Carbon and nitrogen deposition in expanding tissue elements of perennial ryegrass (*Lolium perenne* L.) leaves during non-steady growth after defoliation

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**ABSTRACT**

The effect of defoliation on the deposition of carbon (C) and nitrogen (N) and the contribution of reserves and current assimilates to the use of C and N in expanding leaf tissue of severely defoliated perennial ryegrass (*Lolium perenne* L.) was assessed with a new material element approach. This included $^{13}$C/$^{12}$C- and $^{15}$N/$^{14}$N-steady-state labelling of all post-defoliation assimilated C and N, analysis of tissue expansion and displacement in the growth zone, and investigation of the spatial and temporal changes in substrate and label incorporation in the expanding elements prior to and after defoliation. The relationship between elemental expansion and C deposition was not altered by defoliation, but total C deposition in the growth zone was decreased due to decreased expansion of tissue at advanced developmental stages and a shortening of the growth zone. The N deposition per unit expansion was increased following defoliation, suggesting that N supply did not limit expansion. Transition from reserve- to current assimilation-derived growth was rapid (<1 d for carbohydrates and approximately 2 d for N), more rapid than suggested by label incorporation in growth zone biomass. The N deposition was highest near the leaf base, where cell division rates are greatest, whereas carbohydrate deposition was highest near the location of most active cell expansion. The contribution of reserve-derived relative to current assimilation-derived carbohydrates (or N) to deposition was very similar for elements at different stages of expansion.

Key-words: $^{13}$C; $^{15}$N; (leaf) growth zone; reserves; sink; steady-state labelling.

**INTRODUCTION**

Defoliation is the essential prerequisite for recovery of a grass plant from defoliation. However, the leaf expansion rate of grasses may decrease strongly as a consequence of defoliation (Davidson & Milthorpe 1966a). In a study with perennial ryegrass (*Lolium perenne* L.), this effect was associated with decreases in cell expansion and in the size of the leaf growth zone (Schäufele & Schnyder 2000). Decreased cell production and expansion may be related to limited C and N assimilate supply to the leaf growth zone due to low residual photosynthetic activity, decreased N uptake and assimilation, or low levels of carbohydrate and/or N reserves. Moreover, other factors may also be involved (Schäufele & Schnyder 2000), but have not been studied.

Identification of the actual mechanism(s) of the defoliation-related decrease in leaf expansion requires studies at the level of the leaf growth zone. Few such studies have been conducted and none have investigated substrate fluxes into expanding leaf tissue and its relationship to tissue expansion in defoliated plants. Assimilate flux to growth zones can be assessed in two ways: either by analysing the influx in specified material elements (e.g. expanding cells), which are followed over time (‘material element approach’), or by analysing influx as a function of the position in the leaf growth zone (‘spatial approach’). So far the spatial approach has generally been used and has proved to be most helpful for the study of the assimilate relations of leaf growth zones during phases of steady growth (Schnyder & Nelson 1987; Gastal & Nelson 1994). In these studies, local rates of assimilate deposition in the leaf growth zone are determined from the spatial distribution of growth rates and assimilate contents within the growth zone using the continuity equation (Silk 1984). However, where growth is non-steady – as may be the case after defoliation – the spatial approach is relatively impractical, because it requires determination of changes of growth rates and substance contents in the growth zone with high resolution in both spatial and temporal terms.

Here, we demonstrate and use a new, simpler material approach to the study of C and N deposition in expanding material elements as influenced by severe defoliation of perennial ryegrass. This involved the analysis of tissue expansion and displacement in the growth zone (Schäufele & Schnyder 2000), and investigation of the spatial distribution of C and N contents within the leaf growth zone prior to and at different times after defoliation. The experiment was combined with $^{13}$C/$^{12}$C- and $^{15}$N/$^{14}$N-steady-state labelling of all post-defoliation assimilated C and N, and

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analysis of the isotope composition of C and N in the leaf growth zone in order to assess the contributions of reserve- and current assimilation-derived substrates to C and N deposition in expanding tissue.

MATERIALS AND METHODS

Plant material and growth conditions

Details on plant material and growth conditions have been described previously (Schäufele & Schnyder 2000). Briefly, miniature swards of perennial ryegrass (Lolium perenne L., cv. Modus) were established from seed in 2 L plastic pots filled with washed quartz sand. Swards (n = 60) were kept in two growth chambers throughout the experiment and received 50 mL of a nutrient solution (modified half-strength Hoagland solution with 105 mg N l⁻¹; composition: 2·5 mm KNO₃, 2·5 mm Ca(NO₃)₂, 1 mm MgSO₄, 0·5 mm KH₂PO₄, 0·1 mm Fe-EDTA, 23 μm H₂BO₃, 4·6 μm MnCl₂, 0·4 μm ZnSO₄, 0·16 μm CuSO₄, 0·26 μm H₂MgO₄) four times a day. Irradiance during the 16 h photoperiod was gradually increased from 280 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) at sowing to 630 μmol m⁻² s⁻¹ PPFD on day 18 after sowing. The daily light regime was then held constant until the end of the experiment. Temperature was controlled at 20/16 °C and relative humidity near 75/85% during the light and dark periods.

33 d after seeding, all plants were defoliated at 5 cm stubble height and redistributed randomly among the two cabinets. Defoliation was performed at 3 h before the start of the light period and was immediately followed by the first sampling.

¹³C and ¹⁵N labelling

The C and N assimilated prior to and after defoliation were differentially labelled using the techniques described by Schnyder (1992) and De Visser, Vianden & Schnyder (1997). During the pre-defoliation phase, both chambers received CO₂ from a mineral source (δ = -4·6‰; Buse, Bad Höningen, Germany). The CO₂ partial pressure was maintained near 33 Pa by injection of CO₂ into CO₂-free air that was fed to the cabinets at a rate of 18 m³ h⁻¹. At defoliation, the δ of the CO₂ in one of the two chambers (‘labelling chamber’) was changed to -25·0‰, while the δ was kept constant at -4·6‰ in the other chamber (‘control’). The other growth conditions were kept identical in both chambers. This procedure allowed for the subsequent determination of defoliation- and tissue-specific effects on C isotope discrimination (Δ¹³C) (Schnyder 1992; De Visser et al. 1997).

Also, at defoliation the nitrate in the nutrient solution was enriched to 1·0 atom% ¹⁵N (nitrate derived from KNO₃ and Ca(NO₃)₂) as the sole source of N and the standard nutrient solution contained 0·3662 atom% ¹⁵N using 99 atom percentage ¹⁵N-KNO₃ and -Ca(NO₃)₂ (Isotec Inc., Miamisburg, OH, USA).

Sampling

Plant material was sampled 0, 1, and 2 d after defoliation. Sampling always included two pots per chamber, started 2 h before the onset of the light period and was completed within 4 to 6 h. In each pot at least 10 main tillers were selected randomly. The most rapidly elongating leaf of the main tiller was carefully freed from the surrounding leaf sheaths and cut from the tiller at the base. Leaves were selected only if the ligule was located within 2 mm of the point of attachment of the leaf to the tiller base. A cutting device holding 12 parallel razor blades spaced 3·5 mm was used to dissect the basal 38 mm into 11 segments. Segments of at least 10 leaves per replicate were combined by position. Samples were then stored at -30 °C until freeze-dried, weighed, ground in a ball mill and stored at -30 °C until further use. Sampling of segments occurred in parallel with collection of replicas of the abaxial epidermis along the leaf base of most actively elongating main tiller leaves which were used to assess the growth distribution along the leaf base (Schäufele & Schnyder 2000). Replicas were taken from a paired set of tillers from the same pots.

C and N analysis

Aliquots of dry ground plant material were placed in tin cups (Type 76 9813 26; Lüdi, Flawil, Switzerland) and the C and N contents and isotope compositions determined with an automatic elemental gas chromatograph interfaced to a continuous-flow isotope-ratio mass spectrometer (Roboprep TCD-TraceMass; Europa Scientific, Crewe, UK). The carbon isotope composition of the CO₂ used for labelling was measured with a dual-inlet isotope-ratio mass spectrometer (SIRA 9; VG Instruments, Middlewich, UK). PeeDee Belemnite (PDB)-gauged laboratory standards were used as a reference.

Analysis of C and N isotope data

The C and N isotope data (δ¹³C and atom% ¹⁵N) were used to calculate the weight fractions (fpre and fpost, where fpost = 1 – fpre) of C and N derived from pre- and post-defoliation assimilation. The fraction of pre-defoliation C (fpre) in a segment was obtained from δP = fpreδPC + (1 – fpre)δPL:

\[ f_{pre} = \frac{\delta_P - \delta_{PL}}{\delta_{PC} - \delta_{PL}}. \]

(1)

where δP is the δ of C in a given segment harvested from the labelling chamber, δPL is the δ of the C assimilated from the labelling CO₂, and δPC is the δ of the parallel segment collected from the control chamber. The δPL was not determined experimentally, but was estimated as

\[ \delta_{PL} = \left( \delta_{SO} - \Delta^{13}C \right) / (1 + \Delta^{13}C), \]

(2)

where δSO is the δ of the labelling CO₂, and Δ¹³C is the segment-, replicate- and time-specific C isotope discrimination as determined in the parallel sample collected from the
control chamber (Schnyder & De Visser 1999). It was thus assumed that $\Delta^{13}C$ was the same in both cabinets, because $\Delta^{13}C$ is independent of the $\delta$ of the source CO$_2$ (Deléens, Palvídés & Queiroz 1983; Farquhar & Richards 1984; Evans et al. 1986; Schnyder 1992) and growth conditions in the two cabinets were identical. Indeed, except for isotope composition, analysis of variance indicated no differences among samples collected from the labelling and control cabinets.

The mass of pre-defoliation C per mm leaf length ($C_{pre}$) was calculated as

$$C_{pre} = f_{pre}C,$$

where $C$ is the total C mass per mm leaf length in the sample. N isotope data were evaluated accordingly, but neglected the N isotope discrimination, which was insignificant relative to the enrichment of the nitrate by $^{15}N$ (De Visser et al. 1997).

Assessment of substrate deposition

For day 0, when growth was approximately steady (Schäufele & Schnyder 2000), ‘local’ net rates of C and N deposition [$D; g$ (mm leaf length h)$^{-1}$] in the growth zone were calculated using the one-dimensional form of the continuity equation as described by Silk (1984):

$$D = (dP/dt) + v(dP/dx) + (dv/dx)P,$$

where $P$ is local substance content (C or N; g per mm leaf length), $t$ is time, $x$ is distance from the leaf base, and $v$ is the velocity of displacement (mm h$^{-1}$) of tissue relative to the leaf base. Local velocities of displacement and relative expansion rates [$dv/dx; mm$ (mm h)$^{-1}$] were derived from leaf elongation rate and the spatial distribution of epidermal cell lengths as measured in the same experiment (for details see Schäufele & Schnyder 2000). Local substance contents used for calculation of deposition rates were estimated by first fitting a fourth-degree polynomial regression to the spatial distribution of C (or N) content data as obtained by destructive sampling ($r^2 > 0.99$ for both C and N). For the situation of steady growth it was assumed that local substance contents were constant with time.

Defoliation caused non-steady spatial and temporal distributions of growth rates (Schäufele & Schnyder 2000) and substance contents (see below), invalidating the application of the continuity equation to the data obtained in the present experiment. Therefore, a new ‘material element’ approach (as opposed to a ‘spatial’ approach, see above) was contrived and used to assess substance deposition during the non-steady growth phase following defoliation. This consisted of calculating the deposition of C (and N) in (expanding) material elements of the leaf during one-day intervals [g (element d)$^{-1}$], and is illustrated in Fig. 1. Each element was defined as a cross-sectional segment of the leaf which had a (initial) length of 0.1 mm at the beginning of the one-day interval. Elements were identified by the position $x$, mm relative to leaf base) of their proximal ($p$) and distal ($d$) limits at the beginning and end of a one-day interval. The displacement (i.e. change of position) of $p$ ($x_{p,0} \rightarrow x_{p,1}$) and $d$ ($x_{d,0} \rightarrow x_{d,1}$) experienced during the one-day interval was obtained from the analysis of displacement of cells during the respective intervals (Schäufele & Schnyder 2000). As an element was displaced, its length ($L$, mm per element) increased due to expansion of the tissue which was located between its proximal and distal limits. The length of the element at the beginning ($L_0$) and end of the interval ($L_1$) was obtained as $L_0 = x_{d,0} - x_{p,0}$, and $L_1 = x_{d,1} - x_{p,1}$.

Net C deposition [$\Delta C; g$ (element d)$^{-1}$] in an element during the one-day interval was obtained as $\Delta C = C_1 - C_0$, where $C_I$ and $C_0$ are the masses of C contained in the element at the end ($C_1$) and beginning ($C_0$) of the one-day interval. The latter were obtained by integrating the local C contents between the proximal and distal limit of the element at $t_0$ and $t_1$, respectively. For further explanation see text.

Statistical analysis

Data were analysed with the statistical package SPSS® for Windows® (SPSS Inc., Chicago, IL, USA). Effects of sampling date were evaluated by analysis of variance. The sampling dates were arranged in the cabinets in a randomized complete block design. As only the most rapidly elongating leaf on a tiller was sampled, sampling date effects would not be expected for undisturbed growth: it was shown repeat-
edly that leaf expansion rate and associated metabolism are approximately steady for several days during the period of most rapid elongation, if plants are not disturbed (e.g. Volenec & Nelson 1984; Schnyder & Nelson 1989; Schnyder et al. 1990). Therefore, sampling date effects (i.e. differences in the parameters evaluated on plants sampled at day 0 compared to plants sampled at day 1 and 2) were interpreted as defoliation effects.

Total C and N contents were assessed on four replicates per sampling date with 10 leaves per replicate. To test whether C or N content changed with distance from the leaf base a linear regression was calculated of C or N content versus distance for the region in question. Spatial gradients of C or N content were assumed to be non-existent, if the slope of the linear regression was not significantly different from 0. The fractional contribution of current assimilates was assessed using two (C) or four (N) replicates per sampling date with 10 leaves per replicate.

The standard errors (SE) associated with deposition rate data were calculated using the ‘propagation of errors’ procedure (Bevington 1994), where the function used to calculate the value in question is partially derived with respect to each of its parameters and the absolute values of each partial derivative is multiplied by the SE associated with the respective parameter. The sum of these products is taken as the maximum SE associated with the calculated value, assuming that the individual SE are correlated positively, that is, they work in the same direction.

RESULTS AND DISCUSSION

C and N relations during undisturbed growth

The spatial gradients of C and N contents (mg per mm leaf length) in the leaf growth zone during undisturbed growth were reflected in samples collected at defoliation (day 0). At that time C content decreased strongly with distance from the leaf base up to about 20 mm, where C content was about half of that observed near the base (Fig. 2a). Beyond 20 mm, C content was near constant with distance ($P > 0.1$). C concentration was near constant at about 420 mg per g dry matter throughout the growth zone (data not shown).

The N content also decreased with increasing distance from the leaf base (Fig. 2a). In relative terms the spatial gradient of N content was much greater than that observed for C. The latter was reflected in a strong increase of the C/N (w/w) ratio in biomass with distance from the leaf base (Fig. 2b). In consequence, N concentration (g per g dry matter) also decreased with distance from the leaf base (data not shown). The N concentration was highest close to the leaf base, where cell division occurs (86 mg per g dry matter), and decreased to 37 mg per g dry matter at the distal limit of the leaf growth zone.

These relationships were due to differing spatial patterns of C and N deposition (Fig. 2c). The local rate of N deposition was maximum near the leaf base and decreased strongly towards the distal end of the growth zone. Conversely, C deposition rate reached its maximum at about 10 mm from the leaf base and – relative to N – decreased much less with distance above that position. Although N and C deposition in leaf growth zones have not been compared in previous studies, the present results are consistent with previous findings (N: Gastal & Nelson 1994; Hu & Schmidhalter 1998; C: Hu, Schnyder & Schmidhalter 2000). The high rate of N deposition near the leaf base has been interpreted in terms of a high N demand for synthesis of proteins and nucleic acids in cells undergoing division. In contrast, nitrate concentration was very low in the growth zone (Gastal & Nelson 1994; Hu & Schmidhalter 1998). Thus, the N concentration of 86 mg per g dry mass near the leaf base suggests that more than 50% of the dry matter in the immediate vicinity of the leaf base consisted of amino acids, polypeptides, proteins and nucleic acids.

C deposition results from C import in this heterotrophic tissue (where deposition rate equals the rate of import less the rate of respiration and export, with export being pre-
sumably negligible in this strong sink). Two forms of substrates contribute the bulk of the C deposited in the growth zone: carbohydrates (Schnyder & Nelson 1989) and amino acids (Gastal & Nelson 1994). Estimates of local rates of carbohydrate-C deposition were obtained by subtracting amino-C deposition from total C deposition assuming a C/N (w/w) ratio of 2·6 in amino acids (Fisher & Macnicol 1986; Lefevre, Bigot & Boucaud 1991) supplied to the leaf growth zone (Fig. 2c, dashed line). These data indicate that amino-C must have contributed a very large fraction of the total C deposition near the leaf base. Conversely, carbohydrates contributed almost all of the C deposition near the distal limit of the growth zone, where N deposition (and, hence, amino-C deposition) was very low or nil. Clearly, the ratio of amino acid to carbohydrate deposition changed dramatically along the leaf growth zone. This may have resulted from (i) a change in the relative rates of import of the two substrates and/or (ii) relatively high rates of carbohydrate consumption in respiration near the leaf base, where cell production and N metabolism is active.

The decreases in C and N content along the leaf growth zone probably resulted from dilution of biomass during cell expansion. As cells expand they are displaced away from the leaf base (MacAdam, Volenec & Nelson 1989; Schnyder et al. 1990). Essentially all of the volume increase of growing cells results from the uptake of water. As water enters the expanding cells their contents are diluted. These processes are counteracted by a concomitant uptake of assimilate (cf. Fig. 2c) and consequent synthesis of structural and non-structural biomass (MacAdam & Nelson 1987; Schnyder & Nelson 1987). The present negative gradient of C and N content along the leaf growth zone (Fig. 2a) indicates that as cells moved through the zone of active cell elongation water uptake (and hence volume increase of cells) was stimulated relatively more than assimilate deposition.

Spatial and temporal changes in C and N content as affected by defoliation

The spatial gradient of C and N contents was also analysed at 1 and 2 d after defoliation (Fig. 3a & b). Defoliation had no significant effect on the spatial distribution of C content in the basal 20 mm of the most actively expanding leaves ($P > 0.5$). Conversely, defoliation affected the spatial gradient of C content between 20 and 40 mm from the leaf base: whereas C content was near constant with distance in this zone at defoliation ($P > 0.1$), it increased significantly with distance beyond 20 mm at 1 and 2 d after defoliation ($P < 0.05$).

Cell growth analysis indicated that the length of the leaf growth zone decreased from 33 mm at day 0 to 27 and 21 mm at day 1 and 2, respectively (Schäufele & Schnyder 2000; cf. also arrows given in Fig. 3a). The increase in C content distal to 20 mm at day 1 and 2 was possibly due to this defoliation-related shortening of the leaf growth zone and an associated ‘displacement’ of the zone of secondary wall deposition to a more basal position. Secondary cell wall deposition starts near the distal limit of the leaf growth zone where cells reach their final size. In tall fescue (Festuca arundinacea Schreb.) leaves, this process was accompanied with increasing dry matter content (g per mm leaf length) with increasing distance beyond the distal limit of the leaf growth zone (MacAdam & Nelson 1987; Maurice, Gastal & Durand 1997).

Defoliation had no significant effect on N content near the leaf base ($P > 0.5$; Fig. 3b). However, distal to about 15 mm the N content was higher at 1 and 2 d after defo-
Table 1. Displacement, expansion and associated C and N deposition rates of material elements of the leaf growth zone of *Lolium perenne* as affected by defoliation. Two elements (*a* and *b*) having an initial length of 0·1 mm and located at 5 (*a*) and 11 (*b*) mm from the leaf base were followed during one-day intervals: the day preceding defoliation (day 0: elements *a*0 and *b*0), the first day after defoliation (day 1: *a*1 and *b*1) and the second day after defoliation (day 2: *a*2 and *b*2). The displacement and expansion experienced by elements during the one-day intervals was obtained from data presented by Schäufele & Schnyder (2000). C and N deposition rates were calculated as explained in ‘Materials and Methods’

<table>
<thead>
<tr>
<th>Element</th>
<th>Position at beginning of interval</th>
<th>Position at end of interval</th>
<th>Length at end of interval</th>
<th>Displacement of elements</th>
<th>Expansion of elements</th>
<th>C/N (w/w) ratio of biomass deposited</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mm from leaf base)</td>
<td>(mm from leaf base)</td>
<td>(mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>a</em>0</td>
<td>5·0</td>
<td>14·8</td>
<td>0·39 ± 0·02</td>
<td>0·02 ± 0·40</td>
<td>0·04 ± 0·21</td>
<td>11·8 ± 0·9</td>
</tr>
<tr>
<td><em>a</em>1</td>
<td>5·0</td>
<td>10·6</td>
<td>0·40 ± 0·04</td>
<td>0·03 ± 0·21</td>
<td>0·05 ± 0·19</td>
<td>6·8 ± 0·4</td>
</tr>
<tr>
<td><em>a</em>2</td>
<td>5·0</td>
<td>13·0</td>
<td>0·38 ± 0·07</td>
<td>0·02 ± 0·02</td>
<td>0·03 ± 0·07</td>
<td>8·5 ± 1·2</td>
</tr>
<tr>
<td><em>b</em>0</td>
<td>11·0</td>
<td>30·9</td>
<td>0·33 ± 0·04</td>
<td>0·25 ± 0·05</td>
<td>0·19 ± 0·07</td>
<td></td>
</tr>
<tr>
<td><em>b</em>1</td>
<td>11·0</td>
<td>30·5</td>
<td>0·40 ± 0·22</td>
<td>0·40 ± 0·20</td>
<td>2·40 ± 0·25</td>
<td></td>
</tr>
<tr>
<td><em>b</em>2</td>
<td>11·0</td>
<td></td>
<td>0·20 ± 0·02</td>
<td>0·21 ± 0·03</td>
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</table>

C and N deposition in expanding material elements

The effect of defoliation on C and N deposition in growth zone tissue was investigated using a ‘material element’ approach (cf. ‘Materials and Methods’). A detailed analysis of the C and N relations is presented for two elements of the growth zone (elements *a* and *b*, respectively) (Tables 1, 2 & 3). Elements *a* (*a*0, *a*1, *a*2) for days 0, 1 and 2 after defoliation were located at 5 mm from the leaf base and elements *b* (*b*0, *b*1, *b*2) at 11 mm at the beginning of the one-day intervals for which displacement, expansion and C and N deposition were assessed. In the following, elements *a* and *b* are termed ‘proximal’ and ‘distal’ elements. They were chosen to reflect the developmental heterogeneity of tissue in the leaf growth zone, proximal elements containing cells at (relatively) early and distal elements containing cells at more advanced stages of expansion and development.

During the one-day intervals the elements were displaced from their original position and, simultaneously, they experienced expansion (Table 1). As expected, displacement was always less for proximal than for distal elements. This is because displacement is the result of expansion of all tissue located between an element and the leaf base. Thus, tissue located between the proximal and distal element contributed to the displacement of the distal element, but not to displacement of the proximal element. Distal elements were always displaced beyond the distal limit of the leaf growth zone during the one-day intervals and, hence, completed their expansion during the respective interval. Conversely, proximal elements were still located within the growth zone at the end of the interval (Table 1, Fig. 3a).

Defoliation had no effect on expansion of proximal elements, with the length of elements increasing about four-fold during each one-day interval. In contrast, defoliation had a strong effect on the expansion of distal elements: whereas the length increased three-fold prior to defoliation (element *b*0), it increased 2·5-fold during day 1 (*b*1) and 1·9-fold (*b*2) during day 2 after defoliation (Table 1). This was due to a defoliation-related reduction of cell expansion in the distal part of the leaf growth zone, whereas defoliation had no effect on cell expansion in the basal part of the leaf growth zone (Schäufele & Schnyder 2000).

C deposition was always higher in the proximal than in the distal elements (Table 1). Variation in C deposition was linearly related to expansion of tissue elements, as was seen from calculation of deposition and expansion for a range of other elements of the growth zone tissue (Fig. 4a). However, extrapolating the relationship between expansion (*x*) and C deposition (*y*) yielded a positive intercept with the *y*-axis, showing that C deposition and expansion were not directly proportional in the range studied here. This was due to a larger C deposition per unit expansion for elements at more advanced stages of development, namely, elements located at more distal positions. These were displaced beyond the distal limit of the growth zone within the one-day interval (cf. Fig. 3a and Table 1). Hence,
some of the C deposition in these elements occurred after cessation of elemental expansion. This was especially true for distal elements during day 1 and day 2 after defoliation.

Interestingly, defoliation did not influence the relationship between element expansion and C deposition \((P < 0.05)\). Thus, the reduced C deposition, which was most prominent in the distal element during day 2 (Table 1), was related to reduced expansion of that element. Moreover, as defoliation reduced the length of the leaf growth zone (Fig. 3a) and thus the number of expanding elements (Schäufele & Schnyder 2000), the total mass of C deposited into the growth zone tissue was much less during days 1 and 2 after defoliation than prior to defoliation.

Similar to C, the N deposition was much higher in the proximal than in the distal elements (Table 1). However, the relationship between N deposition and expansion was modified strongly by defoliation, as after defoliation more N was deposited per unit expansion in both proximal and distal elements than before defoliation (Fig. 4b). As a consequence, the ratio of C deposition to N deposition decreased strongly after defoliation. This was particularly evident in the distal elements (Table 1), thus causing the increase in N content distal to 15 mm from the leaf base after defoliation (Fig. 3b).

The present data do not provide evidence for a role of C and N supply in controlling (the decreased) expansion of elements after defoliation: the relationship between elemental expansion and C deposition was essentially the same before and after defoliation, and N deposition was even increased relative to expansion. In an earlier study De Visser et al. (1997) observed a dilution of biomass in the basal part of expanding leaves after defoliation, which was due to (i) a reduced fresh mass of tissue per unit leaf length (spatial dilution) and (ii) a reduced C (but not N) concentration per unit fresh mass (chemical dilution). Although deposition rates were not assessed in that study, the dilution phenomenon suggested that C deposition was decreased relative to expansion after defoliation. Notably, no such dilution occurred in the present study, showing that dilution is not a general response to defoliation. However, to date the mechanisms controlling the defoliation-related dilution of biomass in leaf growth zones have not been studied in any detail.

We have shown previously, that the defoliation-related reduction of leaf elongation rate (~34% during day 2 relative to the rate before defoliation) was partially due to a reduced rate of cell production (Schäufele & Schnyder 2000). It is not clear how defoliation effected the decrease

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**Table 2.** Deposition rates of reserve- and current assimilation-derived C and N in expanding material elements of the leaf growth zone of *Lolium perenne* as affected by defoliation. For further explanation see caption of Table 1

<table>
<thead>
<tr>
<th>Element</th>
<th>day 1</th>
<th>day 2</th>
<th>Element</th>
<th>day 1</th>
<th>day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reserve-derived C ((\mu g \text{ element}^{-1} \text{d}^{-1}))</td>
<td>4.57 ± 0.16</td>
<td>0.96 ± 0.19</td>
<td>2.93 ± 0.18</td>
<td>0.63 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Currently assimilated C ((\mu g \text{ element}^{-1} \text{d}^{-1}))</td>
<td>1.33 ± 0.02</td>
<td>3.58 ± 0.14</td>
<td>1.08 ± 0.02</td>
<td>1.77 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Reserve-derived N ((\mu g \text{ element}^{-1} \text{d}^{-1}))</td>
<td>0.71 ± 0.03</td>
<td>0.27 ± 0.03</td>
<td>0.32 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Currently absorbed N ((\mu g \text{ element}^{-1} \text{d}^{-1}))</td>
<td>0.16 ± 0.01</td>
<td>0.26 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Reserve contribution to total C deposition (fraction)</td>
<td>0.78 ± 0.05</td>
<td>0.21 ± 0.06</td>
<td>0.73 ± 0.08</td>
<td>0.26 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Reserve contribution to total N deposition (fraction)</td>
<td>0.82 ± 0.06</td>
<td>0.50 ± 0.09</td>
<td>0.80 ± 0.09</td>
<td>0.52 ± 0.17</td>
<td></td>
</tr>
</tbody>
</table>

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**Table 3.** Estimated contributions of amino-C and carbohydrate-C (CHO-C) to reserve-derived and current assimilation-derived C accumulation in expanding material elements of the leaf growth zone of *Lolium perenne* as affected by defoliation. Deposition of amino-C was estimated as N deposition times 2.57 (cf. Fisher & Macnicol 1986; Lefevre et al. 1991). Deposition of carbohydrate-C was obtained as total C deposition minus amino-C deposition. For further explanation see caption of Table 1

<table>
<thead>
<tr>
<th>Element</th>
<th>day 1</th>
<th>day 2</th>
<th>Element</th>
<th>day 1</th>
<th>day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/N ((\text{w/w})) ratio in reserve-derived assimilates</td>
<td>6.4 ± 0.5</td>
<td>3.6 ± 1.2</td>
<td>9.2 ± 1.2</td>
<td>5.7 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Reserve-derived CHO-C, fraction of total reserve-derived C</td>
<td>0.60 ± 0.04</td>
<td>0.28 ± 0.18</td>
<td>0.72 ± 0.09</td>
<td>0.56 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>Reserve-derived amino-C, fraction of total reserve-derived C</td>
<td>0.40 ± 0.02</td>
<td>0.72 ± 0.22</td>
<td>0.28 ± 0.04</td>
<td>0.44 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>C/N ratio ((\text{w/w})) in current assimilates</td>
<td>8.3 ± 0.3</td>
<td>13.8 ± 1.0</td>
<td>13.5 ± 0.8</td>
<td>17.7 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Current assimilation-derived CHO-C, fraction of total current assimilation-derived C</td>
<td>0.69 ± 0.04</td>
<td>0.81 ± 0.07</td>
<td>0.81 ± 0.06</td>
<td>0.85 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Current assimilation-derived amino-C, fraction of total current assimilation-derived C</td>
<td>0.31 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Reserve-CHO, fraction of total C deposition</td>
<td>0.47 ± 0.03</td>
<td>0.06 ± 0.04</td>
<td>0.52 ± 0.06</td>
<td>0.15 ± 0.08</td>
<td></td>
</tr>
</tbody>
</table>

in cell production, but it is known that N supply has a strong positive effect on cell production (Volenec & Nelson 1983). However, N concentration in the zone of cell production near the leaf base did not decrease after defoliation (Fig. 3b), indicating that the defoliation-related reduction in cell production was not related to an effect of defoliation on the N supply to the meristem.

**Fractions of pre- and post-defoliation C and N**

Steady-state labelling demonstrated a rapid incorporation of currently fixed C in the basal part of expanding leaves (Fig. 5a). At 1 d after defoliation, approximately 16% of the total C in the basal 40 mm of growing leaves consisted of C derived from current photosynthesis. This fraction increased to approximately 50% at 2 d after defoliation (Fig. 5a). Notably, the change with time in the fractional contribution of currently fixed C to total tissue C differed among zones, with the increase with time being slower near the leaf base than beyond 10 mm from the base. This effect was particularly clear at 2 d after defoliation, when currently fixed C accounted for 42% of the total C in the basal 10 mm of the expanding leaves, and 53% between 10 and 26 mm from the leaf base ($P < 0.05$).

The N absorbed after defoliation accounted for about 9% of total N in the basal 40 mm of expanding leaves at 1 d after defoliation (Fig. 5b). This fraction increased to 21% at 2 d after defoliation (Fig. 5b). Hence, the increase with time in the fractional contribution of currently absorbed N was much slower than that of currently fixed C. Furthermore, zonal differences in the change with time in the fraction of currently absorbed N were stronger than those observed for C. At both 1 and 2 d after defoliation the fractional contribution of currently absorbed N near the base was less than half of that observed distal to 10 mm from the base ($P < 0.05$).

**Deposition of reserve- and current assimilation-derived C and N**

The method used to assess total C and N deposition in expanding elements was also used to assess the contribu-

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**Figure 4.** Relationship between expansion and C deposition rate (a) and N deposition rate (b) for a range of material elements of the growth zone of the most actively expanding leaf of the main tiller of *Lolium perenne* plants during days 0, 1, and 2 following defoliation. All elements were defined as having the same length (0-1 mm) at the beginning of the one-day interval. Expansion–deposition relationships were studied for elements located at 4, 5, ..., 12 mm from the leaf base at the beginning of one-day intervals. The line in (a) is a least squares regression (C deposition = 1.65 + 11.22 × Expansion; $r^2 = 0.84; P < 0.0001$). The most proximal elements are marked by an arrow in (b). Lines connecting neighbouring elements are shown in (b).

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**Figure 5.** Spatial distribution of the fraction of currently assimilated C (a) and N (b) along the basal 38 mm of the most actively expanding leaf of the main tiller of *Lolium perenne* plants at 1 d (●) and 2 d (○) following defoliation. Data points are means of two replicates for C and four replicates for N (10 leaves per replicate). Vertical bars indicate ± 1 SE.
tion of reserve- and current assimilation-derived C and N to the assimilate supply of expanding tissue after defoliation. These data demonstrate a very rapid transition from reserve- to current assimilation-derived C supply to expanding leaf tissue (Table 2). Pre-defoliation C (i.e. C fixed prior to defoliation and redistributed from other plant parts) was the main C source for expanding elements during day 1 after defoliation, providing between 73 and 78% of the total C deposited in elements. During day 2, however, current photosynthesis was the main source of C for expanding elements. Reserves supplied no more than 26% of the total C deposited in expanding elements during day 2. These relationships were very similar in proximal and distal elements.

In addition, the reserve-derived N was the main source of N for expanding elements during day 1 after defoliation: between 80 and 82% of the total N deposited in expanding elements was pre-defoliation N, i.e. N redistributed from other plant parts (Table 2). However, during day 2 about half of the N deposition was derived from current uptake.

It is interesting to note that the fractional contribution of current-assimilation derived C and N to biomass exhibited spatial variation (Fig. 5), although the fractional contribution of current assimilate to C and N deposition was very similar in proximal and distal elements of the growth zone (Table 2). This apparent discrepancy is explained by differences in the relative (or ‘specific’) rates of C and N deposition in different elements of the growth zone tissue. Thus, where the relative rate of deposition was high the pre-defoliation biomass was ‘diluted’ faster by current assimilate, than where the relative rate was low. Elements found close to the leaf base at 1 d after defoliation experienced relatively little expansion during the first day following defoliation, whereas elements located beyond 10 mm had entered (or passed through) the zone of most rapid cell expansion during the previous one-day interval (Schäufele & Schnyder 2000). The relative rate of assimilate influx is highest near the centre of the growth zone where the relative rate of expansion is also highest (Schnyder & Nelson 1987; Schäufele & Schnyder 2000). Therefore, it is not surprising that the fractional contribution of currently assimilated C and N to biomass was highest at positions distal to 10 mm from the leaf base (Fig. 5).

However, it is not self-evident that reserve- and current assimilation-derived assimilates should feed the different parts of the growth zone in a similar way: all reserve-derived substrates must enter the leaf growth zone at the base of the leaf, whereas most of the current assimilation-derived assimilates produced shortly after defoliation probably enter the leaf growth zone at its distal end. In severely defoliated ryegrass – as was the case in the present study – most of the photosynthetically active tissue present shortly after defoliation was associated with the most actively expanding leaves fraction (Schnyder & De Visser 1999). The close similarities in the (spatial) distribution of reserve- and current assimilation-derived assimilate thus suggests that assimilate transport occurred in both directions within the growth zone. This is consistent with results from 14C pulse-labelling studies with F. arundinacea during undisturbed growth (Allard & Nelson 1991).

### The composition of reserve-derived and current assimilation-derived substrate

During days 1 and 2, the C/N ratio in both reserve- and current assimilation-derived substrate deposited was higher in the distal than in the proximal element (Table 3). Thus, both reserve- and current assimilation-derived substrate contributed to maintaining the spatial gradient of C and N contents along the growth zone (Fig. 3a & b). However, the C/N ratio in reserve-derived substrate was lower than the C/N ratio in current assimilates (Table 3). This indicates that the chemical composition of reserve-derived and current assimilate deposited in expanding elements was different, with current assimilate being relatively enriched in carbohydrates, whereas reserve-derived assimilate was relatively enriched in amino acids. Thus, one should expect that amino-C was a significant component of the reserve-derived C deposition in expanding elements. Estimates of the contributions of carbohydrates and amino acids to reserve-derived C supply of expanding tissue were obtained by assuming the same C/N ratio (w/w) of 2.6 in amino acids (Fisher & Macnicol 1986; Lefevre et al. 1991) as used above. These data indicate that carbohydrates were the main source of reserve-derived C deposition during day 1 after defoliation, whereas amino-C was the main source of reserve-derived C deposition during day 2 (Table 3). This corroborates conclusions from earlier investigations where the contribution of carbohydrate-C and amino-C to reserve-derived substrate supply to leaf growth was assessed using less direct methodology (Avic et al. 1996; De Visser et al. 1997; Schnyder & De Visser 1999).

Current assimilate deposited in expanding elements during days 1 and 2 after defoliation were mainly composed of carbohydrates (Table 3). In fact, current assimilation was the dominant source of carbohydrate-C deposition already during day 1 after defoliation (Table 3). During day 2 the current assimilation supplied almost all of the carbohydrate-C deposited in expanding elements.

Clearly, the gross influx (i.e. import) of reserve-derived carbohydrates must have been larger than assessed here, since some of the imported carbohydrates were used in respiration. However, since current assimilation was the main source of carbohydrates we would expect that it also provided the bulk of substrate consumed in respiration. Therefore, reserve carbohydrates were a minor source of C for expanding tissue at least after day 1 from defoliation. This result is in contrast with the conclusions from earlier studies, where an extended period of reserve carbohydrate supply to regrowth in grasses was inferred from the evolution of reserve carbohydrate levels in stubble tissue (e.g. Alberda 1957; Davidson & Milthorpe 1966b; Gonzalez et al. 1989; for a critical discussion cf. De Visser et al. 1997). Conversely, the rapid transition to current-assimilation driven growth as observed here is in accordance with the results of studies, where the incorporation of current assimilates was assessed.
at the scale of the entire regrowing tiller biomass (Schnyder & De Visser 1999), or on the basis of the labelling kinetics of sucrose in the basal part of expanding leaves (Morvan-Bertrand et al. 1999). But the transition was significantly faster (<1 d for carbohydrates, Table 3) than was judged on the basis of the labelling kinetics of growth zone biomass by De Visser et al. (1997; approx. 3 d; cf. also the data presented in Tables 2 & 3 and Fig. 4). This result highlights the fact that transition from reserve- to current assimilation-driven growth can only be assessed accurately by investigating substrate fluxes at the level of the growing tissue as shown here, or by investigating the labelling kinetics of the substrate pools (e.g. sucrose) within the expanding tissue as demonstrated by Morvan-Bertrand et al. (1999).

CONCLUSIONS

This is the first study reporting C and N deposition in ‘expanding’ material elements of leaf growth zones during steady and non-steady growth as affected by defoliation. We believe that the methodology described in this paper will also be useful to study the response of expanding leaf tissue and associated assimilate relations to other types of disturbance or stress. In combination with \(^{13}C/^{12}C\) and \(^{15}N/^{14}N\)-steady-state labelling the present analysis demonstrated a very fast transition from reserve- to current assimilation-driven growth in severely defoliated perennial ryegrass. This transition was faster for carbohydrates (<1 d) than for N (approximately 2 d). The ‘relative’ contribution of reserve-derived carbohydrate and N deposition to total assimilate deposition was very similar for elements at different stages of expansion, that is, at different positions within the leaf growth zone, suggesting that assimilate transport within the growth zone was unrestricted and occurred in both a basipetal and acropetal direction. However, as the relationship between C and N deposition changed strongly along the leaf growth zone (an) unknown factor(s) related to the developmental stage of expanding elements must have controlled the composition of the assimilate that was imported into expanding tissue. The relationship between N deposition and element expansion was increased after defoliation, indicating that N deposition per se was not controlling the defoliation-related decrease in leaf expansion.

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