argue internal regulation of N uptake act as a main determinant of the importance of mobilized long-term stores as source of N for leaf growth.


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## INTRODUCTION

Most plants store C and N. Stores are continuously built-up or mobilized, hence interacting directly with the provision and utilization of plant resources. Allocation of C and N towards, and mobilization from, storage pools has been described in many species, and the effects of changes in growth conditions on such patterns are relatively well known (Millard 1988; Chatterton *et al.* 1989; Chapin, Schulze & Mooney 1990; Volenec, Ourry & Joern 1996). Conversely, the role of mobilized stores in supplying plant C and N growth demand is less clear, particularly regarding vegetative growth of perennial species.

One reason is simply lack of information. Studies of the role of stores require accurate identification of substrates derived from distinct sources, which poses methodological problems. Steady-state labelling techniques proved particularly useful, allowing storage to be defined on a *time* basis and results interpreted in terms of use of resources present in the plant *before* and *after* labelling started (de Visser *et al.* 1997). This does not, however, provide a quantitative partitioning of the sources supplying growth. The reason is tracers often move through a number of pools before being incorporated into growing tissue, which diminishes their usefulness to discriminate specific sources. Analysis of tracers' time-course can help infer the number and kinetics of mixing pools (Moorby & Jarman 1975; Rocher & Prioul 1987). This approach, frequently applied for C and N fluxes in source systems, has received less attention in analyses of sources supplying sinks.

Another reason hindering the understanding of the role of stores is the absence of knowledge about putative ecophysiological determinants. Often, the relative importance of stores as source of substrates for growth tends to be associated with changes in mobilization intensity. However, it may depend as much on these as on responses of actual acquisition and demand rates (Bausenwein *et al.* 2001). Further, C and N metabolisms are closely interrelated: mobilization of organic N implies mobilization of amino-C, C status affects N mobilization and uptake (Thornton *et al.* 2002), and N status affects C assimilation and storage, and possibly mobilization too (Stitt & Krapp 1999). Such interdependent relationships –central to putative mechanisms controlling acquisition, storage, and mobilization of both C and N (Touraine, Clarkson & Muller 1994; Stitt & Krapp 1999; Farrar *et al.* 2000)– need to be integrated in analyses of the role of stores in supplying growth.

The aims of the present study were to develop a method able to distinguish and quantify the sources of C and N supplying leaf growth in grasses, and then use it to analyze the influence of plant status on the relative importance of those sources with the hypothesis that mobilization from stores is of greater importance for leaf growth when acquisition of C or N are more limited. In order to do this, a previously described method for estimating import of C and N substrates into the leaf growth zone (Figure III.1; Lattanzi, Schnyder & Thornton 2004 *i.e.* Chapter II), was further developed to account for labelled and non-labelled components, and coupled to steady-state labelling of C assimilation and N uptake. Time-course of tracer incorporation into imported C and N substrates was then analyzed with compartmental models, with which the size and turnover of the 'substrate' pool supplying leaf growth, and the contribution to it of current assimilation/uptake and mobilization from stores, were estimated.

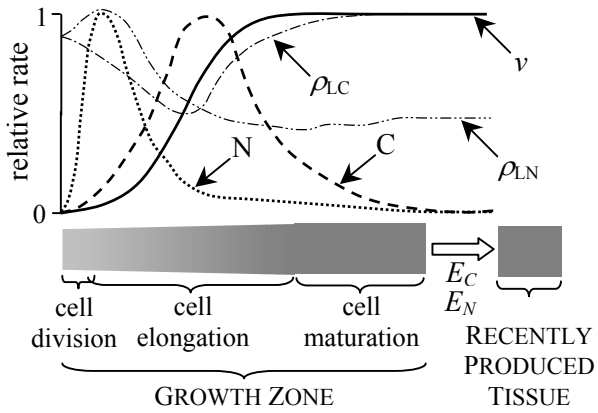


Figure III.1. The leaf growth zone of a grass tiller. Continuous production of cells at basal positions and their subsequent expansion gives rise to a flux of tissue and tissue-bound C and N out of the growth zone ($E_C$ & $E_N$). This export is counterbalanced by import (deposition) of C and N substrates in dividing, elongating and maturating cells. The growth zone can thus be conceived a 'place' where substrates are imported, transformed, and then exported as structurally and functionally differentiated tissue. The velocity of an element moving through the growth zone ($v$) increases until it equals the leaf elongation rate when cell elongation ceases. Lineal density of C and N ($\rho_{LC}$ & $\rho_{LN}$) may increase after cell expansion ceased, but becomes stable near the end of the cell maturation zone in leaves in steady-state growth. Consequently, $E_C$ and $E_N$ can be estimated as leaf elongation rate times C and N lineal density in recently produced tissue. In turn, import rates can be estimated by adding to $E_C$ and $E_N$ the (negative or positive) variation in growth zone C and N mass.

The relative contribution of these sources was evaluated in plants of a $C_3$ (*Lolium perenne* L.) and $C_4$ grass (*Paspalum dilatatum* Poir.) growing undisturbed in mixed stands. Contrasting $C_3/C_4$ balances of

the mixtures were brought about by moderately high (23°C) and low (15°C) temperature regimes. This exploited the differential growth-response of $C_3$ and $C_4$ species to temperature to place *L. perenne* and *P. dilatatum* plants in contrasting hierarchical positions within the stands, and thus produce 'dominant' and 'subordinate' individuals with different rates of C assimilation and N uptake (Table III.1, see also Lattanzi *et al.* 2004 *i.e.* Chapter II).

Table III.1. Plant status at beginning of labelling. *Lolium perenne* and *Paspalum dilatatum* plants grew undisturbed in mixed stands for either eight or ten weeks at 23°C or 15°C, respectively, and thus in dominant and subordinate hierarchical positions. $PPFD_{int}$ indicates the proportion of photosynthetic photon flux density intercepted by the $C_3$ and $C_4$ components within each stand. C assimilation and N uptake rates were measured over one light-period (12 h). Means ±SE (*n*=4 to 12).

| | Stand at 23°C | | Stand at 15°C | |
|---|---|---|---|---|
| | *L. perenne* subordinate | *P. dilatatum* dominant | *L. perenne* dominant | *P. dilatatum* subordinate |
| Height, mm | 485 ±12 | 571 ±14 | 349 ±4 | 255 ±7 |
| C mass, mg plant$^{-1}$ | 572 ±69 | 706 ±90 | 1103 ±108 | 81 ±9 |
| N mass, mg plant$^{-1}$ | 51 ±4.7 | 46 ±7.0 | 71 ±6.0 | 7.5 ±0.8 |
| Leaf expansion duration, d | 13 ±1.0 | 11 ±0.5 | 16 ±1.6 | 23 ±5.7 |
| Leaf life-span, d | 35 ±4.0 | 24 ±2.1 | 43 ±6.4 | 71 ±17 |
| Tillers per plant | 15 ±0.5 | 8 ±0.3 | 29 ±0.9 | 6 ±0.3 |
| $PPFD_{int}$, % | 23 | 77 | 93 | 7 |
| C assimilation, mg plant$^{-1}$ d$^{-1}$ | 25 ±10 | 78 ±17 | 90 ±12 | 3.7 ±1 |
| N uptake, mg plant$^{-1}$ d$^{-1}$ | 0.89 ±0.45 | 1.92 ±0.96 | 1.56 ±0.43 | 0.02 ±0.01 |

## MATERIALS AND METHODS

In the following, we will first describe the labelling facility employed, along with the plant material and growth conditions used to generate stands with contrasting $C_3/C_4$ balances. Then, we will detail the two labelling strategies (and associated sampling schemes) used, and formulae to calculate import of total and labelled C and N into the growth zone. Lastly, we will present the models used to describe tracer kinetics, the assumptions made in solving them, and their (partial) verification.

***$^{13}CO_2$/$^{12}CO_2$ labelling facility***

The equipment and principles involved have been detailed elsewhere (Schnyder *et al.* 2003). In brief, four growth cabinets (E15, Conviron, Winnipeg, Canada) were operated as open gas exchange systems. Air supply was generated by mixing $CO_2$-free air with $CO_2$ of known δ (δ, the deviation of the $^{13}C$ to $^{12}C$ ratio in $CO_2$ from that of the international standard, VPDB). Periodic adjustments of airflow and $CO_2$ concentration in the inlet maintained a constant $CO_2$ concentration of 300 μL $L^{-1}$ within the growth cabinet. Two growth cabinets received $^{13}C$-enriched $CO_2$ (δ -2.9‰, cabinets I & II), and two $^{13}C$-depleted $CO_2$ (δ -47.0‰, cabinets III & IV; both $CO_2$: Messer Griesheim, Frankfurt, Germany).

***Plant material and growth conditions***

Plant material and growth conditions have also been detailed previously (Lattanzi *et al.* 2004, *i.e.* Chapter II). Briefly, mixed *Lolium perenne*/*Paspalum dilatatum* stands were constructed by arranging 178 pots (i.d. 50 mm, length 350 mm) with one seedling of either *L. perenne* or *P. dilatatum* into plastic boxes (0.43 $m^2$). Two such stands were allocated to each of the four growth cabinet.

Plants grew under a 12 h photoperiod, with a photosynthetic photon flux density (PPFD) of 550 μmol photons $m^{-2}$ $s^{-1}$ at canopy height, provided by cool-white fluorescent lamps. Vapor pressure deficit was controlled at 0.5/0.3 kPa (light/dark) in all growth cabinets. An automated irrigation system watered the stands four times a day, flooding the boxes for 30 min with modified half-strength Hoagland's solution (105 mg N $L^{-1}$ supplied only as nitrate). Stands were periodically flushed with distilled water to prevent salt accumulation.

Canopy air temperature was set at 25°C/23°C (light/dark) in two growth cabinets (cabinets II & III), and at 15°C/14°C (light/dark) in the other two cabinets (cabinets I & IV). These temperature regimes resulted in daily average sand temperatures (15 mm below surface) of 23°C and 15°C, respectively.

After eight weeks of growth at 23°C, *P. dilatatum* individuals were larger and taller than their *L. perenne* neighbours. The opposite occurred in mixed stands at 15°C (Table 1). Within each mixture, larger individuals will be referred to as ‘dominant’ plants, the others being ‘subordinate’ plants.

***Partitioning of intercepted PPFD***

On day 0, after either eight (23°C) or ten weeks (15°C) of uninterrupted growth, PPFD and plant's leaf area were recorded at 15 cm increments from top to the bottom of the stands. Intercepted PPFD was then partitioned between the $C_3$ and $C_4$ grass by weighting the fraction of PPFD intercepted at each canopy stratum by the proportional contribution of each species to the leaf area present in that stratum.

***Leaf turnover***

Leaf expansion duration and leaf life-span were estimated from bi-weekly measurements of leaf appearance rate, number of growing leaves, and number of green leaves in a set of 12 tillers per treatment (Davies 1993).

***Labelling strategies and sampling times***

Plants were sampled in a series of six harvests at day 0, 1, 2, 4, 8 & 15 (23°C), or at day 0, 1, 2, 5, 12 & 18 (15°C). There were two labelling strategies associated with these harvests: assimilated C and absorbed N were labelled either *briefly* (last 12 h, *i.e.* the entire photoperiod prior to sampling) or *continuously* (since day 0) before sampling (Figure III.2). In both strategies, C labelling was performed by swapping individual plants between growth cabinets, which resulted in the exposure of plants grown under $^{13}$C-enriched $CO_2$ (δ -2.9‰) to $^{13}$C-depleted $CO_2$ (δ -47.0‰), and *vice versa*. In the case of N, the $^{15}$N-enrichment of the nutrient solution watering cabinets I & II was increased from 0.37 atom% (natural abundance) to 1.1 atom% immediately after the first harvest (Figure III.2).


Figure III.2. The relationship between labelling and sampling times for *briefly* (a) and *continuously* (b) labelled plants. Arrows indicate times of plants' transfers, dotted vertical lines indicate sampling

times (Harvests 1 to 6), and grey shades show the resulting periods over which assimilated C and absorbed N were labelled. All harvests were done immediately after lights went off. Black and white bars in the *time*-axis indicate dark and light periods, and the white asterisk indicates the time when the $^{15}N$-enrichment of the nutrient solution watering cabinets I & II was changed.

In the case of ***briefly* labelled plants**, four plants per species and temperature regime were swapped, during the dark period preceding the labelling photoperiod. Previously, plants were flushed with 0.5 L of distilled water. Importantly, labelling commenced only once lights went on. For C, the reason is obvious. For N, this was so because the first irrigation event after the plants' transfer was scheduled immediately before the start of the light period.

In the case of ***continuously* labelled plants**, 16 plants per species and temperature regime –different from *briefly* labelled ones– were swapped during the dark period after the first harvest: eight plants per species transferred from cabinet II to cabinet III, plus eight from cabinet III to cabinet II (23°C treatment), and the same for cabinets I and IV (15°C treatment, Figure III.2). Previously, stands were flooded with distilled water three successive times.

***Sample collection and analysis***

At each harvest, ten plants per treatment (five per growth cabinet) were sampled from the central part of the stands: four were *continuously* labelled plants, two were *briefly* labelled plants, and four were non-labelled (control) plants. In each plant, the *growth zone*, and an immediately adjacent piece of *recently produced tissue*, were dissected out of two to three growing leaves whose length was approximately half that of the youngest fully expanded leaf present in the tiller (Figure III.1, see Lattanzi *et al.* 2004, *i.e.* Chapter II, for details). The rest of the plant, including root and shoot, was pooled into one sample. After dissection, samples were heated to 100°C for one hour, dried during 48 h at 65°C, weighed and then milled. C and N content and $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios were determined on 1 mg dry matter aliquots using a CHN elemental analyzer (NA1500, Carlo Erba Strumentazione, Milan, Italy) interfaced to a continuous flow isotope mass ratio spectrometer (Delta plus, Finnigan MAT, Bremen, Germany). From isotope ratios, molar fractions were calculated (atom%, *A*). Standards were run every ten samples (SD=0.25‰ for δ, SD=0.0017% for $^{15}N$ atom%).

***Calculation of the proportion of tracer in a sample***

The proportion of C or N (X) tracer in a sample is directly proportional to the content of $^{13}C$ or $^{15}N$ in it. Therefore, the amount of $^{13}C$ or $^{15}N$ in each sample ($A_{spl\ X}$) was expressed as a lineal function of the fractions of labelled and unlabelled C or N ($f_{lab\ X}$ & $f_{unlab\ X}$; see the Appendix for definitions of symbols), and the $^{13}C$ or $^{15}N$ content of analogous samples from control plants ($A_{lab\ X}$ & $A_{unlab\ X}$),

(Eq. III.1a) $$A_{spl\ X} = f_{lab\ X} A_{lab\ X} + f_{unlab\ X} A_{unlab\ X}$$

For example, for a plant swapped from cabinet II to cabinet III, $A_{unlab\ C}$ and $A_{lab\ C}$ correspond to the amount of $^{13}C$ of samples taken from (control) plants continuously grown under $CO_2$ with δ -2.9‰ and $CO_2$ with δ -47.0‰, respectively. $A_{unlab\ C}$ and $A_{lab\ C}$ were determined, for each harvest date, in two plants per species and temperature regime. $A_{unlab\ N}$ was determined, for each harvest date, in four plants per species and temperature regime sampled from cabinets III & IV, *i.e.* irrigated with non-enriched nutrient solution. $A_{lab\ N}$ was not experimentally determined but assumed equal to 1.1%, *i.e.* the $^{15}N$ enrichment of the labelling solution. This implies eventual discrimination effects were ignored. Given the enrichment used, the error introduced in the measurement of $f_{lab\ N}$ was small: less than 1.0% for a 20‰ discrimination.

Since $f_{unlab\ X} = 1 - f_{lab\ X}$, (Eq. III.1a) can be solved for $f_{lab\ X}$

(Eq. III.1b) $$f_{lab\ X} = (A_{spl\ X} - A_{unlab\ X}) / (A_{lab\ X} - A_{unlab\ X})$$

The amount of labelled C or N was then calculated as C or N mass of the sample times the corresponding $f_{lab\ C}$ or $f_{lab\ N}$ value.

***Estimation of C assimilation and N uptake rates***

C assimilation and N uptake were estimated as the total amount of C and N tracer found in whole briefly labelled plants. For C, this is a measure related to daytime C gain, although respiration of non-labelled C is unaccounted for. In the case of N, this measure is close to a net balance between N influx and efflux over the 12 h light period.

***Estimation of import of labelled and non-labelled C and N into the leaf growth zone***

*Briefly labelled plants*

The amount of labelled C or N imported into the growth zone was estimated as the sum of labelled C or N present in the *growth zone* plus labelled C or N present in the piece of *recently produced tissue* (Figure III.1). Clearly, this assumes all imported tracer would still be present in these leaf segments at the end of the 12 h labelling period. This was likely true. On one side, deposition of C and N outside the growth zone is very small relative to deposition within the growth zone (Allard & Nelson 1991; Gastal & Nelson 1994; Maurice *et al.* 1997). On the other, the sampled piece of recently produced tissue corresponded to tissue exported during the last 16 to 19 h (Lattanzi *et al.* 2004, *i.e.* Chapter II). Therefore, all imported tracer would have been initially deposited within the growth zone, and any tracer exported out of the growth zone by tissue-bound efflux during the 12 h labelling period would still be present in the piece of recently produced tissue.

*Continuously labelled plants*

The simple estimation of import of labelled C and N in *briefly* labelled plants could not be extended to *continuously* labelled plants. This is because, after 16 – 19 h, part of the imported tracer would have left the "growth zone + recently produced tissue" segment due to tissue-bound efflux (Figure III.1). A new approach was therefore taken based on a previously presented method to estimate fluxes of C and N through leaf growth zones (Lattanzi *et al.* 2004, *i.e.* Chapter II).

Tissue-bound C and N export out of the growth zone ($E_X$) was estimated over 1 d intervals ($t_{i-1} - t_i$) as the product of leaf elongation rate ($<LER>$, where $<>$ denote 24 h averages) and the density of C and N per unit length in recently produced tissue ($<\rho_{LX}>$). Import of substrates into the growth zone ($<I_X>$) was then estimated by adding the (negative or positive) variation in mass of the growth zone over that time-interval ($G_{X \text{ at } t_i} - G_{X \text{ at } t_{i-1}}$). Thus, for a leaf elongating at 25 mm $d^{-1}$, whose growth zone has a constant mass of 4000 µg C, and whose recently produced tissue contains 80 µg C $mm^{-1}$, C import equals 2000 µg C $d^{-1}$. Respiration was ignored, thus underestimating actual C import by the amount lost through respiration. This measure of import is analogous to a net deposition rate (Allard & Nelson 1991; Maurice *et al.* 1997; Schäufele & Schnyder 2001). Essential prerequisites for the validity of these calculations are the specific definition of the growth stage of sampled leaves, and the precise

location and size of the piece of recently produced tissue where $<\rho_{LX}>$ is determined (see Lattanzi *et al.* 2004, *i.e.* Chapter II, for details and validation).

This method was further developed to estimate the labelled and non-labelled components of C and N fluxes. The amount of labelled C or N imported into the growth zone ($I_{lab\ X}$) was assessed for 1 d intervals as the export of labelled C or N ($E_{lab\ X}$) plus the variation in mass of labelled C or N within the growth zone ($G_{lab\ X}$) over the same time-interval,

(Eq. III.2) $$<I_{lab\ X}> = <E_{lab\ X}> + (G_{lab\ X\ at\ t_i} - G_{lab\ X\ at\ t_{i-1}})$$

Following the previous example, were $f_{lab\ E_X}$ 0.60, and $f_{lab\ G_X}$ 0.55 at $t_{i-1}$ and 0.65 at $t_i$, then import of labelled C would equal 1600 µg C $d^{-1}$ [2000 µg C $d^{-1}$ * 0.60 + 4000 µg C (0.65 – 0.55)]. The amount of non-labelled C or N imported into the growth zone ($I_{unlab\ X}$) is the difference $I_X$ - $I_{lab\ X}$ = 400 µg C $d^{-1}$ [2000 µg C $d^{-1}$ - 1600 µg C $d^{-1}$].

The fraction of labelled C or N in import ($f_{lab\ I_X} = I_{lab\ X}/I_X$) thus results,

(Eq. III.3a) $$<f_{lab\ I_X}> = <E_{lab\ X}> / <I_X> + (G_{lab\ X\ at\ t_i} - G_{lab\ X\ at\ t_{i-1}}) / <I_X>$$

Since $I_C$ and $I_N$, as well as $G_C$ and $G_N$, were constant in time (at a 1 d time-scale, Lattanzi *et al.* 2004, *i.e.* Chapter II), then $E_X = I_X$, and $G_{X\ at\ t_i} = G_{X\ at\ t_{i-1}}$. Hence, $f_{lab\ I_X}$ can be directly estimated as,

(Eq. III.3b) $$<f_{lab\ I_X}> = <f_{lab\ E_X}> + (f_{lab\ G_X\ at\ t_i} - f_{lab\ G_X\ at\ t_{i-1}})\ G_X / <I_X>$$

(Eq. III.3b) explicitly shows that estimation of $f_{lab\ I_X}$ over a 1 d time-interval ($t_{i-1} - t_i$) required estimation of (*i*) $f_{lab\ E_X}$ over the same time-interval, (*ii*) $f_{lab\ G_X}$ at the lower ($t_{i-1}$) and upper ($t_i$) ends of the time-interval, and (*iii*) $G_X / <I_X>$. The former was estimated as the definite integral of non-linear functions fitted to the time-course of the fraction of labelled *X* in samples of recently produced tissue ($RPT$, $<f_{lab\ E_X}> = \int_{i-1}^{i} f_{lab\ RPT_X}$). Similarly, non-linear functions fitted to the time-course of the $f_{lab\ G_X}$ were used to estimated values at $t_{i-1}$ and $t_i$. Values of $G_X / <I_X>$ were taken from Lattanzi *et al.* (2004) (*i.e.* Chapter II).

The five specific 1 d intervals over which $<f_{lab\ I_X}>$ was estimated were defined by the six harvest dates as follows: days 0–1, 1–2, 3–4, 7–8, &20 14–15, and days 0–1, 1–2, 4–5, 11–12, & 17–18 for the 23°C and the 15°C temperature regimes, respectively. To test the accuracy of the method, $<f_{lab\ I_C}>$ and $<f_{lab\ I_N}>$ values estimated through (Eq. III.3b) were compared with those directly measured in sets of *briefly* labelled plants. The regression of estimated *vs.* observed values did not differ from the $y = x$ line ($P > 0.10$, Figure III.3).

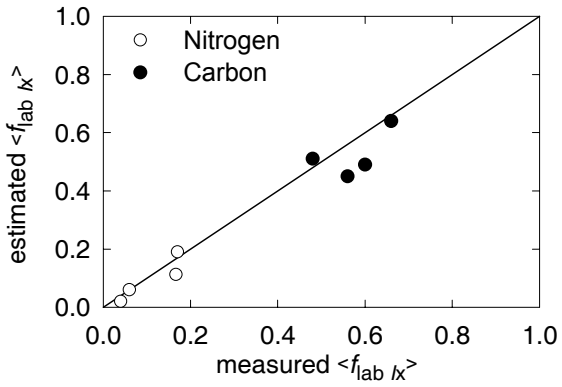


Figure III.3. Comparison between values of the fraction of labelled C or N imported into the growth zone (<$f_{lab\ I_X}$>) measured in *briefly* labelled plants and estimated using (Eq. III.3b). The line indicates the $y = x$ relationship.

***Modelling of time-course of C and N tracer imported into the growth zone***

We propose a two-pool model to describe the time-course of the incorporation of tracer into the growth zone (Figure III.4a), which is formally similar to that used in compartmental analyses of C export from source leaves (*e.g.* Moorby & Jarman 1975; Rocher & Prioul 1987).

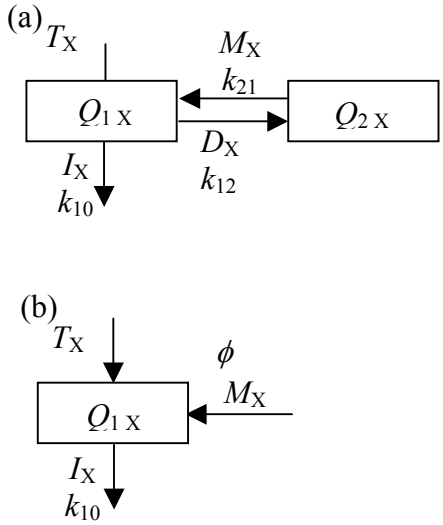


Figure III.4. Two- (a) and one-pool models (b) of sources supplying leaf growth. Newly acquired (*i.e.* labelled) carbon and nitrogen ($X$) first enter $Q_1$ ($T_X$ ). From there, they are either imported into the growth zone ($I_X$), or exchanged with $Q_2$ through deposition ($D_X$) and mobilization ($M_X$). In solving the model, steady-state and first-order kinetics were assumed –that is, pools' size are constant in time, and fluxes are the product of pool size times a rate constant ($k_{10}$, $k_{12}$ & $k_{21}$, numbers referring to donor and receptor pools, respectively). In the one-pool model, $\phi$ is the fraction of non-labelled C or N entering the system, $M_X/(M_X+T_X)$; *i.e.* the proportional contribution of $M_X$ in supplying $Q_1$. In the two-pool model, this corresponds to $k_{12}/(k_{10}+k_{12})$, the average probability for an atom of

being exchanged through $Q_2$ before its import into the growth zone. Models were run with a 0.1 d time-step, but predicted values were then integrated to give 1 d averages. Parameters –estimated by iteratively minimizing the sum of squared errors– were used, along with the rate of total C and N import into the growth zone, to estimate pools' size, turnover rate (the ratio of atoms passing through relative to the total amount present in each pool), and half-time ($t_{0.5}$, the average time an atom will reside in a pool = 0.693/turnover rate). In the two-pools model, the turnover rate of $Q_1$ is $k_{10}+k_{12}$, while turnover rate of $Q_2$ is simply $k_{21}$. In the one-pool model, the turnover rate of the only pool is $k_{10}$.

Newly acquired (*i.e.* labelled) C and N first enter $Q_1$. From there, C and N can be either imported into the growth zone or exchanged with $Q_2$. Assuming first-order kinetics –that is, fluxes are the product of pools size times a rate constant ($k_{10}$, $k_{12}$ & $k_{21}$, numbers referring to donor and receptor pools)– the rate of change of each compartment with respect to time ($t$) is given by,

(Eq. III.4a) $$dQ_1/dt = T + k_{21}\, Q_2 - (k_{10} + k_{12})\, Q_1$$

(Eq. III.4b) $$dQ_2/dt = k_{12}\, Q_1 - k_{21}\, Q_2$$

Assuming the system is in steady-state –that is, $dQ_1/dt = dQ_2/dt = 0$– pools' sizes are given by,

(Eq. III.5a) $$Q_1 = T / k_{10}$$

(Eq. III.5b) $$Q_2 = (T / k_{10})\, (k_{12} / k_{21})$$

and fluxes by,

(Eq. III.6a) $$T = I$$

(Eq. III.6b) $$M = k_{21}\, Q_2 = I\, (k_{12} / k_{10}) = k_{12}\, Q_1 = D$$

where $I$ is the measured import rate, and $k_{10}$, $k_{12}$, $k_{21}$ are fitted parameters.

In some cases, a simpler one-pool model was proposed, in which labelled and non-labelled C and N enter $Q_1$ and from there are imported into the growth zone (Figure III.4**Error! Reference source not found.**b). Hence, the one-pool model is the special case of the former where $Q_2$ becomes infinitely large and $k_{21}$ infinitely small.

Assuming first-order kinetics,

(Eq. III.7) $$dQ_1/dt = T + M - k_{10}\, Q_1$$

Then, under steady-state assumption,

(Eq. III.8) $$Q_1 = T / k_{10}$$

(Eq. III.9a) $$T = I\, \phi$$

(Eq. III.9b) $$M = I\, (1-\phi)$$

where $I$ is the measured import rate, and $k_{10}$ and $\phi$ are fitted parameters. Note that $\phi$ equals $k_{12}/(k_{10}+k_{12})$ in the two-pools model.

Models were implemented in MODELMAKER® (v 4.0, Cherwell Scientific Ltd., UK), a software to simulate dynamic models. Differential equations were solved using the 4th-order Runge-Kutta numerical method, with a step-size of 0.01 d. Predicted $f_{\text{lab } I_C}$ and $f_{\text{lab } I_N}$ were integrated over 1 d intervals, thus to produce data comparable to measured values (*i.e.* $<f_{\text{lab } I_C}>$ & $<f_{\text{lab } I_N}>$). A built-in optimization function was used to estimate the values of fitted parameters by iterative minimization of the sum of squared differences between measured and predicted $<f_{\text{lab } I_C}>$ & $<f_{\text{lab } I_N}>$.

*Verification of assumptions*

The models hold the assumptions customary of compartmental analyses. Two are explicit: (*i*) the system is steady, (*ii*) fluxes obey first-order kinetics. Besides, it is implicitly assumed that (*iii*) pools are homogeneous and well-mixed, and (*iv*) $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic discrimination in exchanges can be neglected.

Regarding the first assumption, previously presented data from this same experiment (Lattanzi *et al.* 2004) and from other studies (Allard & Nelson 1991; Gastal & Nelson 1994) show that growth of the most rapidly elongating leaf had indeed a manifest steady-state dynamics when considered at 1 d intervals. Leaf elongation proceeded at a steady rate, and variations in $\rho_{LC}$ and $\rho_{LN}$ and in $G_C$ and $G_N$ were minor, hence $E_C$ & $E_N$, and $I_C$ & $I_N$, were virtually constant (Lattanzi *et al.* 2004, *i.e.* Chapter II). Further, import of C and N assimilated/absorbed over the 12 h light period –*i.e.* import of labelled C and N in *briefly* labelled plants– was approximately constant. Therefore, not only total C and N import but also their source-composition appeared stable (see below). It should be stressed that steady-state is here defined at a 1 d time-scale. Diurnal cycles most likely occurred (Yoneyama *et al.* 1987; Schnyder & Nelson 1988b) and are not considered by the model.

Eventual effects of non-continuous incorporation of tracer, arising from diurnal cycles in C assimilation/N uptake, were assessed assuming import of C and N into the growth zone ($I_X$) constant over the day, but tracer import ($T_X$) occurring only over the 12 h light periods. Optimized solutions indicated consistent reductions of about 25% in size and half-time of $Q_{1C}$ in all modelled situations. However, these were unstable, probably due to a limited dataset. Including a similar diel cycle in N

uptake had no effect upon $Q_{1N}$ parameters. Since general responses were unchanged, and because of a lack of quantitative support for eventual day/night changes in C and N import rates, we chose to retain the previous optimized values, observing that $Q_{1C}$ size and half-time would be overestimated.

Assumption (*ii*) is, in a strict sense, probably false. However, support for its practical validity has been found repeatedly (Prosser & Farrar 1981; Yoneyama *et al.* 1987; see Farrar 1990 for a discussion).

Assumption (*iii*) is perhaps the models' most drastic simplification. Probably, neither $Q_1$ nor $Q_2$ are homogeneous and well-mixed pools, but comprise a set of biochemically and/or spatially distinct compartments. Further compartmentalization, however, did not improve goodness of fit of the model, although this may reflect a limited number of data points, or a short time-span of the experiment.

Assumption (*iv*) was most likely true, for fractionation during C and N transport and conversion are small compared to $^{13}C$ and $^{15}N$ enrichments used.

***Error estimation and statistics***

The SE associated with the determination of the total and proportional amount of labelled and non-labelled C or N in the flux imported into the growth zone was estimated by Gaussian error propagation. Following Eq. (3b), the SE of $<f_{\text{lab } I_X}>$ includes the SE $<f_{\text{lab } E_X}>$, the SE of the a difference in $f_{\text{lab } G_X}$, and the SE of $G_X/<I_X>$ : SE of $<f_{\text{lab } I_X}>$ = SE $<f_{\text{lab } E_X}>$ + 2 SE $f_{\text{lab } G_X}$ $G_X/<I_X>$ + $\Delta f_{\text{lab } G_X}$ $SE_{G_X/I_X}$.

Models were assessed by, and selection of alternative models based on ANOVA. Ideally, partitioning the residual mean square into 'lack of fit' and 'pure error' terms would provide an objective basis for choosing between alternative models. In the present case, however, lack of true time replicated $<f_{\text{lab}}>$ values prevented this. Hence, the two-pool model was preferred over the one-pool model whenever it reduced residual mean square at least by half. Importantly, in the present case, lack of time replication does not imply repeated measures, for variables were estimated on different experimental units (*i.e.* plants) at different times.

In non-linear regression, the SE of a fitted parameter is of very limited value in assessing its significance. This is because the usually non-normal distribution of errors renders strongly asymmetric confidence intervals. Thus, SE of fitted parameters in the one- and two-pools models (*i.e.* rate constants and $\phi$) are given only for informative purposes. Rather, their usefulness resides in their use,

along with the correlation matrix, to compute the SE of predicted $<f_{lab}>$ values and of derived quantities biologically meaningful, such as $Q_1$ and $Q_2$ sizes and half-times, and of the contribution of $M_X$ to $I_X$ ($\phi = M_X/(T_X+M_X)$) (see Ross 1981 for a discussion). Note that SE of $Q_1$ and $Q_2$ sizes also involved SE of total C and N import.

## RESULTS

### *Import of carbon and nitrogen into the growth zone, and tracer import in briefly labelled plants*

Sequential analysis of leaf elongation rate and of lineal density of C and N along the immature part of leaves showed that import of C and N into the leaf growth zone ($<I_C>$ & $<I_N>$; see the Appendix for symbols definition) was approximately constant along the experimental period. This was true for both species (*L. perenne* and *P. dilatatum*) and growth conditions (mixed stands at 23°C and 15°C). At several times during the experimental period, C assimilation and N uptake were labelled over the photoperiod immediately preceding sampling (*briefly* labelled plants). This demonstrated that the contributions of recent C assimilation and N uptake were also constant during the experimental period (Figure III.5). Such a constancy suggests a system in steady-state with respect to fluxes of C and N, and the contributions of recently assimilated C and absorbed N.

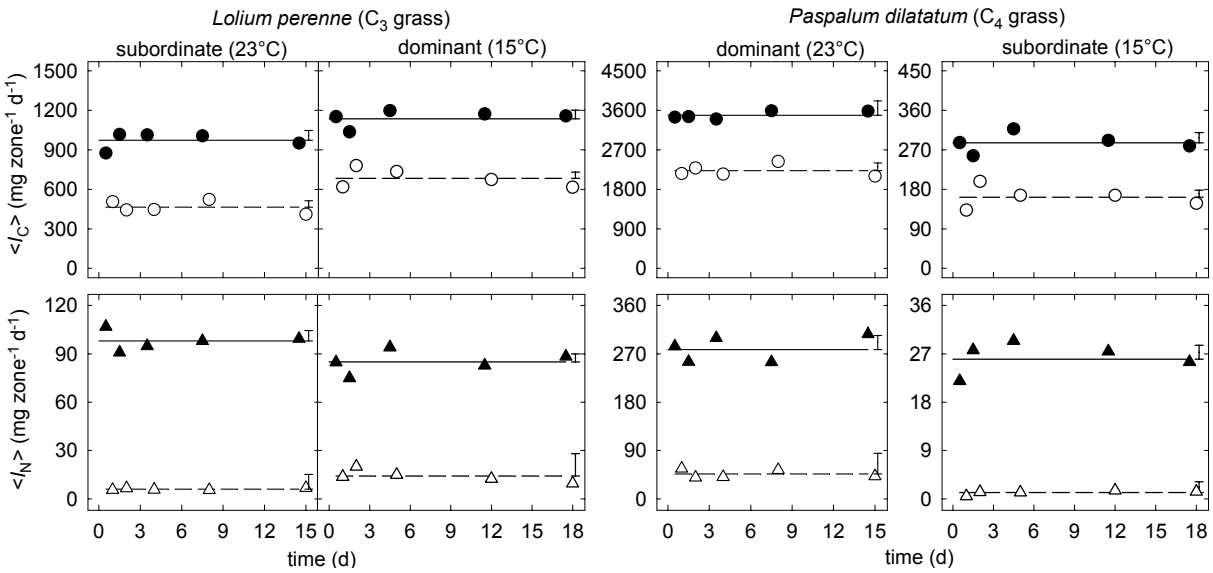


Figure III.5. Import of total (●▲) and *briefly* labelled (Δ○) carbon ($<I_C>$, ●○) and nitrogen ($<I_N>$, ▲Δ) substrates into the leaf growth zone. Growth conditions as for Table III.1. Lines indicate average values. Error bars are 1 SE of import values.

Besides their stability, a remarkable feature of the results was the contrasting behaviour of C and N substrates. Between 48 and 64% of the C imported over a day (*i.e.* 24 h) derived from C assimilated during the previous 12 h light period. Conversely, N taken up over the same period contributed only 3 to 17% to total daily N import. Therefore, in all plants, the relationship between resource acquisition and use was much tighter for C than N substrates.

### *Modelling of tracer time-course in* continuously *labelled plants*

*Continuous* steady-state labelling was applied to follow the saturation kinetics of label in the different C and N pools which served as sources for leaf growth. Continuous labelling thus meant that C and N tracer entered the plants over the whole 15- (23°C) or 18-day experimental period (15°C). The results demonstrated that the proportion of tracer in imported C and N substrates ($<f_{\text{lab } I_C}>$ & $<f_{\text{lab } I_N}>$) was not stable as in *briefly* labelled plants, but characterized by rapid increases followed by either slower increases or complete stabilization (Figure III.6).

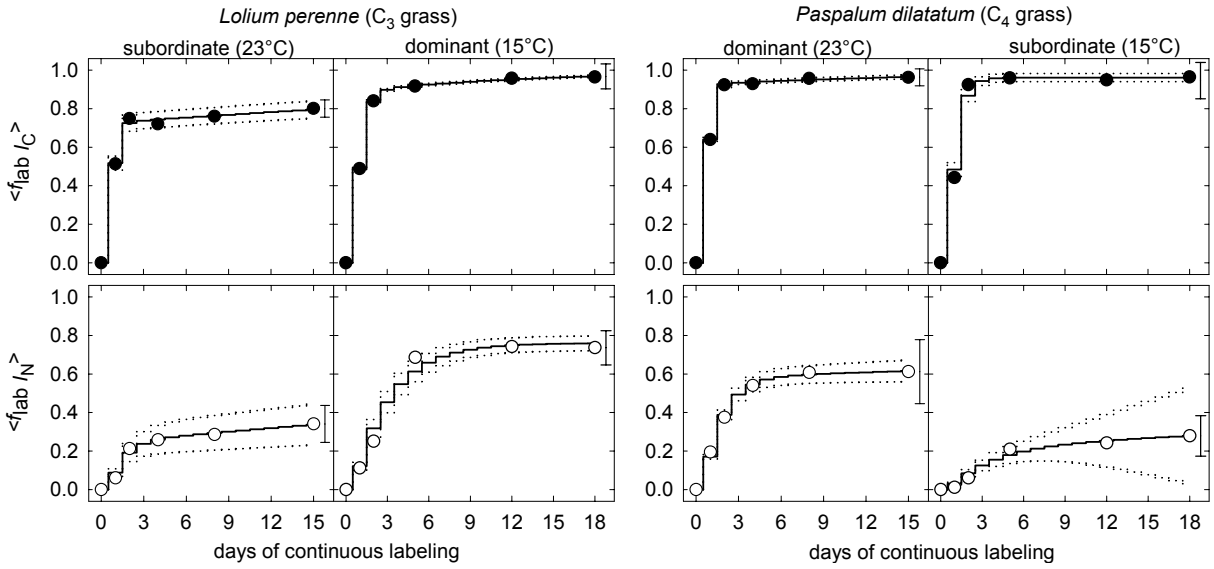


Figure III.6. The proportion of labelled carbon (●, $<f_{\text{lab } I_C}>$) and nitrogen (○, $<f_{\text{lab } I_N}>$) in substrates imported into the leaf growth zone of *continuously* labelled plants estimated with Eq. (3b). Growth conditions as for Table III.1. Lines show models' predictions (—) ±SE of predicted values (---). Error bars indicate ±SE of estimated values.

In six out of the eight situations, a two-pools model adequately described this biphasic pattern. The model consisted of a 'substrate' pool ($Q_1$) supplying the growth zone. $Q_1$ was in turn supplied by

newly acquired C or N, and by mobilization from a 'storage' pool ($Q_2$). Mass transfers were governed by first-order rate constants ($k_{10}$, $k_{12}$ & $k_{21}$, numbers referring to donor and receiver pools, respectively). In two cases ($<f_{\text{lab } I_C}>$ in *P. dilatatum* subordinate & $<f_{\text{lab } I_N}>$ in *L. perenne* dominant plants), the data fitted well to a one-pool model, a reduced case of the former where $k_{21}$ becomes infinitely small (Figure III.4**Error! Reference source not found.**). Optimized and derived model parameters are given in Table III.2.

Very generally, models described well the time-course of $<f_{\text{lab } I_C}>$ & $<f_{\text{lab } I_N}>$, as indicated by the close agreement, in all situations, of values estimated by Eq. (3b) and model predictions (Figure III.7). SE of predicted values (Figure III.6), as well as of derived parameters (Table III.2), were within reasonable limits, with coefficients of variation below 20% for $Q_1$, and close to 35% for $Q_{2C}$. However, $k_{21\,N}$ had comparatively large SE, which clearly reflected on the SE of its derived parameter, $Q_{2N}$ (Table III.2).

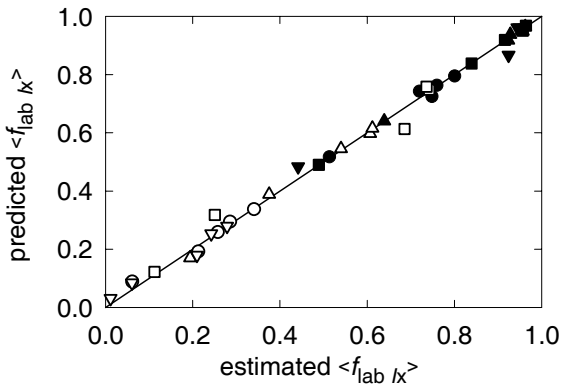


Figure III.7. Comparison between estimated [Eq. (3b)] and predicted (model) values of the fraction of labelled C ($<f_{\text{lab } I_C}>$, ●■▲▼) and N into the growth zone ($<f_{\text{lab } I_N}>$, ○□Δ∇) for *continuously* labelled *L. perenne* (●○■□) and *P. dilatatum* plants (▲Δ▼∇) at 23°C (●○▲Δ) or 15°C (■□▼∇). Growth conditions as for Table III.1. The line indicates the $y = x$ relationship.

Table III.2. Model optimization results. Total carbon or nitrogen import into the growth zone ($I_X$) and first-order rate constants ($k_{10}$, $k_{12}$ & $k_{21}$, numbers refer to donor and receptor pools, respectively) fitted to two- or one-pool models describing the time-course of carbon and nitrogen tracer incorporation into substrates supplying leaf growth (Figure III.4). Derived parameters: pools' size ($Q_1$ & $Q_2$) and half-time ($t_{0.5}$), and the contribution of $M_X$ to $I_X$ [$\phi = M_X/(M_X+T_X)$]. Means ±SE ($n=6$). For comparison, $Q_1$ and $Q_2$ are shown as proportion of tiller mass, and of C assimilation and N uptake rates. Growth conditions as for Table III.1.

| | | *L. perenne* | | *P. dilatatum* | | *L. perenne* | | *P. dilatatum* | |
|---|---|---|---|---|---|---|---|---|---|
| | | 23°C subordinate | 15°C dominant | 23°C dominant | 15°C subordinate | 23°C subordinate | 15°C dominant | 23°C dominant | 15°C subordinate |
| | | ..... carbon ..... | | | | ..... nitrogen ..... | | | |
| $I$ | µg d$^{-1}$ | 972 ±75 | 1136 ±66 | 3488 ±327 | 286 ±23 | 98 ±6.5 | 85 ±5.0 | 278 ±26.4 | 26 ±2.6 |
| $k_{10}$ | d$^{-1}$ | 2.4 ±0.29 | 1.7 ±0.031 | 2.8 ±0.10 | 1.6 ±0.19 | 0.23 ±0.054 | 0.37 ±0.066 | 0.43 ±0.034 | 0.071 ±0.015 |
| $k_{12}$ | d$^{-1}$ | 0.91 ±0.16 | 0.19 ±0.016 | 0.22 ±0.031 | - | 0.66 ±0.24 | - | 0.30 ±0.051 | 0.22 ±0.067 |
| $k_{21}$ | d$^{-1}$ | 0.028 ±0.013 | 0.080 ±0.011 | 0.055 ±0.020 | - | 0.035 ±0.023 | - | 0.010 ±0.013 | 0.020 ±0.14 |
| $Q_1$ | µg | 401 ±57 | 681 ±42 | 1224 ±123 | 177 ±25 | 419 ±101 | 232 ±44 | 645 ±79 | 369 ±89 |
| $Q_2$ | µg | 12976 ±6771 | 1628 ±273 | 4939 ±2008 | - | 7879 ±6147 | - | 19485 ±25853 | 4037 ±27807 |
| $t_{0.5}$ $Q_1$ | d | 0.21 ±0.02 | 0.37 ±0.01 | 0.23 ±0.01 | 0.43 ±0.05 | 0.77 ±0.21 | 1.89 ±0.34 | 0.95 ±0.08 | 2.39 ±0.57 |
| $t_{0.5}$ $Q_2$ | d | 25 ±12 | 9 ±1.1 | 13 ±4.7 | - | 20 ±13 | - | 69 ±91 | 35 ±238 |
| $\phi$ | % | 27 ±4.3 | 10 ±0.8 | 7 ±1.0 | 4 ±2.1 | 74 ±8.4 | 24 ±3.8 | 41 ±4.5 | 76 ±6.9 |
| $Q_1$/tiller | | 0.011 | 0.018 | 0.014 | 0.013 | 0.12 | 0.10 | 0.11 | 0.29 |
| Acquisition/$Q_1$ | | 4.2 | 4.6 | 8.0 | 3.4 | 0.14 | 0.23 | 0.37 | 0.01 |
| $Q_2$/tiller | | 0.34 | 0.04 | 0.06 | - | 2.3 | - | 3.4 | 3.2 |

*Model validation*

Formally analogous models able to identify $Q_1$ and $Q_2$ with biochemically- and spatially-defined metabolites have been evaluated by comparing predicted and estimated values (*e.g.* sucrose in leaves, Rocher & Prioul 1987). In the present study, the system was too complex to expect such direct correspondences, because there are several intermediaries with unknown spatial compartmentalization between $CO_2$ assimilation/$NO_3^-$ uptake and subsequent import of C and N into the leaf growth zone, mainly as sucrose and amino acids (Schnyder & Nelson 1988b; Gastal & Nelson 1994).

As an alternative, sizes of $Q_1$ and $Q_2$ were compared to tiller average C and N mass. This revealed $Q_{1C}$ represented between 1.1 and 1.8% of tiller C mass. Since $I_C$ was underestimated due to unaccounted respiration, $Q_{1C}$ values should have been ~30% higher (Volenec & Nelson 1984). Conversely, $Q_{1N}$ represented 10 and 30% of tiller N mass (Table III.2).

Thus, the smaller relative size of $Q_{1C}$ than $Q_{1N}$ was a common feature in all plants. But the magnitude of this difference varied greatly. Dominant plants had relatively larger $Q_{1C}$ than subordinate plants. The opposite was true for $Q_{1N}$. As a result, the ratio of $Q_{1C}$ to $Q_{1N}$ ($Q_{1C:N}$) discriminated well subordinate from dominant plants (1.0 *vs.* 2.9 in *L. perenne*; 0.5 *vs.* 1.9 in *P. dilatatum*). Strictly comparable data is not available from other studies. However, from data of Farrar (1990) it is possible to calculate that the pool supplying C for shoot growth in three $C_3$ grasses constituted between 1.4 and 5.8% of plant C mass. Similarly, Yoneyama *et al.* (1987) provide enough data to compute a pool supplying protein synthesis in *Brassica campestris* leaves equal to 14% of total plant N.

$Q_2$ values were reasonable for C, though somewhat high in subordinate *L. perenne* plants. But $Q_{2N}$ values were two- to three-times greater than average N mass of tillers (Table III.2). Clearly, despite the models' good statistical behaviour, $Q_{2N}$ values were unrealistic. Knowledge of N cycling within grasses indicates that a delay might occur between tracer incorporation, *e.g.* into growing tissue, and its subsequent mobilization, *e.g.* from senescing tissue (Robson & Deacon 1978; Millard 1988; Thomas 1990). This would result in a non-uniform distribution of tracer within $Q_2$. To test whether such a divergence from assumed homogeneous well-mixed pools would affect estimated pools' size, a lag-time between tracer incorporation in $Q_2$ and subsequent mobilization was included in the model. Optimization yielded lag-times close to 14 d for plants at 23°C, and of 16.7 d for plants at 15°C. These values were close to leaf expansion durations (Table III.1), which agrees with net N mobilization

starting near the time a leaf is fully expanded (Robson & Deacon 1978). Consideration of a delay strongly reduced the size of $Q_{2N}$ to 74 to 87% of tiller N mass (*c.f.* Table 2). Importantly, the lag-time had only minor effects on size of $Q_1$ (<3%), and on the contribution of mobilization from $Q_{2N}$ ($\phi$, <4%).

Taken together, these results indicate $Q_1$ size and half-time, and $\phi$, *i.e.* parameters derived from $k_{10}$ and $k_{12}$, were adequately estimated. The small sensitivity to eventual violations of non-verified assumptions, which was due to a high correlation between $k_{10}$ and $k_{12}$ (C: r=0.77, N: r=0.91), lends further confidence to these estimations (see Ross 1981). On the other hand, $Q_2$ size and half-time were less accurately estimated, particularly for N, and should therefore be interpreted with more caution. The existence of a lag-time between tracer deposition and mobilization in $Q_2$ seems worth of further study, but data at longer time-scales are necessary for stable model solutions.

***The age of imported substrates, and the sources supplying them***

Models were used to determine the time elapsed between C and N tracer entering the plant and its arrival at the growth zone. This yields an age-profile of imported substrates, where import of non-labelled C or N at the end of the experimental period can be described as older than 15 d (23°C) or 18 d (15°C). Additionally, in the two-pools model, the total amount of C and N tracer derived from each days' assimilation/uptake was further separated into the fraction derived from mobilization from $Q_2$ and that only cycling through $Q_1$ (Figure III.8).

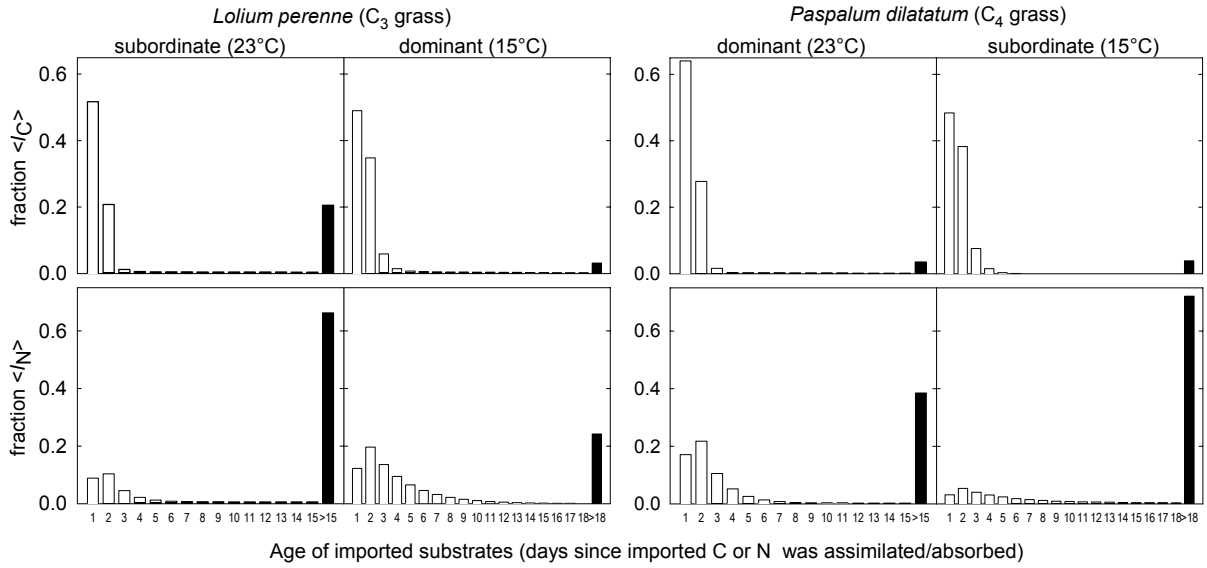


Figure III.8. Age of substrates imported into the leaf growth zone. Import of carbon and nitrogen (<$I_C$> & <$I_N$>) is shown as a function of the number of days elapsed between assimilation/uptake and subsequent deposition in the growth zone. For each day, the white part of the bar indicate substrates that cycled only through $Q_1$, while the dark part refers to substrates exchanged with $Q_2$ prior to import (see Figure III.4). Growth conditions as for Table III.1.

In all treatments, imported C derived mostly from very recent photosynthesis. A high proportion, 50 to 65%, corresponded to C that reached the growth zone within 12 h of entering the plant (day 1). The remaining C was chiefly imported during the next day. Opposite to C results, the importance of very recent N uptake was small: N derived from same day uptake (day 1) represented less than one-sixth of total imported N. Imported N derived in more or less similar proportions from last 3- (23°C) to 5-days uptake (15°C). Since virtually all this C and N cycled only through $Q_1$, this pool effectively acted as a short-term store; 30% to 50% of $Q_1$-derived C, and 66 to 87% of $Q_{1N}$-derived N, were imported after 12 h of its assimilation/uptake (*c.f.* Figure III.8). Since most of this C was imported over the dark and light periods following its assimilation, stores within $Q_{1C}$ probably served to buffer day/night cycles. In the case of N, with half-times greater then 0.8 d, storage within $Q_{1N}$ would be also involved in moderating day-to-day variations in supply.

Mobilization from $Q_2$ supplied less than 10% of imported C. $Q_{2C}$ half-times, ~10 d, were close to leaf expansion duration (Table III.1). The exception were subordinate *L. perenne* plants, where $Q_2$ provided 27% of imported C and had a half-time twice as long, and more similar to that of $Q_{2N}$ (Table III.2). Conversely, the contribution of mobilized N was variable, but relevant in all plants (24 to 75%). In subordinate plants, N mobilized from $Q_2$ clearly was the preponderant source, supplying three-

quarters of imported N. In dominant plants, it was more important in the $C_4$ than in the $C_3$ grass (Table III.2). Half-times of $Q_{2N}$ were relatively long, but these must be interpreted with caution, for they implied unrealistically large pools' sizes. Consideration of a lag-time, which could have caused such behaviour, reduced half-times to 8 d (subordinate *L. perenne*), 10 d (subordinate *P. dilatatum*) and 19 d (dominant *P. dilatatum*) (*c.f.* Table III.2). When added to the 14 to 16 d lag-times, these values become closer to leaf life-spans (Table III.1).

## DISCUSSION

### *On the approach*

This paper presents a novel approach for analyzing the contribution of distinct sources in supplying C and N substrates for growth. It involves three steps: (*i*) estimating C and N import into growth zones, (*ii*) determining their labelled fraction under steady-state labelling of C assimilation and N uptake, and (*iii*) analyzing the time-course of these fractions with compartmental models. The first is an extension of a previously presented method, based on well-established knowledge of C and N fluxes within growth zones (Lattanzi *et al.* 2004, *i.e.* Chapter II). Steady-state $^{13}CO_2/^{12}CO_2$ and $^{15}N/^{14}N$ labelling (de Visser *et al.* 1997; Schnyder *et al.* 2003), as well as modelling of tracer time-courses (Moorby & Jarman 1975; Prosser & Farrar 1981), are established techniques too. New is their concerted use to quantitatively resolve the sources supplying growth.

Compared to prior labelling studies, this approach imports several advancements. First, deposition of C and N in the growth zone is solely driven by the demand of dividing, expanding and maturing cells (Schnyder & Nelson 1988b; Allard & Nelson 1991; Gastal & Nelson 1994). Hence, confounding effects of processes others than these, which also affect tracer content of leaves but bear no direct association with their growth –*e.g.* carbohydrate and amino acids storage in either growing, mature or senescent leaves– are obviated.

The second advantage derives from explicitly addressing tracer mixing. In doing so, the assumptions customary to compartmental analysis are made. Some of these are of difficult validation, such as that of homogeneous well-mixed pools (see Materials and Methods). Indeed, aggregating C and N metabolism into one- or two-pools is a simplification that is almost certainly invalid. But with models, the question is whether assumptions are sufficiently fulfilled to make them useful (Prosser & Farrar

1981). In this sense, we think the approach renders a more meaningful interpretation of tracer fluxes than assuming close correspondences between labelled and non-labelled substrates and the sources supplying them. For instance, in six out of eight modelled situations, newly acquired (*i.e.* labelled) C and N mixed with a storage pool ($Q_2$). As a result, the fraction of imported tracer deriving from *current assimilation/uptake* varied from virtually 1.0 for tracer imported within 1 d of its assimilation/uptake, to less than 0.5 for tracer imported 3 d (C), or 5 to 10 d (N), after its assimilation/uptake (Figure III.8). In these cases, assuming invariant relationships would have been misleading. The approach also reveals the importance of analyzing time-courses; in dominant plants, the contribution of labelled N to total N imported over the first 7 d was similar in both species (51 *vs.* 52%), but mobilization from $Q_{2N}$ was actually supplying 24% of imported N in *L. perenne*, and 41% in *P. dilatatum* (Table III.2).

Finally, use of a modelling approach allowed to realize that the substrate pool ($Q_1$) acted as a short-term store, and that the ratio of C- to N-substrates indicated plant C/N status (see below). Such insights would have otherwise been difficult to gain.

A major restriction in applying this approach is the requirement of systems in steady-state. Therefore, the approach would need to be further developed for analyzing sudden changes in studied variables. Yet, even in this case, information from the steady-state situation would provide the necessary 'base line' against which responses could be compared.

***Nature and importance of the 'growth-substrate' pool,* $\mathbf{Q_1}$**

As the pool directly supplying the growth zone, $Q_1$ is analogous to a growth-substrate, *i.e.* compounds readily available for tissue synthesis. A distinct feature of $Q_1$ was, precisely, its contrasting kinetics for C and N substrates, *i.e.* how "readily" available they were. In all cases, $Q_{1C}$ had shorter half-time than $Q_{1N}$ (Table III.2).

At first glance, such difference might be thought related to a greater proximity, either physical or metabolic, of $CO_2$ fixation than of $NO_3^-$ uptake to subsequent import of sucrose and amino acids into the growth zone. Short-term labelling studies do show photoassimilates can reach the leaf growth zone within 10 to 20 min, whereas it would take at least 1 h for newly absorbed N to do the same (*c.f.* Cooper & Clarkson 1989; Allard & Nelson 1991; Thorpe & Minchin 1991; Hayashi *et al.* 1997). But

this cannot be a significant determinant of observed 0.5 to 2 d shorter half-times of $Q_{1C}$ than $Q_{1N}$. The nature of such differences must therefore be related to kinetics of storage components included within $Q_1$. Indeed, analysis of Figure III.8 revealed that $Q_1$ acted not only as a gateway for substrates in transit to the growth zone, but also as a short-term store (see Results). $Q_{1C}$ represented less than 2% of tiller C mass, and was small compared to C gain, while $Q_{1N}$ constituted 10 to 30% of tiller N mass, and represented several days of N uptake (Table III.2), further supporting the idea of a greater storage component within $Q_{1N}$ than $Q_{1C}$.

We are aware of no other comparative study of C and N growth-substrates kinetics with which present results could be compared. Compartmental analyses of source systems –*i.e.* photosynthetic leaves, active roots– have consistently indicated two distinct pools: a rapidly exchanged cytoplasmic/apoplastic transport pool, and a vacuolar/chloroplastic storage pool of slower turnover. For C, half-times <2 h, and of 12 to 25 h, are typical for the former and the latter, respectively (Rocher & Prioul 1987; Farrar 1990 & refs therein). Once loaded into the phloem, C flows directly to sinks with minor storage along the path (Borland & Farrar 1988). Conversely, source-metabolism of N is more tortuous, as nitrate and amino acids are subject to storage in both roots and shoot. For root nitrate-N, half-times <15 min and of 4 to 7 h are typical for transport and vacuolar pools (Devienne, Mary & Lamaze 1994b & refs therein). Amino acids often account for a substantial part of soluble N, but there is hardly any data on their kinetics. A study in *B. campestris* suggests half-times <35 min, and of 3 to 20 h, for transport and storage pools (Yoneyama *et al.* 1987). Further, xylem/phloem cycling amino acids, a general feature in $C_3$ and $C_4$ species (Touraine *et al.* 1994; Engels & Kirkby 2001), could act as a labile reserve. But whether it is a small reserve cycling fast, or a larger one cycling slowly is not clear (Cooper & Clarkson 1989).

Contrasting these values with $Q_1$ half-times (Table III.2) leads to three conclusions. First, it corroborates that $Q_{1C}$ and $Q_{1N}$ included storage components; neither $Q_{1C}$ nor $Q_{1N}$ labelling kinetics were as fast as expected if accounted solely by transport pools. Second, differences of 0.5 to 2 d between $Q_{1C}$ and $Q_{1N}$ half-times can be explained by only differences in the kinetics of C and N storage (not transport) pools. Thus, $Q_{1C}$ half-times <0.5 d resemble a flux-weighed average of transport and storage pools in source leaves. But $Q_{1N}$ half-times ≥0.9 d suggest N-tracer entered storage pools more than once before reaching the growth zone. This possibility is confirmed by

Yoneyama *et al.* (1987); here, each N atom taken up during the light period was exchanged 0.68 to 0.84 times with nitrate or amino acids storage pools prior to its incorporation into leaf protein. When absorbed during the night, values increased to 1.00 and 1.46.

Third, a more important storage component within N than C growth-substrates is highly expectable, independently of species or growth conditions. Such inherently different kinetics of C and N growth-substrates may be a consequence of contrasting buffering requirements. For obvious reasons, C acquisition has abrupt diurnal cycles. Whereas N uptake is likely to experience more drastic changes at time scales of days, which is due to the pulsed and patchy nature of external N availability (Chapin *et al.* 1990). Plants deprived from N are able to maintain unaltered growth rates for several days. By using supply-interruption cycles, Macduff & Bakken (2003) estimated this buffer capacity to be between 0.5 to 3 d, which compares favorably with $Q_{1N}$ half-times (Table III.2).

***The role of mobilized stores in supplying leaf growth***

Stores were an integral part of the supply of C and N substrates for leaf growth in all plants, providing about half of C and more than 80% of N imported into the leaf growth zone (Figure III.8). Knowledge of what controls the importance of stores is thus necessary for understanding substrate-supply for growth.

We hypothesized the importance of mobilized stores in supplying leaf growth increases whenever resource acquisition becomes limited. This was not the case for C. Subordinate plants had low C assimilation rates, closely related to their reduced light capture (Table III.1). But in general, C older than 2 to 3 d contributed less than 10% to C import, corroborating the notion that leaf growth relies on recent assimilates (Anderson & Dale 1983, but see below), and short-term stores were important in supplying leaf growth in both dominant (well-lit) and subordinate (shaded) plants (Figure III.8). This substantiates results from experiments with *Arabidopsis thaliana* starchless mutants, which showed that the relationship between daily starch turnover and plant growth is not affected by light intensity (Schulze *et al.* 1991). Although the actual contribution of C stores to growth was not assessed in that study, its consideration together with present results strongly suggests that the importance of short-term C stores in supplying growth is closely associated with the need of buffering day/night cycles,

and independent of C assimilation rates. Similar responses of *P. dilatatum* and *L. perenne* suggest analogous mechanisms in starch- ($C_4$) and sucrose/fructan-storing ($C_3$) grasses.

In exception to this general pattern, mobilization provided 27% of imported C in subordinate *L. perenne* plants (Table III.2). In vegetative grasses, there are two potential sources of old C: carbohydrates stores in sheath bases and stems (Thom, Sheath & Bryant 1989; Chatterton *et al.* 1989), and amino-C derived from protein turnover. Compared to dominant *L. perenne* plants, the higher contribution of old C observed in subordinate plants seems strictly associated with differences in mobilized amino-C (Table III.3).

Table III.3. Estimated contribution of carbohydrates (CHO-C) and amino-C to imported C. Amino-C import was estimated as N import times an assumed C:N ratio of 2.6 (w $w^{-1}$) for imported N compounds (see Schnyder & de Visser 1999; Amiard *et al.* 2004). Estimation of $Q_2$-derived amino-C further assumed that there was a strict association between unlabelled C and unlabelled N within organic-N compounds. Growth conditions as for Table III.1. (*n.e.*: not estimated).

| Import of: | | *L. perenne* | | *P. dilatatum* | |
|---|---|---|---|---|---|
| | | 23°C subordinate | 15°C dominant | 23°C dominant | 15°C subordinate |
| Total C | µg $d^{-1}$ | 972 | 1136 | 3488 | 286 |
| Total Amino–C | µg $d^{-1}$ | 255 | 221 | 723 | 68 |
| Amino–C/Total C | | 0.26 | 0.19 | 0.21 | 0.24 |
| $Q_{2C}$ Total C | µg $d^{-1}$ | 265 | 117 | 251 | 11 |
| $Q_{2C}$ Amino-C | µg $d^{-1}$ | 188 | 53 | 298 | 51 |
| $Q_{2C}$ Amino-C/$Q_{2C}$ C | | 0.71 | 0.45 | 1.2 | 4.6 |
| $Q_{2C}$ Amino-C/Total C | | 0.19 | 0.05 | *n.e.* | *n.e.* |
| $Q_{2C}$ CHO-C/Total C | | 0.08 | 0.06 | *n.e.* | *n.e.* |

This is also suggested by a longer half-time of $Q_{2C}$ in subordinate *L. perenne*, close to that of $Q_{2N}$ and to the leaf life-span. Hence, when long-term N stores contribute greatly to N import, old amino-C could become an important source of C for leaf growth in $C_3$ grasses. Interestingly, Thornton *et al.* (2004) found an important contribution of old C to root exudates, which includes a substantial amount of amino-C. better understanding of C supply to growth processes clearly requires more information on amino-C fluxes.

Notably, this flux of old C was absent in subordinate *P. dilatatum* plants, even though long-term N stores supplied an equally significative part of imported N. In fact, calculations in the $C_4$ grass indicated more $Q_2$-derived amino-C than the total amount of $Q_2$-derived C, and thus an unrealistic higher than 100% contribution of amino-C (Table III.3). The reason for this difference between *L. perenne* and *P. dilatatum* is not clear. Varying the assumed C to N ratio affected only slightly estimated values. Hence, the fault probably resides in assuming a strict relationship between unlabelled C and unlabelled N within organic-N compounds. Rubisco synthesis and degradation do not occur simultaneously in $C_3$ species, but they do in (some) $C_4$ species (Millard 1988; Esquivel, Ferreira & Teixeira 2000 & refs therein). Such difference could incorporate more 'new' C along with 'old' N into exported amino acids during transaminations in $C_4$ than $C_3$ species.

Stores provided most of imported N in all treatments. However, this similarity hid contrasting responses. In dominant plants, short-term stores were important, particularly in *L. perenne* (Figure III.8 & Table III.2). The comparatively low importance of mobilization from $Q_{2N}$ plus the short half-time of $Q_{1N}$ determine that, in fact, N import in these plants depended strongly on continuous uptake. Conversely, leaf growth in subordinate plants of both species became largely independent of external N, as 75% of imported N derived from $Q_{2N}$.

There is no indication that dominant and subordinate plants would have used qualitatively different N sources. The lag-time between incorporation and mobilization of N-tracer in $Q_{2N}$ and the close relation between the half-time of this pool and leaf life-span (see 'Results') suggest mobilization of long-term N stores was associated with leaf turnover in all plants. This agrees with long-term N stores in grasses being largely accounted for by export of amino acids from senescing leaves (Millard 1988; Feller & Fischer 1994; Bausenwein *et al.* 2001).

Interestingly, $Q_{1C:N}$ was closely and negatively correlated with the fractional contribution of $Q_{2N}$ to $I_N$, accommodating differences between plants growing in contrasting hierarchical positions, and also between *L. perenne* and *P. dilatatum* dominant plants (Figure III.9). This indicates that the importance of long-term stores in supplying N for leaf growth was related to the relative abundance/scarcity of C and N substrates. There is now convincing evidence for nitrate uptake being controlled by plant N demand through the regulatory activity of C and N metabolites indicators of plant C/N status

(Touraine *et al.* 1994; Stitt & Krapp 1999). Therefore, a possible explanation for Figure III.9 is that such a mechanism is also a determinant of the importance of long-term N stores for leaf growth. Support for this possibility is lent by the fact that the partitioning on N uptake between dominant and subordinate plants within each stand was fairly similar to that of intercepted PPFD, and hence of C assimilation (*c.f.* Table III.1), which suggests uptake rates of subordinate plants were limited by their lower C/higher N plant status (Gastal & Saugier 1989). Crucially, these differences were not caused by variations in N external availability, for all plants had access to full nutrient supply.

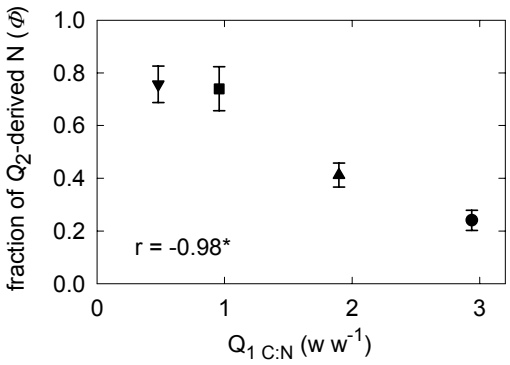


Figure III.9. Correlation between the ratio of C to N growth substrates ($Q_{1C:N}$) and the relative contribution of long-term stores ($Q_{2N}$) to N import into the leaf growth zone, $\phi$ [$M_X/(T_X+M_X)$]. Data from *L. perenne* (●■) and *P. dilatatum* plants (▲▼) growing in mixed stands at 23°C or 15°C, and thus in dominant (●▲) or subordinate positions (■▼). Error bars indicate ±SE. (* P<0.01).

In suggesting a causal link between the importance of long-term stores and the *internal* capacity for N acquisition, this explanation is consistent with our working hypothesis. Importantly, the importance of N stores may not necessarily increase (as often assumed) were N uptake reduced by a limiting *external* availability. In fact, growth rate and N demand rapidly adapt one-to-one to external N supply over a wide range of relative N addition rates, and s*tay-green* mutants unable to mobilize chlorophyll-binding proteins grew less than the wild-type only at the lowest N addition rates (Macduff, Raistrick & Humphreys 2002). A corollary of plant C/N status controlling both N acquisition and the importance of long-term stores is that, whenever possible, a plant will acquire and use new N rather than stores (and increase its growth rate). This might not be so in all grasses. Farrar (1990) suggested the inherent

difference between slow- and fast-growing species is their use of C stores. A differential use of nutrients stores seems a plausible possibility, worth of experimental test.

## CONCLUSIONS

A novel approach to study the sources of C and N supplying leaf growth is presented. Compared to previous analysis, it imports a more mechanistic definition of the process studied, an increased accuracy with which it is measured, and a more meaningful interpretation of tracer fluxes. By using such an approach, we were able to show that stores were a critical part of the supply of C and N substrates for leaf growth in both *L. perenne* ($C_3$) and *P. dilatatum* ($C_4$). Long-term carbohydrate stores were of generally little relevance for leaf growth in these undisturbed plants, and short-term C stores had an important role buffering light/dark cycles in all plants. Hence, no evidence was found of a causal link between C acquisition and the importance of C stores in supplying leaf growth. Long-term N stores were important in supplying leaf growth in all situations, but particularly more in plants where growth was more C- than N-limited and whose N uptake capacity was lower. It is proposed that a common mechanism regulates N acquisition and use of N stores.

# Chapter IV. GENERAL AND SUMMARIZING DISCUSSION

The present study focused on the analyses of the import of C and N substrates into leaf growth zones and its use for leaf tissue production. Thus, in Chapter II, the extent and control of putative plasticity in the relationship between fluxes of C and N entering and leaving the growth zone, and the amount and quality of produced tissue were explored. Conversely, in Chapter III the emphasis was on the origin of imported C and N; specifically, the role of current assimilation/uptake and mobilization from short- and long-term storage pools in supplying leaf growth.

**A novel approach for studying C and N fluxes in leaf growth zones**

Achieving these aims required accurate estimations of fluxes of tissue and tissue-bound C and N in the leaf growth zone (efflux and deposition), since it is there where the conversion of substrates into structure –*i.e.* growth– takes place. For this purpose, available approaches appeared either too coarse to capture expected responses, *e.g.* classical growth analysis (*e.g.* Hunt 1978), or impractically refined, *e.g.* deposition patterns within the growth zone (*e.g.* Silk 1984). Therefore, the first step was to develop and test an intermediate approach for analysing processes and responses of whole leaf growth zones.

The method involved (*i*) determining the size of the growth zone, (*ii*) measuring the rate of mature tissue area (or length) production and the C and N density on an area (or length) basis of recently produced (still enclosed) tissue thus to estimate tissue-bound C and N efflux out of the growth zone (Eq. II.3a & b, p. 12). Importantly, this efflux is generated solely by deposition associated with cell expansion and maturation demands. The tissue is still enclosed within the whorl, not exposed to light, and thus not active photosynthetically. Consequently, (*iii*) C and N substrate import can be estimated by adding to export the (positive or negative) variation in C and N mass of the growth zone it-self (Eq. II.2, p. 12). The accuracy of the method was tested by comparing estimated and measured C and N import and export rates on published datasets. A good agreement was evident for both dry matter and N import and export fluxes in *Festuca arundinacea* and *L. perenne* plants (Figure II.2), which lent confidence to the estimated values.

An important issue concerning C fluxes is that respiration is not considered in the analysis. Therefore, import rates are underestimated by the amount of C respired, and are thus equivalent to *net* deposition

rates (*e.g.* Schnyder & Nelson 1989; Allard & Nelson 1991; Maurice *et al.* 1997). Respiration could be incorporated by direct measurement of respiration rates of the growth zone (*e.g.* Moser, Volenec & Nelson 1982). Alternatively, respiration rates can be approximated by assuming a constant proportion of maintenance respiration, and then estimating growth respiration based on the biochemical composition of produced tissue and the corresponding efficiencies of substrate conversion (*e.g.* Volenec & Nelson 1984).

Basically, the developed method constitutes a mass balance of C and N in the growth zone. Coupled to steady-state $^{13}CO_2/^{12}CO_2$ and $^{15}NO_3^-/^{14}NO_3^-$ dual labelling, these mass balances were further decomposed into 'labelled' and 'non-labelled' components (Eq. III.2, p.35). The time-course of C and N tracers incorporation into imported substrates (Eq. III.3a & b, p. 35) were thus determined, and subsequently analyzed with compartmental models (Figure III.4, p. 36). This yielded information on the number and kinetics of distinct pools supplying import into the growth zone, and hence on their relative importance in supporting leaf growth. Compared to previous labelling studies, this analysis represented a significant improvement on the interpretation of tracer fluxes, allowing to infer fluxes of interest –say current assimilates, and mobilization from short- and long-term stores– from those actually measured –say, labelled C.

Labelling techniques are becoming a certain possibility for field studies. A major problem for the application of the present approach in field experiments are the strongly non-steady growth conditions, mainly driven by temperature changes. This will difficult the determination of import fluxes of labelled and non labelled fractions, and will affect basic assumptions made in compartmental modelling. Regarding the former, Figure IV.1 clearly shows that direct sampling of leaf growth zone tissue might be a useful proxy for estimating the fraction of labelled C or N in exported tissue. But this is not the case for that in imported substrates. The reason for the difference between $f_{\mathrm{lab}\ G_X}$ and $f_{\mathrm{lab}\ I_X}$ is the turnover of mass in the growth zone (Eq. III.3b, p.35). Alternatively, $f_{\mathrm{lab}}$ of specific compounds, such as sucrose and free amino acids, might provide adequate proxies for imported C and N.

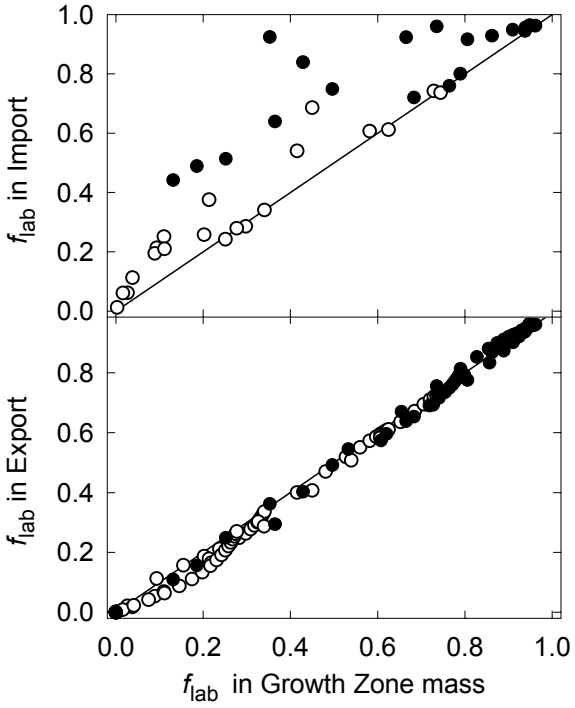


Figure IV.1. Relationship between the $f_{lab}$ of C (●) and N (○) in import and export and that in the growth zone.

**The relationship between substrates import, tissue production, and leaf growth zone C status**

Defoliation is a most common event in the life of a grass plant. Therefore, the ability to regenerate lost leaf area (*i.e.* re-foliation) is an essential requirement for grass survival. Yet, it is a poorly understood process (Richards 1993). In Chapter II, the existence and putative controls of flexibility in the relationship between substrate import and tissue efflux was investigated. A great potential flexibility was evident in the relationship between substrate import and tissue efflux. This plasticity, allowed leaf area expansion to proceed virtually unaffected following severe defoliation for at least 2 d in dominant *L. perenne* plants (Figure II.4, p.16). This was accomplished by displacing into the light already existent –mature– leaf tissue by strongly reducing the amount of C (and less so N) deposition per unit of exported tissue. Use of internal C stores and changes in the density of produced tissue were both involved in this response. Thus, an amount of leaf tissue area –probably big enough to support the leaf growth zone C demands (Volenec & Nelson 1984)– was rapidly exposed to the light and became photosynthetically active.

These results confirm previous studies (*e.g.* Davidson & Milthorpe 1966; Morvan-Bertrand *et al.* 2001) in indicating a role for C stores located within growth zone in sustaining regrowth. Furthermore, they also demonstrate a quantitatively more important role of associated substantial and sustained changes in the 'quality' of produced tissue (as measured by decreases in its C and N density) in supporting leaf expansion under C shortage. A lower C status of the growth zone prior to defoliation brought about by growth in low hierarchical positions within the stand, resulted in a lesser buffer capacity and hence more rapid and abrupt decreases in leaf area expansion rates following defoliation. Partly, this was simply due to lack of substrates (*i.e.* stores) to sustain export (Figure II.5, p.18). But also, and more importantly, this was associated with a reduced ability of the growth zone to adapt (*i.e.* decrease) the C density of newly produced tissue (Figure II.8, p. 19).

Most of C storage within leaf growth zones occurs as fructans in temperate $C_3$ grasses (Davidson & Milthorpe 1966; Volenec & Nelson 1984; Morvan-Bertrand *et al.* 2001). However, $C_4$ species and $C_3$ grasses of Gondwanian origin are unable to synthesize fructans (Chatterton *et al.* 1989). These species might therefore be at a disadvantage regarding buffer capacity. Likewise, species with inherently thinner or less dense leaves might be less able to adapt to sudden C shortage. Indeed, this was observed in *P. dilatatum*, which showed lower levels of WSC within the growth zone and lesser density, and had a lesser buffering capacity (Figure II.10, p.24). Notably, changes in the density of produced tissue were far less marked for N for C. A current hypothesis on the control of leaf area expansion concerns, precisely, a regulation of the amount of N allocated per unit produced tissue area (Grindlay 1997). Corroborating intrinsic differences between $C_3$ and $C_4$ species, and the control and determination of leaf N content on a tissue area basis are certainly worth of further research.

**The sources supplying leaf growth**

The patterns of accumulation and mobilization of C and N stores are relatively well-known (Millard 1988; Chatterton *et al.* 1989; Chapin *et al.* 1990). But just how important mobilized substrates are for growth remains poorly understood. In Chapter III the role of C and N substrates derived from mobilization from short- and long-term stores in supplying leaf growth, as opposite to those derived from current C assimilation and N uptake, were investigated in undisturbed plants. Specifically, an association between the ability of plants to acquire external resources *vs.* the importance of internal

stores was hypothesized. Such a link was not evident for C substrates. In all plants –$C_3$ or $C_4$, dominant or subordinate– assimilates less than 2 d old were the main source supplying leaf growth. Approximately half of these were delivered within 12 h of its assimilation; and the rest over the following 24 h (Figure III.8, p.46). Therefore, short-term stores played a (roughly) equally important role in buffering day/night cycles in both well-lit plants with high photosynthetic rates, and in shaded plants with limited assimilation. The age-profile of N substrates imported into the leaf growth zone differed from that of C and was highly heterogeneous depending on the treatment. First, N taken up over the last 3 to 5 d made similar contributions to imported N. Second, subordinate plants were much more dependent on mobilized long-term stores than dominant plants (Figure III.8, p.46 & Table III.2, p.43).

Differences between the age-composition of imported C and N substrates (in the order of days) can not be explained by physical or metabolic distances between assimilated/absorbed $CO_2$ and $NO_3^-$ and imported sucrose and amino acids (*c.f.* Cooper & Clarkson 1989; Allard & Nelson 1991; Thorpe & Minchin 1991; Hayashi et al. 1997). Therefore, there is a greater probability for absorbed N than assimilated C to be stored before reaching the growth zone, associated with the existence of several, successive, storage pools between $NO_3^-$ uptake and amino acid import (*e.g.* vacuolar $NO_3^-$ in roots and shoots, chloroplastic, vacuolar and xylem-phloem cycling reduced N). Since such differences between C and N are intrinsic to plant metabolism, the shorter dependence of leaf growth on C than N stores observed in defoliation studies (*e.g.* Avice *et al.* 1996; de Visser *et al.* 1997) might in fact be inherent rather than a direct response to defoliation.

A close negative correlation was evident between the importance of N stores in supplying leaf growth and the C to N status of the plant, accommodating not only differences between subordinate and dominant plants, but also those between the $C_3$ and $C_4$ grass (Figure III.9, p.53). In turn, these responses were associated with far lower N uptake rates in subordinate than dominant plants in both *P. dilatatum* and *L. perenne*. Therefore, a link between current acquisition and importance of (long-term) stores did appear for N substrates. To our knowledge, this is the first report suggesting a putative mechanism controlling the role of mobilized stores in supplying growth. But further testing in studies specially planned to address an association between N uptake capacity and the role of N stores are needed to confirm or reject more completely this hypothesis.

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# APPENDIX

| *Symbol* | *Description* | *Units* |
|---|---|---|
| $C$ | Carbon | |
| $N$ | Nitrogen | |
| $X$ | Any element (here, carbon or nitrogen) | |
| *WSC* | Water soluble carbohydrates | |
| $\delta$ | The deviation of the $^{13}C$ to $^{12}C$ ratio in $CO_2$ from that of the international standard, VPDB | |
| $A$ | Molar fraction of $^{15}N$ or $^{13}C$ | |
| <*variable*> | denotes 24 h averages | |
| $f_{lab}$, $f_{unlab}$ | Fraction of labelled, fraction of non-labelled | |
| $G$ | Leaf growth zone (cell division, expansion and maturation zones) | |
| *RPT* | Recently produced (structurally and functionally differentiated) leaf tissue | |
| *LER,* $L_L$ | Leaf elongation rate | mm $d^{-1}$ |
| *LAER,* $L_A$ | Leaf area expansion rate | $mm^2$ $d^{-1}$ |
| $G_X$, $G_C$, $G_N$ | Mass of X, C or N in the growth zone | µg |
| $\rho_{LX}$, $\rho_{LC}$, $\rho_{LN}$ | Density of X, C or N in recently produced tissue (on a lineal basis) | µg $mm^{-1}$ |
| $\rho_{AX}$, $\rho_{AC}$, $\rho_{AN}$ | Density of X, C or N in recently produced tissue (on an area basis) | µg $mm^{-2}$ |
| *SLA* | Specific leaf area | $m^2$ kg $C^{-1}$ |
| $N_L$ | Leaf N content | g N $m^{-2}$ |
| $I_X$, $I_C$, $I_N$ | Import (net deposition) into the growth zone of X, C or N | µg $d^{-1}$ |
| $E_X$, $E_C$, $E_N$ | Export (tissue-bound efflux) out of the growth zone of X, C or N | µg $d^{-1}$ |
| $t$ | Time | d |
| $k_{10}$, $k_{12}$, $k_{21}$ | First order rate constants (fluxes *I, D*, and *M*, respectively) | $d^{-1}$ |
| $T_X$, $T_C$, $T_N$ | Incorporation of labelled X, C or N into the system | µg $d^{-1}$ |
| $M_X$, $M_C$, $M_N$ | Incorporation of non-labelled X, C or N into the system (one-pool model)<br>Transfer from $Q_2$ to $Q_1$ of X, C or N (two-pools model) | µg $d^{-1}$ |
| $D_X$, $D_C$, $D_N$ | Transfer from $Q_1$ to $Q_2$ of X, C or N | µg $d^{-1}$ |
| $\phi$ | The relative contribution of $M_X$ to $I_X$. Parameter directly fitted in the one-pool model, or equal to $k_{12}/(k_{10}+k_{12})$ in the two-pools model | % |
| $Q_1$, $Q_2$ | Compartment sizes ('substrate', and 'stores') | µg |
| $t_{0.5}$ | Half-time | d |
| $Q_{1C:N}$ | Ratio of $Q_{1C}$ to $Q_{1N}$ | |

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## ACKNOWLEDGMENTS

A Ph.D. is far more than a thesis. In this case, it has been four and half years of my life. I would like to thank the people I met during this time for things they did but were not supposed to do.

To Hans Schnyder, for his confidence in me, and the support he gave me at all times. I appreciate this trust more than anything else. I can not remember a single occasion when I had felt him other than as an (incredible patient) advicer.

To Barry Thornton. Honestly, I can't tell whether I am more grateful to him for being such a kind supervisor, or for making me feel at home in Aberdeen. I suppose those experienced with Scottish wheather will easily understand why I feel inclined for the latter.

To all people at the Lehrstuhl für Grünlandlehre for their help and kindness. In particular, to Rudi & Wolfgang, who made the vagaries and mystic of operating gas exchange chambers & an IRMS understable to me. To Melitta, who deals with German bureaucracy in a 'gaucho way': she rides it, whether they like or not.

To the friends I made at the Lehrstuhl für Grünlandlehre, the 'early' ones, Katja, Rudi & Wofgang, and the 'late-comers', Arnoud, Agustín & Monika (moje malinká a zelená žabička). We shared lot of times I shall not forget.

To my room-mates at Freising and Aberdeen, Uli (echt Schwabisch), Tanja (echt Frankonian), Zivayi (a 100% black wwman), Ela (Polish? French?), the two Frederics, Christian and Fabien (all Frenchs, but of the good ones, eh?), Alfonso (Spanish), Mani (best cook of Pakistani chiabatis), Philip, Julia & Anne (three Bavarians who I never saw eating Weißwurst). I had a wonderful time living with you.

Finally, to my dear friends at Argentina & To my family. No need for specifying reasons here, are there?